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Suppression of cell adhesion and spreading activities of fibronectin by arginine-specific ADP-ribosyltransferase from chicken polymorphonuclear leukocytes

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Abstract

Arginine-specific ADP-ribosyltransferase present in secretory granules of chicken polymorphonuclear leukocytes (so-called heterophils) was shown to be released into the extracellular space by secretagogues (Terashima et al., J. Biochem. 120 (1996) 1209–1215). In the present work, we examined fibronectin as an extracellular target protein of the released transferase. Fibronectin was ADP-ribosylated by purified transferase and stoichiometry of ADP-ribose incorporation into fibronectin was 1.0 mol/mol of fibronectin. Cell adhesion and spreading assays revealed that ADP-ribosylation of fibronectin markedly inhibited the adhesion activity of fibronectin. A proteolytic peptide map of ADP-ribosylated fibronectin demonstrated that the modification occurs in the cell binding domain of fibronectin. ADP-ribosylation of the RGD peptide suggests that the RGD sequence is the modification site in the domain. ADP-ribosylation of fibronectin in plasma means that fibronectin can probably serve as the substrate for extracellularly released ADP-ribosyltransferase in vivo. Thus, in the extracellular space, ADP-ribosyltransferase released from polymorphonuclear leukocytes may perhaps be involved in regulation of cell adhesion process by interfering with the activity of fibronectin. 0167-4889/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.

Keywords: ADP-ribosyltransferase; ADP-ribosylation; Fibronectin; Cell adhesion; RGD

1. Introduction

Arginine-specific ADP-ribosylation is a post-translational modification in which the ADP-ribose moiety of NAD is transferred to the guanidino group of arginine residues in various proteins and peptides. In eukaryotes, several arginine-specific ADP-ribosyltransferases (ADPRTs) were detected, including rabbit skeletal muscle [1,2], human neutrophils [3], mouse cytotoxic T-cells [4], and chicken polymorphonuclear leukocytes [5,6]. The former three transferases are located on the cell surface via the glycosylphosphatidylinositol (GPI) anchor, and mouse cytotoxic T-cell ADPRT was shown to be released by the stimulation of anti-CD3, interleukin 2 or

Abbreviations: ADPRT, ADP-ribosyltransferase (EC 2.4.2.31); FN, fibronectin; PMSF, phenylmethanesulfonyl fluoride; PCA, perchloric acid; TCA, trichloroacetic acid

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phorbol myristate acetate [7]. The skeletal muscle ADPRT and cytotoxic T-cell ADPRT modify adhesion molecules integrin α7 and lymphocyte function-associated molecule-1 (LFA-1), respectively [8,9]. These findings suggest an extracellular ADP-ribosylation and involvement of the modification in the control of cell adhesion.

In chicken polymorphonuclear leukocytes, arginine-specific ADPRT is localized in cytoplasmic secretory granules, and is released by secretagogues, such as calcium ionophore or opsonized-zymosans [10]. Thus, we asked whether extracellularly released chicken ADPRT could modify the extracellular matrix protein, such as fibronectin (FN), a ligand for the integrin family. Fibronectin is a major component of extracellular matrices and is present as an adhesive glycoprotein dimer, of which the monomer has a molecular mass of 235 kDa. It is also present in plasma at high concentration (0.3 mg/ml). Fibronectin interacts with integrins, collagen, heparin, and fibrin, and is involved in adhesion, morphological change, and migration of cells [11]. The fibronectin molecule is composed of type I, II, and III domains, identified as repeating amino acid motifs in the primary structure. The site in FN to promote cell attachment is present in one of the type III domains, FIII10. The Arg-Gly-Asp (RGD) sequence in the domain has been shown to be the minimal cell recognition sequence and thus to be critical for interaction with integrins [12–14]. We show here that chicken arginine-specific ADPRT modifies FN in plasma, and that the modification inhibits the cell adhesion activity of FN.

2. Materials and methods

2.1. Materials

Chickens were obtained from a local slaughterhouse. [adenylate-^{32}P]NAD (29.6 TBq/mmol) was purchased from New England Nuclear. Gelatin-Sepharose and heparin-Sepharose were from Amersham Pharmacia Biotech. RGD peptide and thermolysin were from Sigma. TIG-3 (JCRB0506) and BALB/3T3 (JCRB9005) cells were obtained from Health Science Research Resources Bank (HSRRB), Japan.

2.2. ADP-ribosylation of FN

Fibronectin was purified from chicken plasma as described by Hayashi and Yamada [15]. Purified FN was incubated with 50 mM Tris-HCl (pH 9.0), 5 mM dithiothreitol, 50 μM [^{32}P]NAD (3.7 kBq/tube), and appropriate amounts of ADPRT purified from chicken polymorphonuclear cells [5] in a total volume of 200 μl at 25°C for the indicated time. The reaction was terminated by adding 10% TCA and radioactivity in the acid-insoluble fraction was measured by filter assay, or analyzed by SDS-PAGE and autoradiography.

2.3. Cell adhesion assay and phase contrast microscopic study

Cell adhesion assay and phase contrast microscopic study using TIG-3 or BALB/3T3 cells were done as described in elsewhere [16]. Fibronectin (10 μg) was incubated alone or with NAD (1 mM), ADPRT (10 ng), or both, in 50 mM Tris-HCl (pH 9.0) and 5 mM dithiothreitol in a plastic tube at 25°C for 2 h in a total volume of 100 μl. We confirmed that 1 mol of ADP-ribose was incorporated into 1 mol of fibronectin under these conditions. With each mixture or PBS, the surface of 96-well flat bottomed plates was coated and kept at 4°C, overnight. TIG-3 or BALB/3T3 cells cultured in minimal essential medium (MEM) containing 10% fetal calf serum (FCS) were plated on the well at the concentration of 10^5/ml. Attached cells were stained with 1 mg/ml methylene blue and intensity of the color was quantified directly by absorbance at 650 nm. Before staining, the shape of the attached cells was observed using phase-contrast microscopy.

2.4. Proteolytic peptide map

Purified FN (10 μg) was incubated with 50 mM Tris-HCl (pH 9.0), 5 mM dithiothreitol, 50 μM [^{32}P]NAD (37 kBq/tube) and purified ADPRT (10 ng) for 30 min at 25°C in a total volume of 50 μl. ADP-ribosylation was terminated by adding 1 mM novobiocin, a potent inhibitor of arginine-specific ADPRT [17], and ADP-ribosylated FN was incubated with thermolysin (1 μg) according to Sekiguchi and Hakomori [18] at 25°C. After 2 h,

thermolysin (2 μg) was further added to the reaction mixture and incubated for an additional 2 h. The proteolytic reaction was terminated by adding 5 mM ethylenediaminetetraacetic acid (EDTA) and SDS-PAGE sample buffer, and the digested products were analyzed by SDS-PAGE (12.5% polyacrylamide) and autoradiography.

2.5. ADP-ribosylation of FN in chicken plasma

Fresh chicken plasma (800 μl) collected with 1 mM EDTA and 0.2 mM PMSF was incubated with 50 μM [^{32}P]NAD (110 kBq), 2 mM ADP-ribose and 500 ng of purified ADPRT for 1 h at 25°C in a total volume of 1 ml. The plasma (800 μl) was also incubated with 20 μM [^{32}P]NAD (110 kBq) and isolated chicken polymorphonuclear leukocytes (5×10^7) [5] in the presence of 5 μM A23187 and 1 mM $CaCl_2$ [10], for 30 min at 37°C in a total volume of 1 ml. After the incubation, 10% PCA-insoluble fraction of 100 μl of the reaction mixture was analyzed by SDS-PAGE followed by autoradiography. The remainder of each mixture was centrifuged at $600\times g$ for 10 min and the supernatant was applied to a gelatin-Sepharose column [15] equilibrated with buffer A (10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 5 mM EDTA) and incubated at room temperature for 1 h. After washing twice with the buffer, the bound proteins were eluted with 1 ml of buffer A containing 4 M urea, precipitated with 10% TCA (w/v), and analyzed by SDS-PAGE and autoradiography.

3. Results

3.1. ADP-ribosylation of purified FN

To determine if FN is modified by arginine-specific ADPRT from chicken polymorphonuclear leukocytes, FN purified from chicken plasma was incubated with [^{32}P]NAD in the absence or presence of the purified ADPRT, and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1A, FN incubated together with [^{32}P]NAD and ADPRT was radiolabeled; radiolabeling did not occur in the absence of ADPRT. These results indicate that purified chicken arginine-specific ADPRT apparently modified FN. The modification occurred stoichiometrically, and 1 mol of ADP-ribose was incorporated into 1 mol of FN molecule (Fig. 1B). Further addition of the enzyme or NAD, or prolonged incubation failed to extend the modification. The ADP-ribose–FN linkage was labile to NH_2OH, but resistant to $HgCl_2$ [19] (data not shown). These results confirm the ADP-ribosylation of FN on an arginine residue.

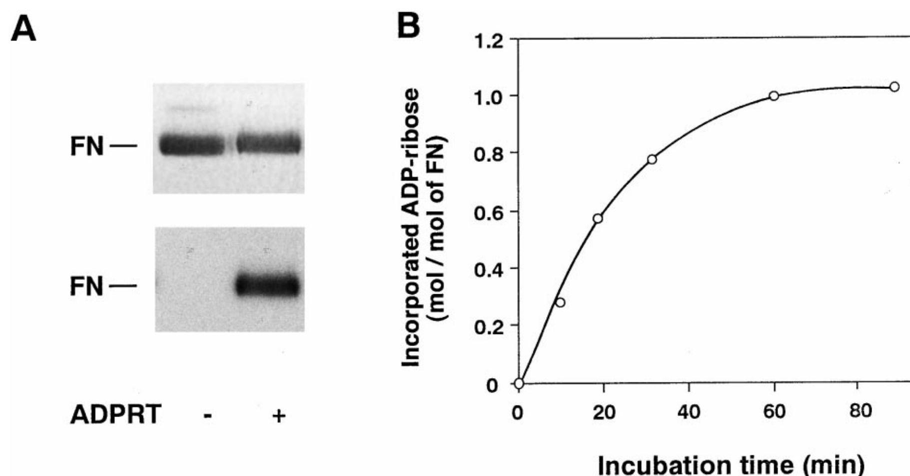

Fig. 1. (A) ADP-ribosylation of purified FN. FN (5 μg) was incubated in reaction mixture containing 100 μM [^{32}P]NAD, with or without purified chicken ADPRT (10 ng) at 25°C for 10 min and subjected to SDS-PAGE followed by protein staining (upper) and autoradiography (lower). (B) Stoichiometry of ADP-ribose incorporated into FN. Purified FN (2 μg) was incubated in the reaction mixture containing 50 μM [^{32}P]NAD and ADPRT (5 ng) at 25°C for the indicated time. The incorporation of ADP-ribose into the FN was determined by filter assay.

3.2. Cell adhesion and spreading activities of ADP-ribosylated FN

Next, we asked if ADP-ribosylation of FN would affect its potential to adhere to cells. FN incubated alone or with NAD, ADPRT, or both, was coated on the plates and adhesion activity of each FN towards TIG-3 cells was determined by staining of cells attached to the plate with methylene blue. As shown in Fig. 2, treatment of FN together with NAD and ADPRT remarkably decreased cell adhesion, while FN preincubated alone or either with NAD or ADPRT adhered to the cells. Phase contrast microscopy also clearly showed that TIG-3 cells adhered and extended on the FN-coated plate, exhibiting a spread out morphology (Fig. 3A). Cells not adhering to the ADP-ribosylated FN-coated plate, had a circular morphology (Fig. 3B). The experiments described above were also carried out using BALB/3T3 cells and similar results were obtained (data not shown). These studies indicate that the ADP-ribosylation of FN decreased cell adhesion and spreading activities.

3.3. ADP-ribosylation may occur in the cell binding domain of FN

It was reported that the RGD sequence in the cell binding domain of FN is the main contributor to the FN–cell interaction [16,17]. We then looked to see if the modification site locates in the cell binding domain. When [^{32}P]ADP-ribosylated FN was digested with thermolysin, radiolabeling was observed at the 105 kDa fragment, which contains cell binding domain [18,20] (Fig. 4). Moreover, ADP-ribosylation of RGD peptide was confirmed by reverse-phase HPLC (data not shown). These findings support the notion of a modification of the RGD sequence in the cell binding domain. We propose that inhibition of cell adhesion and spreading activities of FN by ADP-ribosylation may be attributed to modification of the RGD sequence in the cell binding domain.

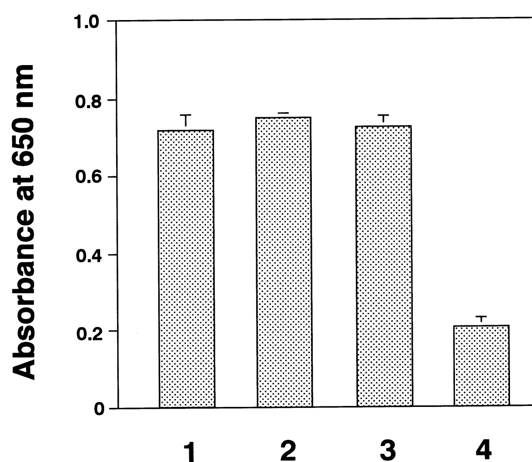

Fig. 2. Effect of ADP-ribosylation on cell adhesion activity of FN. Cell adhesion assays of FN (10 μg) preincubated alone (1), or with NAD (2), ADPRT (3) or NAD and ADPRT (4) were done as described in Section 2. The data are the mean ± S.D. of three experiments conducted in triplicate.

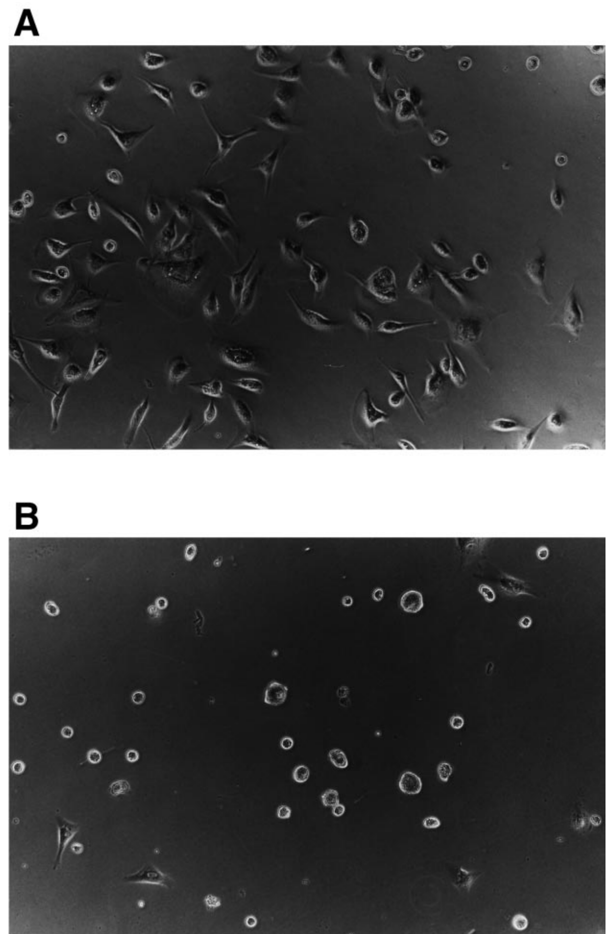

Fig. 3. Effect of ADP-ribosylation on cell spreading activity of FN. Phase contrast micrographs of TIG-3 cells cultured for 2 h on plastic plates coated with 10 μg/ml of FN preincubated (A) alone, or (B) in the presence of NAD and ADPRT at ×400 magnification.

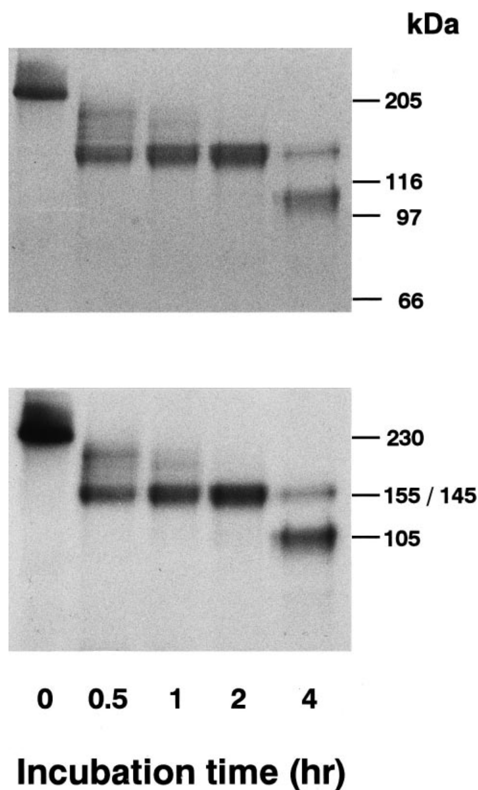

Fig. 4. Proteolytic peptide map of ADP-ribosylated FN. [^{32}P]ADP-ribosylated FN was digested with thermolysin as described in Section 2, and fractionated with SDS-PAGE followed by Coomassie brilliant blue staining (upper) and autoradiography (lower).

3.4. ADP-ribosylation of FN in chicken plasma

To observe if FN could serve as the substrate for the released ADPRT from chicken polymorphonuclear leukocytes [10], chicken plasma was incubated with [^{32}P]NAD and purified ADPRT, and then placed on to a gelatin-Sepharose column to isolate the FN [18]. The acid-insoluble fraction of proteins eluted with 4 M urea from the column was analyzed by SDS-PAGE. After autoradiography, the radioactivity was detected at a band with a molecular mass of 230 kDa, which corresponds in size to FN (Fig. 5, lane 1). The 67 kDa protein appeared in Coomassie brilliant blue staining was probably contaminated albumin. When chicken polymorphonuclear leukocytes were stimulated with 5 μM A23187 and 1 mM $CaCl_2$ in chicken plasma in the presence of [^{32}P]NAD, labeling of FN was also observed (Fig. 5, lane 2). These data suggest that ADP-ribosylation of FN might possibly occur in vivo.

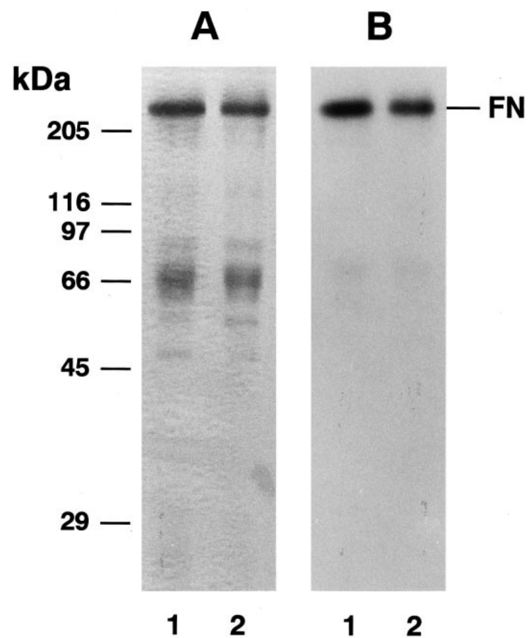

Fig. 5. ADP-ribosylation of FN in plasma. Fresh chicken plasma was incubated with 50 μM [^{32}P]NAD and purified ADPRT (lane 1), or with 20 μM [^{32}P]NAD and chicken polymorphonuclear leukocytes in the presence of secretagogues (lane 2), as described in Section 2, and applied to a gelatin-Sepharose column. The eluate with 4 M urea was analyzed using SDS-PAGE followed by protein staining (A) and autoradiography (B).

4. Discussion

Fibronectin serves as a target for glycosylation, phosphorylation, sulfation, and acylation [13,21–23]. Although the glycosylation of FN protects the protein against proteolysis and provides useful cell-type-specific markers [21], the significance of other post-translational modifications of FN is unknown. We found that ADP-ribosylation of FN decreases its cell adhesion and spreading activities, perhaps through a modification of the RGD sequence in the cell binding domain of FN. This is apparently the first report demonstrating that FN is ADP-ribosylated and we suggest that this modification may have a role in regulating the cell adhesion activity of FN.

Fibronectin is a glycoprotein present in extracellular matrices and in plasma, and plays important roles in a variety of biological processes. Fibronectin binds heterodimeric integrins, including $\alpha_5\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ via its cell binding domain containing FIII10. Occupation of these integrin receptors

with extracellular matrix proteins, such as FN, leads to tyrosine phosphorylation of the proteins and cytoplasmic alkalinization, which leads to activation, proliferation or interleukin production in lymphocytes, and proliferation or migration in fibroblasts [24].

A synergic region FIII9 was found to be required for the biological activity of FN [25,26]. Moreover, crystal structure of FIII7–FIII10 of human FN indicates that FIII9 and FIII10 are on the same surface of FN and easily access to a single integrin molecule [27]. Thus, ADP-ribosylation may directly obscure the protruding RGD sequence on the cell binding domain or indirectly disrupt the 'synergy' effect between FIII9 and FIII10, thereby possibly inhibiting FN–cell interactions. The precise mechanism, by which ADP-ribosylation decrease the cell adhesion activity of FN, and the site of ADP-ribosylation are now being examined.

ADP-ribosyltransferases, attaching to the outer surface of skeletal muscle myoblasts and mouse cytotoxic T-cells via the GPI-anchor, have been shown to modify integrin $\alpha7$ [8] and LFA-1 [9], respectively. These modifications occurring on the cell surface might inhibit cell aggregation or transmembrane signaling through an as yet to be determined mechanism. ADP-ribosyltransferase of chicken polymorphonuclear leukocytes is released into extracellular spaces by secretagogues [10]. ADP-ribosylation of FN by stimulated polymorphonuclear leukocytes in plasma suggests a possible modification in vivo. Human neutrophils also possess arginine-specific ADPRT activities in cells [3]. Thus, ADPRTs dispersed into extracellular space, might regulate extracellular matrix functions, by modifying FN in cellular processes, such as cell attachment, migration, and proliferation, during inflammation or wound healing.

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