Poly(ADP-ribose) Modulates the Properties of MARCKS Proteins†

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ABSTRACT: In mammalian cells, the formation of DNA strand breaks is accompanied by synthesis of poly(ADP-ribose). This nucleic acid-like homopolymer may modulate protein functions by covalent and/or noncovalent interactions. Here we show that poly(ADP-ribose) binds strongly to the proteins of the myristoylated alanine-rich C kinase substrate (MARCKS) family, MARCKS and MARCKS-related protein (also MacMARCKS or F52). MARCKS proteins are myristoylated proteins associated with membranes and the actin cytoskeleton. As targets for both protein kinase C (PKC) and calmodulin (CaM), MARCKS proteins are thought to mediate cross-talk between these two signal transduction pathways. Dot blot assays show that poly(ADP-ribose) binds to MARCKS proteins at the highly basic effector domain. Complex formation between MARCKS-related protein and CaM as well as phosphorylation of MARCKS-related protein by the catalytic subunit of PKC are strongly inhibited by equimolar amounts of poly(ADP-ribose), suggesting a high affinity of poly(ADP-ribose) for MARCKS-related protein. Binding of MARCKS-related protein to membranes is also inhibited by poly(ADP-ribose). Finally, poly(ADP-ribose) efficiently reverses the actin-filament bundling activity of a peptide corresponding to the effector domain and inhibits the formation of actin filaments *in vitro*. Our results suggest that MARCKS proteins and actin could be targets of the poly(ADP-ribose) DNA damage signal pathway.

The members of the myristoylated alanine-rich C kinase substrate (MARCKS)[1] family are protein kinase C (PKC) substrates that are essential for brain development and postnatal survival (*1*−*3*). The family comprises two members: MARCKS is an ubiquitous 30-kDa protein whereas MARCKS-related protein (MRP) is a 20-kDa protein abundant in brain and reproductive tissues. Changes in the expression, phosphorylation state, and/or subcellular localization of MARCKS proteins are concomitant with major cellular events such as neurosecretion, motility, phagocytosis, and mitogenesis. MARCKS proteins have been proposed to regulate the structure of the actin cytoskeleton and/or the concentration of free calmodulin (CaM) (for reviews, see refs *4* and *5*). MARCKS proteins contain three conserved domains: (a) The N-terminus is myristoylated and is involved in membrane binding (*6*, *7*). (b) The MARCKS homology 2 domain contains a hexapeptide with sequence identity to the large cation-independent mannose-6-phosphate receptor; its function is unknown. (c) The effector domain (also called the phosphorylation site domain) is a highly basic domain, containing 24−25 residues, that mediates all known interactions of MARCKS proteins. It binds to membranes containing negatively charged phospholipids (*8*−*13*). It binds to CaM in a calcium-dependent manner (*14*−*16*). It cross-links actin filaments (*17*).[2] It is phosphorylated by PKC at two and three serine residues in MRP and MARCKS, respectively (*15*, *16*, *18*, *19*). This modification is reversed by phosphatases (reportedly phosphatase 1, phosphatase 2A, and calcineurin), which can dephosphorylate MARCKS both in vivo (*20*) and in vitro (*21*, *22*).

The multiplicity of interactions taking place at the effector domain results in competition between the various partners of MARCKS proteins: Phosphorylation regulates the properties of MARCKS proteins by inhibiting their interactions with membranes (*6*), with CaM (*14*, *15*, *23*), and with actin filaments (*17*).[2] Also, Ca^{2+}/CaM inhibits the phosphorylation of MARCKS proteins by PKC (*16*, *24*, *25*) as well as the interaction with actin (*17*).[2] That these competitive interactions also take place in the cell is suggested by several in vivo studies (*26*−*30*), providing the basis for a function of MARCKS proteins in cross-talk between the CaM and PKC signal transduction pathways (for a review, see ref *31*).

Phosphorylation/dephosphorylation cycles have been recognized as the major mechanism by which the functions of MARCKS proteins are regulated (*4*, *5*). Several reports

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[1] Abbreviations: CaM, calmodulin; CaM_D, dansylated CaM; MARCKS, myristoylated alanine-rich C kinase substrate; MMS, methyl methanesulfonate; MRP, MARCKS-related protein; NAD, nicotinamide adenine dinucleotide; poly(A), polyadenylic acid; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; PKM, catalytic subunit of PKC.

[2] Wohnsland, F., Schmitz, A. A. P., Steinmetz, M. O., Aebi, U., and Vergères, G., manuscript in preparation.

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suggest, however, that other modifications might also have regulatory functions. (a) Although myristoylation of proteins is generally believed to be an irreversible co-translational event (*32*), a pool of unmyristoylated MARCKS (*33*, *34*) and a demyristoylation activity (*35*) have been detected in cytosolic fractions of brain. Since we have recently demonstrated that purified MARCKS proteins can be efficiently myristoylated posttranslationally (*16*), acylation/deacylation cycles could potentially regulate the function of MARCKS proteins. (b) Carbohydrate moieties have been detected on electroblots of MARCKS purified from rat brain (*36*). Although MARCKS proteins contain consensus sequences for N-glycosylation (Gln-Gly-Ser in MARCKS, Gln-Gly-Thr in MRP), the significance of this observation remains unclear since these proteins are not translocated into the lumen of the endoplasmic reticulum. The authors have therefore proposed that MARCKS belongs to a class of nonclassical cytoplasmic glycoproteins (*37*). A confirmation of the presence of carbohydrates on MARCKS and an analysis of the stoichiometry of this modification and of its effects on the properties of the protein remain to be presented. (c) MARCKS can be radiolabeled in an ADP-ribosylation reaction in a brain homogenate (*38*). Interestingly, ADP-ribosylation has been recognized as an important regulatory mechanism of the structure and dynamics of the cytoskeleton (*39*, *40*). Bacterial toxins as well as endogenous mono-ADP-ribosyltransferases modify actin (*41*) but also neuromodulin (*42*, *43*) and Rho (*44*, *45*), two proteins involved in the regulation of the actin cytoskeleton. In this respect, Chao et al. (*38*) have proposed that mono-ADP-ribosylation might regulate the interactions of MARCKS with the actin cytoskeleton. However, as for N-glycosylation, data on the specificity and stoichiometry of this reaction and on its effect on the properties of MARCKS proteins still need to be demonstrated.

Poly(ADP-ribose) is a variably sized homopolymer of up to 200 ADP-ribose residues assembled into a complex structure with several branching points. It has been found in all eukaryotic cells except yeast, and it is covalently linked to the nuclear enzyme poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) (*46*). The regulation of poly(ADP-ribose) synthesis involves the specific binding of poly(ADP-ribose) polymerase to DNA strand breaks, activation of its catalytic activity, and covalent attachment of the polymers to the enzyme itself. This automodification reaction converts the enzyme into a protein carrying up to 28 acidic polymers, thereby drastically changing the microenvironment of the DNA break (*47*). The acidic polymers (two negative charges per residue) can engage in strong noncovalent interactions with other proteins (*48*, *49*). It has been proposed that these interactions are an integral part of a DNA damage signal mechanism (*50*).

Recent studies show the presence of specific polymer-binding motifs in a structurally and functionally diverse group of proteins (*50*). Computer-assisted sequence analysis revealed that such a motif is present in the effector domain of MARCKS proteins. Essential elements of this motif are at least three hydrophobic amino acids spaced at 6 and 7 positions apart and a combination of flanking and intervening clusters of positively charged amino acids (*50*).[3] This finding has prompted us to investigate whether poly(ADP-ribose) binds to MARCKS proteins. In this report, we demonstrate that free polymers as well as PARP-bound polymers bind stoichiometrically and with high affinity to the effector domain of MARCKS proteins. Furthermore, this interaction completely inhibits most of the properties of MRP, including its binding to CaM and membranes and its phosphorylation by the catalytic subunit of PKC (PKM). Finally, we show that poly(ADP-ribose) also impairs actin polymerization in vitro.

EXPERIMENTAL PROCEDURES

Materials. Murine myristoylated MRP was expressed in *E. coli* and purified using a mild procedure that does not require heating and acid treatment (*13*). Human myristoylated MARCKS was expressed in *E. coli* and purified as described (*15*). The plasmids containing the genes coding for murine MRP (pET3d−F52M1) and human MARCKS (pET3d−FH80K) were kind gifts of Dr. Perry Blackshear, Duke University, Durham, NC. The design, expression in *E. coli*, and purification of MRPHis (a MRP protein modified at the C-terminus with an His tag that allows purification on a nickel column) and MRPΔ(83−113)His (an His-tagged MRP protein in which the effector domain has been deleted) will be described elsewhere.[4] Rabbit muscle actin, purified from acetone powder (*51*), was a gift from Dr. Michel Steinmetz (Maurice E. Müller Institute, University of Basel). Labeling of actin with pyrene was performed according to ref *52*. PKM was obtained from Calbiochem; bovine brain dansylated CaM (CaM_D) and ADP-ribose were from Sigma; and polyadenylic acid (poly(A)) was from Fluka. A peptide whose sequence corresponds to the effector domain of murine MRP (KKKKKFSFKKPFKLSGLSFKRNRK) (residues 86−109) was synthesized by the solid-phase method and purified to >95% purity (AMS Biotechnology Ltd., Lugano, Switzerland). A second peptide whose sequence corresponds to the effector domain of bovine MARCKS (KKKKKRFSFKKSFKLSGFSFKKNKK) (residues 150−174) was synthesized and purified as described previously (*10*). The amino acid composition and the concentration of proteins and peptides were determined by amino acid analysis. Protein-attached poly(ADP-ribose) was synthesized using purified calf thymus poly(ADP-ribose) polymerase and NAD^+ as previously described (*53*, *54*). For radioactive labeling of the polymers, [^{32}P]-NAD^+ (45 Ci/mmol; New England Nuclear) was used. Free polymers with a mean polymer size of 40 residues were detached from the automodified polymerase by hydrolysis at pH 12 and purified by extraction of the protein in phenol:chloroform (*54*). To prevent loss of the polymers, the microcentrifuge tubes used for all experiments were siliconized (*55*).

ADP-Ribose Polymer Blot Assay. Dot blot assays were performed as described (*56*). Briefly, equimolar amounts of proteins or peptide (25 and 125 pmol) were spotted on a nitrocellulose membrane in a volume of 5 μL and air-dried. The membranes were then incubated for 1 h with 10 mL of TBST (10 mM Tris, 0.15 M NaCl, and 0.05% Tween 20,

[3] Pleschke, J. M., Kleczkowska, H. E., and Althaus, F. R., manuscript in preparation.

[4] Schmitz, A. A. P., and Vergères, G., manuscript in preparation.

pH 7.4) containing 1.0 nmol of total ADP-ribose (270 000 cpm [^{32}P]). The membranes were then washed with three changes of TBST, dried, and exposed to an X-ray film for detection. For competition experiments, 14 nmol of poly-(A) was included.

Interaction of MRP and of the Effector Peptide with CaM$_D$. Binding of MRP and of the effector peptide to CaM was investigated with CaM$_D$ as described previously (*16*). Briefly, 50 nM MRP or effector peptide was incubated with 30 nM CaM$_D$ in 0.4 mL of a solution containing 10 mM Tris, pH 7.4, 100 mM NaCl, 0.1 mM $CaCl_2$, and 0.001% Triton X-100. The fluorescence emission increase resulting from the binding of MRP or the effector peptide to CaM$_D$ was measured in the presence of increasing concentrations of poly(ADP-ribose) in 5 mm quartz cuvettes (Hellma) at 480 nm (λ_{ex}: 340 nm) at 20 °C in a Jasco FP-777 spectrofluorometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan). To correct for the contribution of poly(ADP-ribose) and CaM$_D$ to the signal, the same experiments were performed in the absence of MRP or effector peptide. As a control, the same experiments were performed with poly-(A) instead of poly(ADP-ribose). The amount of poly(A) used was doubled as compared to poly(ADP-ribose) in order to compensate for the 2-fold difference in net charge of the corresponding monomers.

Phosphorylation of MRP and of the Effector Peptide by PKM. Phosphorylation of MRP and of the effector peptide by PKM was performed as described previously (*16*). Briefly, 1 μM MRP or effector peptide was incubated in the presence of increasing concentrations of poly(ADP-ribose) with 0.5 nM PKM at 30 °C in 20 μL of a solution containing 10 mM Tris, pH 7.4, 100 mM NaCl, 0.1 mM $CaCl_2$, 6 mM $MgCl_2$, and 0.1 mM [γ-^{32}P]-ATP (0.2 μCi). The reaction was stopped after 20 min by the addition of 5 μL of SDS sample buffer and heating at 95 °C for 3 min. MRP or the effector peptide was separated from excess [γ-^{32}P]-ATP on SDS polyacrylamide gels, and the extent of phosphorylation was determined on a PhosphorImager (Molecular Dynamics).

Binding of MRP to Silica Beads Coated with a Lipid Bilayer. Binding of MRP to membranes was performed using 30-μm silica beads coated with a phospholipid bilayer composed of 80% phosphatidylcholine and 20% phosphatidylserine (Transil, NIMBUS GmbH, Leipzig) (*57*), as follows: 100 nM MRP was incubated for 1 h at room temperature under vigorous shaking with Transil in the presence of increasing concentrations of poly(ADP-ribose). The amount of Transil was chosen such that the final total lipid concentration was 1 mM, as determined by phosphate analysis (*58*). The incubation was performed in a final volume of 50 μL in 10 mM MOPS, pH 7.4, 0.1 mM EGTA, 100 mM NaCl, 1 mM DTT, and 0.001% Triton X-100. Membrane-bound MRP was then separated from the free protein by spinning the suspension in a microcentrifuge for 1 min at 10 000*g*. Both supernatant and pellet were mixed with SDS sample buffer, and the equivalent of one-third of the total fraction (maximal 33 ng of MRP) was loaded on a 12.5% SDS polyacrylamide gel, blotted onto a nitrocellulose membrane, and detected by ECL (Amersham) using an anti-MRP polyclonal antibody and a peroxidase-conjugated secondary antibody (Sigma).

Polymerization of Actin Filaments. The analysis of the effect of the effector peptide of MRP on the structure of actin filaments was performed according to a procedure that will be described elsewhere.[2] Briefly, 10 μL of a solution containing 5 μM monomeric actin was polymerized at room temperature in F-buffer (2.5 mM imidazole, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.2 mM ATP) in the presence or absence of 2 μM effector peptide and/or 2 μM poly(ADP-ribose). After 2.5 h, the solutions were transferred onto carbon-coated copper grids, negatively stained with 2% uranyl acetate, and examined using a Philips 400 electron microscope at 80 kV acceleration voltage.

For depolymerization experiments, 4 μM 80% pyrene-labeled actin was polymerized in F-buffer for 2.5 h in the presence or absence of equimolar amounts of poly(ADP-ribose). Depolymerization was induced by 30-fold dilution of the actin solution with G-buffer (F-buffer minus KCl and $MgCl_2$). Fluorescence of the pyrene moiety (λ_{ex} = 365 nm; λ_{em} = 407 nm) was recorded as a function of time.[2]

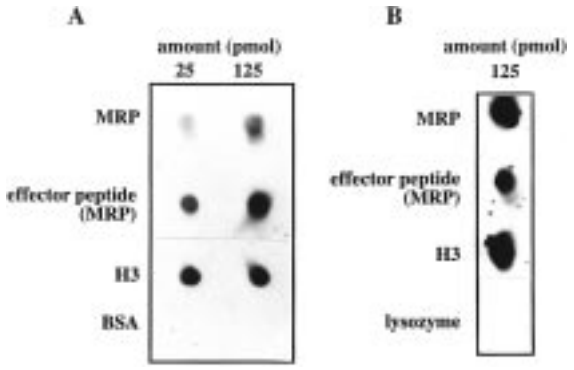

FIGURE 1: Poly(ADP-ribose) binds to MRP. The indicated amounts of proteins and peptides were blotted onto nitrocellulose and incubated with [^{32}P]-poly(ADP-ribose). (A) Binding of free poly-(ADP-ribose). Lane 1, MRP; lane 2, effector peptide of MRP; lane 3, histone H3; lane 4, BSA. The data are representative of *n* independent experiments (n = 5). (B) Binding of poly(ADP-ribose) covalently associated with PARP. Lane 1, MRP; lane 2, effector peptide of MRP; lane 3, histone H3; lane 4, lysozyme (n = 2).

RESULTS

Binding of Poly(ADP-ribose) to MRP. In the experiments shown in Figure 1A, the binding of free ADP-ribose polymers to MRP and to the MRP effector peptide was determined using a polymer blot assay. The results were positive for both, and the autoradiographic signals were comparable to the one obtained with histone H3, which served as a positive control (*56*). No binding was detectable with bovine serum albumin (negative control).

The experiments described in Figure 1A were repeated with polymers covalently bound to PARP (Figure 1B). With the automodified polymerase, the same results are obtained: Binding of the PARP-bound polymers to histone H3 (lane 3), but not to lysozyme (lane 4), is observed. Also, the protein-bound polymers bind to MRP (lane 1) and to its effector peptide (lane 2).

Specificity of Binding. The lack of binding of poly(ADP-ribose) to lysozyme, a cationic protein at neutral pH, indicates that the interaction of poly(ADP-ribose) with MRP is selective. To further demonstrate this specificity, a series of experiments were performed. First, poly(ADP-ribose) does not bind to MRPΔ(83−113)His, a mutated form of MRP in which the effector domain has been removed by site-directed mutagenesis (Figure 2A, lane 3). This lack of

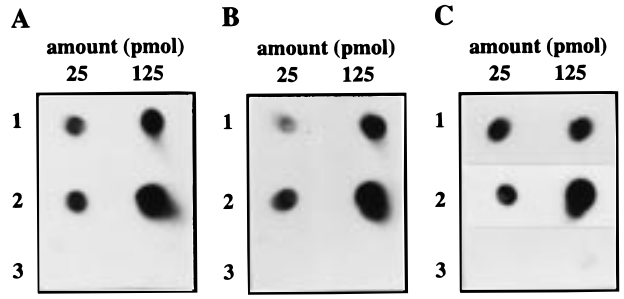

FIGURE 2: Poly(ADP-ribose) binds to the effector domain of MARCKS proteins. The indicated amounts of proteins and peptides were blotted onto nitrocellulose and incubated with [^{32}P]-poly(ADP-ribose) ($n = 2$). (A) Binding of poly(ADP-ribose) to the effector domain of MRP. Lane 1, MRP; lane 2, MRPHis; lane 3, MRPΔ-(83−113)His. (B) Binding of poly(ADP-ribose) to MRP in the presence of an excess of poly(A). Lane 1, MRP; lane 2, MRPHis; lane 3, MRPΔ(83−113)His. (C) Binding of poly(ADP-ribose) to the effector domain of MARCKS. Lane 1, MARCKS; lane 2, effector peptide of MARCKS; lane 3, lysozyme.

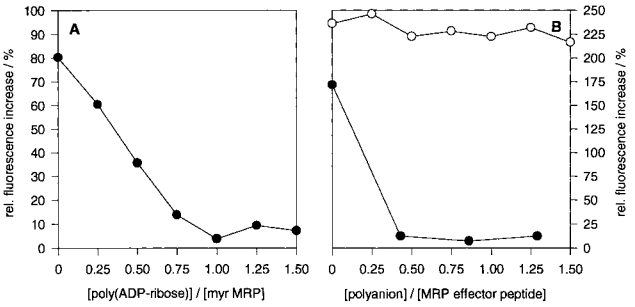

FIGURE 3: Poly(ADP-ribose), but not poly(A), inhibits binding of MRP to CaM_D. Effect of poly(ADP-ribose) and poly(A) on the fluorescence of the MRP·CaM_D complex. [CaM_D] = 30 nM. (A) Binding of MRP to CaM_D. [MRP] = 50 nM [$n = 3$; closed circles, poly(ADP-ribose)]. (B) Binding of the effector peptide of MRP to CaM_D. [MRP effector peptide] = 50 nM [$n = 2$; closed circles, poly(ADP-ribose), open circles, poly(A)].

binding is not due to the fact that we have modified the C-terminus of MRP with an His tag since MRPHis is recognized by poly(ADP-ribose) (lane 2) as well as wild-type MRP (lane 1). Second, a 7-fold excess of poly(A) over [^{32}P]-poly(ADP-ribose) does not reduce the signal (Figure 2B, lanes 1 and 2), demonstrating that the binding of poly-(ADP-ribose) to MRP is not simply due to the polyanionic nature of the polymers. Third, Figure 2C shows that poly-(ADP-ribose) binds to MARCKS, the other member of the MARCKS family (lane 1), and to its effector peptide (lane 2) but not to lysozyme (lane 3).

Taken together, the results presented in Figures 1 and 2 demonstrate that free and PARP-bound polymers specifically bind to the effector domain of MARCKS proteins. Poly-(ADP-ribose) could consequently regulate the interactions of MARCKS proteins with CaM, PKC, membranes, and actin. We have therefore set up experiments to test this hypothesis.

Poly(ADP-ribose) Blocks the Interaction of MRP with CaM. Binding of MRP to CaM_D increases the fluorescence intensity of the dansyl group by a factor of 1.8−2.5, providing a convenient assay to monitor the interactions of these molecules (*15*). Using this assay, we have previously demonstrated that myristoylated MRP binds to CaM_D with high affinity ($K_d = 4$ nM) (*16*). Figure 3A shows that poly-(ADP-ribose) inhibits the fluorescence enhancement resulting

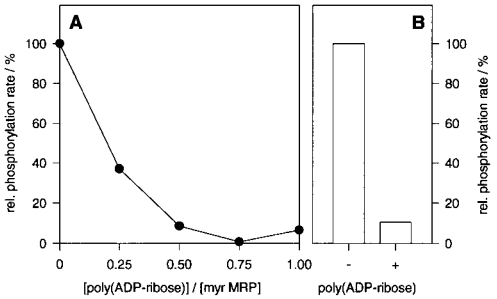

FIGURE 4: Poly(ADP-ribose) inhibits the phosphorylation of MRP by PKM. Effect of poly(ADP-ribose) on the relative rate of phosphorylation of MRP by PKM. [PKM] = 0.5 nM. (A) Phosphorylation of 1 μM MRP by PKM in the absence or presence of increasing amounts of poly(ADP-ribose) ($n = 3$). (B) Phosphorylation of 1 μM MRP effector peptide by PKM in the absence or presence of equimolar amounts of poly(ADP-ribose) ($n = 2$).

from the binding of MRP to CaM_D, indicating complex disruption by poly(ADP-ribose). Poly(ADP-ribose) also efficiently inhibits the binding of CaM_D to the effector peptide of MRP (Figure 3B). Interestingly, half-maximum inhibition is obtained at ratios of poly(ADP-ribose)/MRP or poly(ADP-ribose)/effector peptide close to 0.5, suggesting that a stoichiometric complex takes place between MRP and the polymers. That poly(ADP-ribose) can efficiently inhibit complex formation between 50 nM MRP or effector peptide and 30 nM CaM_D suggests that poly(ADP-ribose) binds to MRP with high affinity. Note also that the same amounts of ADP-ribose (not shown) or poly(A) (Figure 3B, open circles) do not disrupt the MRP·CaM_D complex, showing that the effect is specific for poly(ADP-ribose).

Poly(ADP-ribose) Inhibits Phosphorylation of MRP by PKM. The constitutively active PKM phosphorylates two serine residues in the effector domain of myristoylated MRP with high affinity ($[S]_{0.5} = 3.5$ μM), positive cooperativity ($n_H = 2.5$), and a turnover number of 130 min^{-1} (*16*). Figure 4A demonstrates that poly(ADP-ribose) dramatically reduces the rate of phosphorylation of MRP. Poly(ADP-ribose) inhibits phosphorylation of 1 μM MRP with a half-maximum concentration of 0.25 μM, again indicating a strong binding of poly(ADP-ribose) to MRP. Finally, in agreement with the results obtained with the effector peptide in the dot blot assay (see Figures 1 and 2) as well as in the binding assay with CaM_D (see Figure 3), equimolar concentrations of poly-(ADP-ribose) also fully inhibit the interactions of PKM with the effector peptide (Figure 4B).

Poly(ADP-ribose) Inhibits the Binding of MRP to Membranes. We have previously demonstrated that MRP binds to sucrose-loaded vesicles containing 20% negatively charged phospholipids with a partition coefficient of (2−5) × 10^4 M^{-1} (*13*). Since the effector domain also mediates the interactions of MARCKS proteins with membranes (*6*, *7*), poly(ADP-ribose) might modulate the partitioning of MRP between the aqueous and the membrane phase. We have therefore investigated the effect of poly(ADP-ribose) on the binding of MRP to membranes containing 80% phosphatidylcholine and 20% phosphatidylserine. To simplify the assay, we have replaced the 100-nm sucrose-loaded vesicles with 30-μm silica beads coated with a single lipid bilayer (Transil) containing the same percentage of negatively charged phospholipids (*57*). Due to their size and density, these beads settle to the bottom of the reaction tube easily,

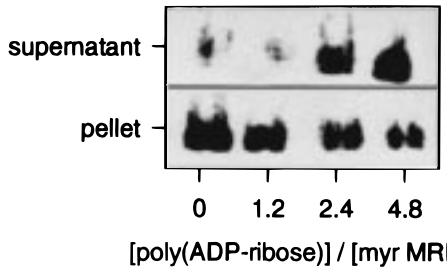

FIGURE 5: Poly(ADP-ribose) inhibits binding of MRP to membranes. Effect of poly(ADP-ribose) on the binding of 100 nM MRP to Transil beads coated with a bilayer containing 80% phosphatidylcholine and 20% phosphatidylserine (1 mM total phospholipids). Following incubation of MRP with Transil, membrane-bound MRP was separated from free MRP by centrifugation. The protein in the pellet and in the supernatant was detected by Western blotting ($n = 2$).

avoiding a centrifugation at 100 000*g* to separate membrane-bound MRP from free MRP. Assuming that the partition coefficient determined for the binding of MRP to sucrose-loaded vesicles is also valid for Transil beads, nearly all MRP should be associated with the beads at a total phospholipid concentration of 1 mM. Accordingly, we find that MRP binds efficiently to the beads at this lipid concentration as judged by its presence in the pellet after centrifugation (Figure 5). Adding increasing amounts of poly(ADP-ribose) decreases the fraction of MRP associated with the pellet and simultaneously increases the fraction of protein in the supernatant, demonstrating that complexation of MRP by poly(ADP-ribose) inhibits its binding to membranes. We have estimated by scanning densitometry that a 4.8-fold excess of polymer of ADP-ribose over MRP removes as much as 50% MRP from the membrane. That the polymers do not completely inhibit the binding of MRP to membranes might be explained by our observation that the myristoyl moiety also contributes to the interaction of MRP with membranes by inserting into the bilayer (*13*).

Poly(ADP-ribose) Impairs the Formation of Actin Filaments. Hartwig et al. (*17*) have shown that MARCKS cross-links actin filaments in vitro. Since a strong actin filament bundling activity was observed with a peptide corresponding to the effector domain of MARCKS, this domain was proposed to be responsible for the actin cross-linking activity of the intact protein. Figure 6 shows that the effector peptide of MRP also induces a strong bundling of the actin filaments (compare panels A and B).[2] However, in the presence of poly(ADP-ribose), the effector peptide cannot form actin bundles (Figure 6C). More importantly, we could not detect "normal" actin filaments in these preparations. This observation suggests that poly(ADP-ribose) not only impairs the interactions of MRP with actin filaments but might also *directly* affect actin polymerization. This conclusion is confirmed by experiments in which actin was incubated in polymerization buffer with poly(ADP-ribose) in the absence of the effector peptide: Figure 6D shows that poly(ADP-ribose) alone prevents the formation of "normal" actin filaments. Although polyanions such as nucleoside phosphates or oligomers of acidic amino acids disaggregate actin bundles into single filaments (*60*), these molecules were not reported to prevent the formation of single actin filaments. This suggests that the structural effect observed in Figure 6D is not simply due to the polyanionic nature of poly(ADP-ribose). Note that poly(ADP-ribose) forms particles that can also be observed by electron micrography (Figure 6E) (*59*). How these structures differ from the ones observed with poly(ADP-ribose) in the presence of actin remains to be determined (compare panel E with panels C and D).

That poly(ADP-ribose) destabilizes actin−actin interactions could also be observed kinetically in depolymerization experiments: upon dilution below the critical concentration of actin monomers, pyrene-labeled actin polymers disassemble more rapidly in the presence than in the absence of poly(ADP-ribose) (data not shown).

DISCUSSION

The results presented in this paper clearly demonstrate that poly(ADP-ribose) binds to MARCKS proteins. That this binding is selective is supported by several observations: First, experiments with peptides corresponding to the effector domain of MARCKS proteins as well as with a mutated form of MRP in which the effector domain has been deleted clearly identify this domain as the site of interaction with the polymers. Second, ADP-ribose or the polyanion poly(A) cannot compete with poly(ADP-ribose) for its binding to the effector domain of MRP. Third, poly(ADP-ribose), but not poly(A), inhibits binding of MRP effector peptide to CaM_D (see Figure 3B). Fourth, poly(ADP-ribose) does not bind to other basic proteins including lysozyme (see Figures 1 and 2), proteinase K, and cytochrome *c* (*49*). Finally, poly(ADP-ribose) binds to the basic C-terminal domain of histone H1 but neither to the basic globular domain nor to the basic N-terminal domain of the same protein (*49*).

We have also shown that near equimolar concentrations of poly(ADP-ribose) can fully inhibit the interactions of MRP with its cellular partners CaM, PKM, and membranes. Although our assays do not allow us to determine the affinity of poly(ADP-ribose) for MRP, the observation that equimolar concentrations of poly(ADP-ribose) can efficiently disrupt the strong MRP·CaM complex ($K_d = 4$ nM; *16*) indicates that poly(ADP-ribose) binds very tightly to MRP. Consequently, the affinity of poly(ADP-ribose) for MRP should be sufficient to allow the formation of a complex in vivo, provided these molecules have the same subcellular localization.

Since MARCKS proteins are mostly associated with the cytosolic leaflet of cellular membranes (*4*, *5*, *7*) and since PARP is considered as a nuclear enzyme (*61*, *62*), the problem of the colocalization of these molecules is certainly the most critical aspect to consider in estimating the physiological relevance of our observations. Several reports indicate that poly(ADP-ribose) might have a function in the cytosol. First, the presence of poly(ADP-ribose) in the cytosol has been claimed based on the observation that an oncogenic temperature-sensitive form of p53, which exhibits a cytoplasmic subcellular localization at 37 °C, is not only tightly associated with PARP but is also heavily poly(ADP-ribosylated) (*63*). Work from our laboratory also shows that p53 is a target for poly(ADP-ribose) binding and that the p53 function is also affected by these polymers (*64*). Second, cytoplasmic forms of PARP have been reported in a number of cell extracts including free ribosomes and polysomes of Hela cells (*65*), spermatocytes (*66*, *67*), and free messenger ribonucleoprotein particles in mouse plasmacytoma (*65*). Third, for poly(ADP-ribose) to appear in the cytosol, the

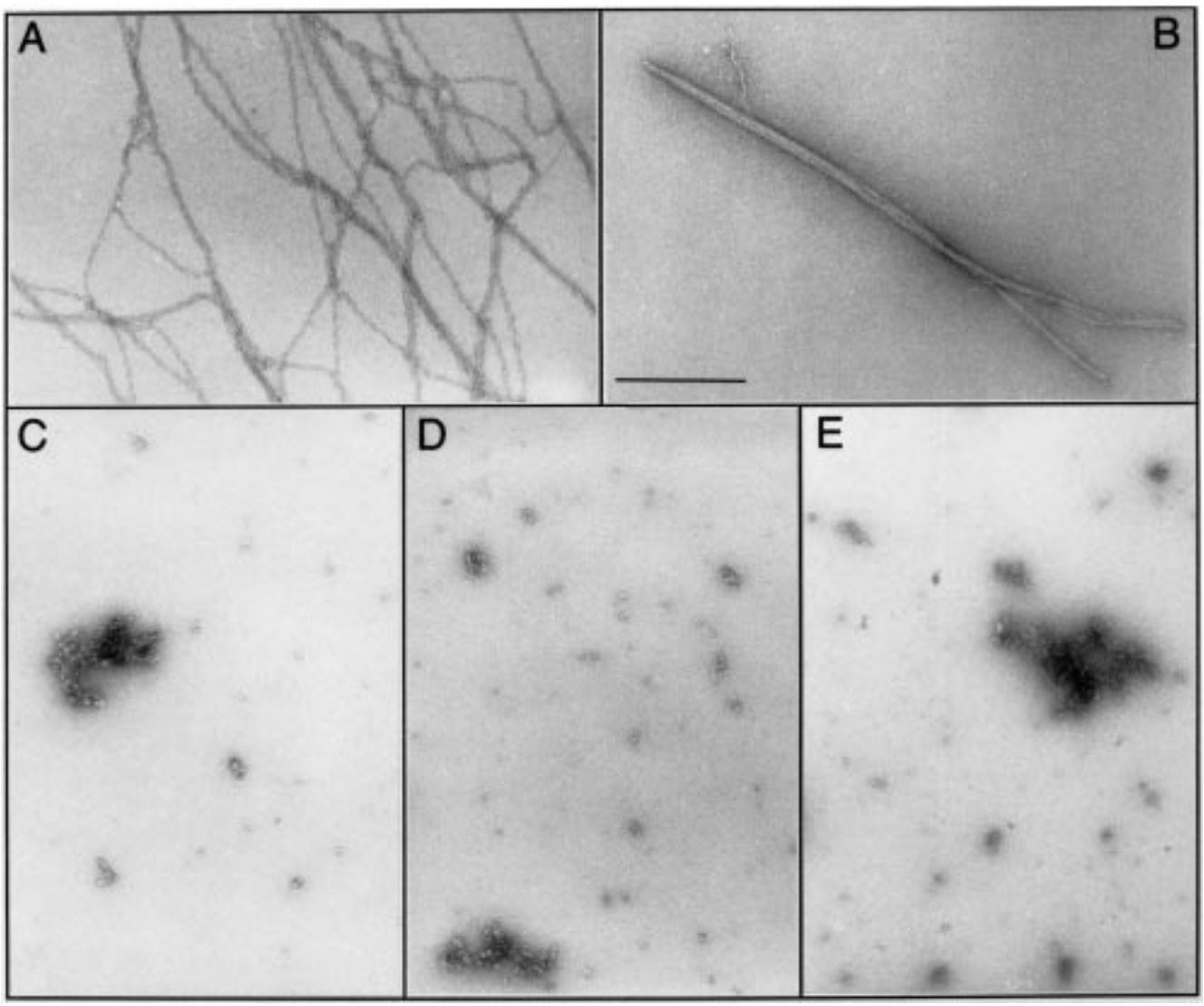

FIGURE 6: Poly(ADP-ribose) inhibits actin filament polymerization. Electron micrographs of negatively stained actin (5 μM) in the presence or absence of the effector peptide of MRP (2 μM) and/or poly(ADP-ribose) (2 μM) ($n = 3$). (A) Actin alone. (B) Actin + effector peptide. (C) Actin + effector peptide + poly(ADP-ribose). (D) Actin + poly(ADP-ribose). (E) Poly(ADP-ribose) alone. The bar represents 300 nm.

cytosolic presence of PARP is not required: PARP-bound polymers might well be cleaved in the nucleus through the endoglycosidic action of poly(ADP-ribose) glycohydrolase (*68-70*) and transported, either passively or actively, through the nuclear pores to fulfill a cytosolic function.

Although there is no direct in vivo evidence for a role of polyADP-ribosylation in the biology of MARCKS proteins, one report suggests that such an interaction might take place: Methyl methanesulfonate (MMS), a tumorgenic DNA alkylating agent (*71*, *72*), inhibits the PKC-dependent phosphorylation of MARCKS in cultured NIH 3T3 cells (*73*). The data presented by these authors supports a model in which MMS affects MARCKS phosphorylation by regulating the activity of phospholipase C and, consequently, the activity of PKC. Since MMS also induces the activation of PARP (*74*), an attractive, nonexclusive, alternative model would be that newly synthesized ADP-ribose polymers bind to the effector domain of MARCKS proteins, thereby inhibiting their phosphorylation by PKC.

In this paper, we have also shown that poly(ADP-ribose) inhibits the formation of actin filaments, suggesting that poly(ADP-ribosylation) can also potentially regulate the structure and/or the dynamics of the cytoskeleton. As for MARCKS proteins, a direct link between activation of PARP and the organization of the actin cytoskeleton has not been established. It is, however, interesting to note that drugs that inhibit actin polymerization in vitro, such as cytochalasin-D (*75*) and tolytoxin (*76*), induce nuclear and DNA fragmentation in vivo. These phenotypes are hallmarks of apoptosis, a cellular event in which the role of PARP is widely recognized (for reviews, see refs *61*, *62*, and *77*). Although mice lacking PARP develop normally (*78*), recent data show that inactivation of PARP, either by deletion of the gene or by inhibition with aminobenzamide, produces a giant cell phenotype with multiple nuclei (*50*).[5] It is thus tempting to suggest that poly(ADP-ribosylation) might directly act on actin in vivo to regulate the dynamics of the cytoskeleton.

In conclusion, this paper shows that—in vitro—poly(ADP-ribose) effectively modulates the properties of MARCKS proteins as well as inhibits the polymerization of actin. Keeping in mind that poly(ADP-ribose) is part of DNA damage signaling and that actin and MARCKS proteins have

[5] Müntener, C., and Althaus, F. R., unpublished results.

vital cellular properties, poly(ADP-ribose) could switch off the "normal" function of these proteins as an early response of the cell to DNA damage (*50*).

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