ADP-ribosylation of integrin $\alpha 7$ modulates the binding of integrin $\alpha 7\beta 1$ to laminin

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The extracellular domain of integrin $\alpha 7$ is ADP-ribosylated by an arginine-specific ecto-ADP-ribosyltransferase after adding exogenous NAD^+ to intact C2C12 skeletal muscle cells. The effect of ADP-ribosylation on the structure or function of integrin $\alpha 7\beta 1$ has not been explored. In the present study, we show that ADP-ribosylation of integrin $\alpha 7$ takes place exclusively in differentiated myotubes and that this post-translational modification modulates the affinity of $\alpha 7\beta 1$ dimer for its ligand, laminin. ADP-ribosylation in the 37-kDa 'stalk' region of $\alpha 7$ that takes place at micromolar NAD^+ concentrations increases the binding of the $\alpha 7\beta 1$ dimer to laminin. Increased *in vitro* binding of integrin $\alpha 7\beta 1$ to laminin after ADP-ribosylation of the 37-kDa fragment of $\alpha 7$ requires the presence of Mn^{2+} and it is not observed in the presence of Mg^{2+} . In contrast, ADP-ribosylation of the 63-kDa

N-terminal region comprising the ligand-binding site of $\alpha 7$ that occurs at approx. 100 μM NAD^+ inhibits the binding of integrin $\alpha 7\beta 1$ to laminin. Furthermore, incubation of C2C12 myotubes with NAD^+ increases the expression of an epitope on integrin $\beta 1$ subunit recognized by monoclonal antibody 9EG7. We discuss our results based on the current models of integrin activation. We also hypothesize that ADP-ribosylation may represent a mechanism of regulation of integrin $\alpha 7\beta 1$ function in myofibres *in vivo* when the continuity of the membrane is compromised and NAD^+ is available as a substrate for ecto-ADP-ribosylation.

Key words: ADP-ribosylation, integrin, laminin, muscle, myoblast, myotube.

INTRODUCTION

Mono-ADP-ribosylation is a post-translational modification of proteins in which the ADP-ribose moiety is transferred from NAD^+ to a specific amino acid in a target protein [1,2]. The reaction is catalysed by a group of enzymes called ARTs (mono-ADP-ribosyltransferases). The family of ARTs includes many bacterial toxins (e.g. cholera, pertussis, diphtheria and botulinum toxin) [3–5], as well as poorly characterized eukaryotic ARTs.

The first eukaryotic ART, ART1, was purified and cloned from rabbit skeletal muscle [6,7]. Subsequently, six other ART-related genes were identified [8–12]. Most of the eukaryotic ARTs catalyse the transfer of ADP-ribose to the guanidino group in arginine residues in proteins, as well as in free arginine. ARTs show a restricted pattern of expression, with ART1 being predominantly expressed in skeletal and cardiac muscle [6,11]. In mouse skeletal myoblasts cultured *in vitro*, ART1 is undetectable before myoblast differentiation, and it is strongly up-regulated in differentiated myotubes [13]. ART1 is a cell-surface protein anchored in the membrane by a GPI (glycosylphosphatidylinositol) tail [6,7]. Interestingly, other members of the eukaryotic ART family are also ecto-enzymes, either GPI-anchored in the membrane or secreted to the extracellular space [11].

During the search for protein substrates of ART1 in mouse myoblastic cell line C2C12, integrin $\alpha 7$ was identified as the major protein that was ADP-ribosylated after adding exogenous NAD^+ to intact, differentiated C2C12 myotubes [13,14]. ADP-ribosylation of integrin $\alpha 7$ was specific, occurred rapidly and required micromolar concentrations of NAD^+ [13,14]. Integrin $\alpha 7$ is composed of the N-terminal 'heavy' chain (X1 or X2 splice variant of ~ 100 kDa) and the C-terminal 'light' chain (A or B splice variant of ~ 33 or ~ 38 kDa respectively; see Figure 1B)

[15–18]. The two chains are connected by a disulphide bond and can be separated upon reduction. Integrin $\alpha 7$ forms a dimer with integrin $\beta 1$ and binds to laminin, an ECM (extracellular matrix) protein [19,20]. Interestingly, in cytotoxic T-cells that express high levels of ecto-ADP-ribosyltransferase activity, two other members of the integrin family, αL and $\beta 2$ [forming the LFA-1 (lymphocyte function-associated molecule-1) dimer], were also found to be ADP-ribosylated [21,22].

The effect of ADP-ribosylation on the structure and function of integrin $\alpha 7\beta 1$ has not been explored. Since ADP-ribose represents a bulky, negatively charged group that can potentially change the conformation of a polypeptide chain, we hypothesized that ADP-ribosylation in the extracellular domain of integrin $\alpha 7$ may affect its ligand binding affinity directly. The results presented in the present paper suggest that ADP-ribosylation of integrin $\alpha 7$ indeed modulates the affinity of $\alpha 7\beta 1$ dimer for its ligand, laminin. While ADP-ribosylation in the middle 37-kDa region of $\alpha 7$ taking place at micromolar NAD^+ concentrations increases the binding of the $\alpha 7\beta 1$ dimer to laminin *in vitro*, ADP-ribosylation in the 63-kDa N-terminal fragment of $\alpha 7$ at approx. 100 μM NAD^+ inhibits its binding to laminin. Incubation of C2C12 myotubes with NAD^+ increases the expression of an epitope on integrin $\beta 1$ subunit recognized by mAb (monoclonal antibody) 9EG7. We discuss our results based on the current models of integrin activation [23–25].

MATERIALS AND METHODS

ADP-ribosylation in intact C2C12 cells

C2C12 myoblasts were cultured in six-well plates in growth medium [DMEM (Dulbecco's modified Eagle's medium)

Abbreviations used: ART, mono-ADP-ribosyltransferase; CHO, Chinese-hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's PBS; ECM, extracellular matrix; GPI, glycosylphosphatidylinositol; LFA-1, lymphocyte function-associated molecule-1; mAb, monoclonal antibody.

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supplemented with 10 % (v/v) foetal bovine serum] at 37°C in the presence of 5 % CO₂ under humidified atmosphere. To induce differentiation, confluent cells were transferred to differentiation medium [DMEM containing 2 % (v/v) horse serum]. After 3–5 days in differentiation medium, when many myotubes were present, cells were washed and incubated for 30 min in DPBS (Dulbecco's PBS) containing 0.5 mM MgCl₂, 1 mM CaCl₂, 1 mM ADP-ribose and 0–100 µM [*adenylate*-³²P]NAD⁺ (specific activity 0.3–0.6 Ci/mmol). As reported previously, C2C12 myotubes contain an active ecto-phosphodiesterase that efficiently converts ADP-ribosylarginine in integrin α7 into (phospho)-ribosylarginine [14]. The presence of 1 mM free ADP-ribose during the ADP-ribosylation reaction, and subsequently in cell lysis buffer, inhibited the degradation of ADP-ribosylarginine.

Binding of integrin α7β1 to laminin columns

EHS (Engelbreth–Holm–Swarm) laminin (Invitrogen) was dialysed against buffer containing 0.1 M NaHCO₃, pH 8.3, and 0.5 M NaCl, and then coupled to CNBr-activated Sepharose (Amersham Biosciences; 3 mg of laminin/ml of bed volume) according to the manufacturer's instructions. Following labelling of differentiated C2C12 cells with [³²P]NAD⁺, cells were lysed with lysis buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 50 mM octylglucoside, 1 mM MnCl₂, 1 mM ADP-ribose, 1 mM *N*-ethylmaleimide, 1 mM AEBSE [4-(2-aminoethyl)benzenesulphonylfluoride hydrochloride], 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin A (1 ml of lysis buffer/well). The lysate was centrifuged for 20 min at 15 000 *g*, and the supernatant (2 ml, unless indicated otherwise) was applied to laminin or to the control columns (0.1 ml bed volume). In the experiment shown in Figure 2(B), CHO (Chinese-hamster ovary) cells stably transfected with rat integrin α7X1B or α7X2B ([26]; a gift from S. J. Kaufman, University of Illinois, Urbana, IL, U.S.A.) were used instead of C2C12 cells. After washing the columns with 10 ml of lysis buffer and elution with 200 µl of elution buffer (lysis buffer containing 20 mM EDTA instead of MnCl₂), proteins were analysed by SDS/PAGE under reducing conditions, followed by autoradiography or Western blotting with anti-(integrin α7A) or anti-(integrin α7B) antibodies (a gift from S. J. Kaufman).

Determination of the stoichiometry of ADP-ribosylation of integrin α7

To determine the amount of radioactive ADP-ribose associated with integrin α7, total cell lysates or laminin column eluates were analysed by SDS/PAGE under reducing conditions; a portion of the gel containing integrin α7 was left unfixed and unstained, another portion containing molecular-mass markers was stained with Coomassie Blue. Two 3-mm gel slices containing the 100-kDa heavy chain of integrin α7 were excised and analysed by liquid-scintillation counting. The number of moles of ADP-ribose was calculated based on the known specific activity of [³²P]NAD⁺ used during the ADP-ribosylation reactions. The number of moles of the 100-kDa integrin α7 heavy chain in laminin column eluates was estimated after silver-staining of the gels, using BSA as a standard. The number of moles of integrin α7 in total cell lysates was determined after Western blotting with anti-α7A or anti-α7B antibodies, using known amounts of laminin-purified integrin α7 as standards and assuming that α7A and α7B isoforms each contributed 50 % to the total integrin α7 pool [16–18].

Immunostaining of cells with mAb 9EG7

Confluent C2C12 cells grown on tissue culture plastic in six-well plates were incubated for 4 days in differentiation medium. Cells

were washed and incubated for 30 min at 25°C in DPBS containing 0.5 mM MgCl₂, 1 mM CaCl₂, 1 mM ADP-ribose, and 0, 10 or 100 µM NAD⁺. Cells were then washed once and incubated for 30 min in Tyrode's buffer (5 mM Hepes/KOH, pH 7.4, 12 mM NaHCO₃, 150 mM NaCl, 2.6 mM KCl, 5 mM glucose, 0.5 mM MgCl₂ and 1 mM CaCl₂) in the presence of 3 µg/ml mAb 9EG7 (BD Pharmingen), followed by washing, and incubation in Tyrode's buffer, 1 mM ADP-ribose and 7.5 µg/ml of rhodamine-conjugated anti-rat IgG antibody. In a parallel experiment, cells were incubated for 30 min in Tyrode's buffer containing 1 mM ADP-ribose and 1 mM MnCl₂, then for 30 min in the same buffer supplemented with 3 µg/ml mAb 9EG7. Cells were then washed and incubated with the secondary antibody, as described above. After washing, cells were analysed immediately using an Axiovert 200 inverted fluorescent microscope.

Immunoblotting

Samples were resolved by SDS/PAGE and transferred on to a nitrocellulose membrane. Membranes were blocked in DPBS containing 3 % (w/v) dried milk and 0.3 % (v/v) Tween 20, then incubated with a primary antibody in blocking buffer, followed by incubation with a horseradish-peroxidase-labelled secondary antibody and detection with a chemiluminescence detection method (West Pico, Pierce). The following dilutions of primary antibodies were used: anti-α7A, 1:2000; anti-α7B, 1:2000; anti-myogenin (clone F5D; Santa Cruz Biotechnologies), 1:50; anti-MyoD1 (clone 5.8A; Lab Vision), 1:500; anti-p130 (clone 10; BD Biosciences), 1:1000; anti-tubulin (clone DM 1A; Sigma), 1:40 000.

RESULTS

Two different fragments of integrin α7 are ADP-ribosylated in intact C2C12 myotubes after adding exogenous NAD⁺

After incubation of intact differentiated C2C12 cells with [*adenylate*-³²P]NAD⁺ and analysis of total cell lysates by SDS/PAGE and autoradiography, most of the ³²P-label was previously found associated with a single protein of ~100 kDa [13,14]. Based on the mobility of the radiolabelled protein (100/140 kDa under reducing/non-reducing conditions respectively, see Figure 1B), its binding to a laminin column, co-immunoprecipitation with anti-(integrin β1) antibody, and direct amino acid sequencing, it was shown previously that the radiolabelled protein was the heavy chain of integrin α7 [13,14]. The removal of the radiolabel after treatment of cells with snake venom phosphodiesterase (an indication of ADP-ribosylation) or 1 M NH₂OH, pH 7.0 (an indication of an ADP-ribosylarginine linkage), or inhibition of the labelling by pre-treatment of cells with phosphatidylinositol-specific phospholipase C demonstrated that the modification of integrin α7 involved ADP-ribosylation of an arginine residue, and it was catalysed by the GPI-anchored ART1 enzyme [13].

Partial trypsinization of purified integrin α7 revealed that at 5 µM NAD⁺, a single 37-kDa fragment was ADP-ribosylated, while at 100 µM NAD⁺, an additional fragment of 63 kDa was modified as well (Figures 1A and 1B). This result is consistent with an earlier report that the major ADP-ribosylation site (or sites) that is ADP-ribosylated at low NAD⁺ concentrations (≤ 5 µM) is located in the central region of α7, and that at higher NAD⁺ concentrations additional site(s) in the 63-kDa N-terminal fragment of α7 are ADP-ribosylated [14].

It was shown previously that the expression of ADP-ribosyltransferase activity and the appearance of the 100-kDa ³²P-labelled band correlated well with C2C12 cell differentiation [13].

Figure 1 Two different fragments of integrin $\alpha 7$ are ADP-ribosylated after adding exogenous NAD $^{+}$ to intact C2C12 myotubes

(A) Differentiated C2C12 cells were incubated for 30 min in DPBS in the presence of 5 μM or 100 μM [adenylate- ^{32}P]NAD $^{+}$, as indicated. Integrin $\alpha 7\beta 1$ was then purified on laminin-affinity columns, as described in the Materials and methods section. The eluates from the laminin columns were incubated for 10 min with the indicated amounts of trypsin, followed by SDS/PAGE under reducing conditions and autoradiography. The 100-kDa heavy chain of integrin $\alpha 7$ is indicated by an arrow, the 37-kDa tryptic fragment of the heavy chain that is ADP-ribosylated at both 5 and 100 μM NAD $^{+}$ is indicated by a large asterisk, the 63-kDa fragment that is ADP-ribosylated at 100 μM NAD $^{+}$, but not at 5 μM NAD $^{+}$, is denoted by a small asterisk. Positions of the molecular-mass markers (in kDa) are shown on the left. (B) Schematic diagram showing the location of the ADP-ribosylated fragments in integrin $\alpha 7$. Proteolytic processing by an intracellular protease at the RRRRE site in the extracellular domain leads to generation of the heavy chain of 100 kDa (X1 or X2 splice variant) and the light chain of 33/38 kDa (A or B splice form respectively) that are connected by a disulphide bond and separated upon reduction. The 63-kDa and 37-kDa fragments of the heavy chain generated by trypsin cleavage at the RRQ site correspond to the radiolabelled tryptic fragments in (A). (C) ADP-ribosylated integrin $\alpha 7$ resides in differentiated myotubes and not in reserve cells. Left-hand panel, after ADP-ribosylation had been completed, differentiated myotubes (M) and non-differentiated reserve cells (R) were separated by brief and mild trypsinization of cells incubated with 2% (v/v) horse serum (HS) for 4 days. Reserve cells are characterized by down-regulation of MyoD expression and up-regulation of the retinoblastoma-related p130 protein, and differentiated myotubes are characterized by sustained expression of MyoD and low expression of p130 [27]. For comparison, the expression levels of MyoD and p130 are also shown in proliferating C2C12 cells grown in 10% FBS. Tubulin is a gel loading control. Right-hand panel, ^{32}P -ADP-ribosylated $\alpha 7$ (arrows) is present in myotubes and not in reserve cells. Notice that since the separation of

Differentiating C2C12 cells represent, however, a heterogeneous population of cells that contain a pool of differentiated, multi-nucleated myotubes and a pool of non-differentiated, quiescent 'reserve' cells characterized by down-regulation of the expression of MyoD and up-regulation of the retinoblastoma-related p130 protein [27]. Whereas the exact localization of ART1 is not known, integrin $\alpha 7$ is expressed in both myotubes and reserve cells. Since differentiating C2C12 cells are fully confluent, regardless of its localization, ART1 enzyme should have access to integrin $\alpha 7$ in both myotubes and reserve cells. To determine whether ADP-ribosylation of integrin $\alpha 7$ by ART1 takes place in myotubes and/or reserve cells, the two populations of cells were separated by mild trypsinization after the ADP-ribosylation reaction had been completed, and the extent of ^{32}P -labelling of $\alpha 7$ was examined. As shown in Figure 1(C), nearly all ADP-ribosylated integrin $\alpha 7$ was found to be associated with the myotube fraction and not with non-differentiated reserve cells, suggesting a role for ADP-ribosylation in regulating integrin $\alpha 7$ function in differentiated muscle cells.

Integrin $\alpha 7\beta 1$ -laminin affinity *in vitro* assay

To evaluate the effect of ADP-ribosylation of integrin $\alpha 7$ on the affinity of $\alpha 7\beta 1$ dimer for its ligand, we decided to incubate C2C12 myotubes with different concentrations of NAD $^{+}$, to extract membrane proteins from cells, and then to examine the binding of integrin $\alpha 7\beta 1$ to laminin-1 affinity columns. First, we determined a linear range of the assay. As shown in Figure 2(A), when integrin $\alpha 7$ was extracted from C2C12 cells grown in six wells or fewer in a six-well plate and loaded on 0.1 ml columns containing 0.3 mg of laminin, the amount of integrin $\alpha 7$ retained on the column was directly proportional to the amount of $\alpha 7$ loaded on the columns. Thus, in the experiments shown in Figures 3–6, integrin $\alpha 7$ was extracted from C2C12 cells in two wells in six-well plates for each column-binding assay.

To determine the extent of $\alpha 7$ binding to laminin columns, we wanted to analyse column inputs and eluates by Western blotting using anti-integrin $\alpha 7$ antibody. Differentiated C2C12 cells express both X1 and X2 splice variants of integrin $\alpha 7$ heavy chain and both A and B splice variants of the light chain [17,18]. Since the only anti- $\alpha 7$ antibodies available to us that were suitable for Western blotting recognized either the A or the B form of the light chain [that could connect to either X1 or X2 variant of the heavy chain where the ADP-ribosylation site(s) were located], and since it is not known whether ADP-ribosylation involves the X1 or the X2 isoform, or both, it was important to determine whether the X1 and X2 variants bound equally well to laminin columns. Although it was reported previously that $\alpha 7\text{X1B}$ - and $\alpha 7\text{X2B}$ -transfected HEK-293 (human embryonic kidney) cells adhered equally well to laminin-1-coated surfaces [28], in a solid-state binding assay, the recombinant soluble $\alpha 7\text{X2B}\beta 1$ dimer bound more readily to laminin-1 than the $\alpha 7\text{X1B}\beta 1$ dimer [29]. In the present study, we utilized CHO cells stably transfected with the integrin $\alpha 7\text{X1B}$ or $\alpha 7\text{X2B}$ isoform. As shown in Figure 2(B), the X1B and X2B variants bound equally well to laminin columns, validating the use of the column-binding assay to assess the effect of ADP-ribosylation on the laminin-binding affinity of $\alpha 7\beta 1$.

myotubes and reserve cells involves trypsinization, the 37-kDa cleavage product is observed in the myotube fraction in addition to the native 100-kDa heavy $\alpha 7$ chain. T (total) represents the sum of the R and M fractions. Positions of molecular-mass markers are shown on the left (in kDa).

Figure 2 Column binding as an assay to evaluate the affinity of integrin $\alpha 7\beta 1$ for laminin

(A) Determination of the linear range of the assay. C2C12 cells grown in six-well plates and incubated in differentiation medium for 4 days were lysed with buffer containing 1 mM Mn^{2+} . Cell lysates obtained from three, six or 12 wells were loaded on laminin columns (0.1 ml columns, 3 mg of laminin/ml of bed volume), columns were washed and eluted with buffer containing 20 mM EDTA. Column eluates were analysed by SDS/PAGE under reducing conditions and Western blotting with antibodies recognizing either the A isoform (upper left panel) or the B isoform (upper right panel) of the integrin $\alpha 7$ light chain. The intensities of the bands corresponding to $\alpha 7A$ or $\alpha 7B$ isoforms (in arbitrary units, a.u.) were measured by densitometry and plotted against the number of wells used for purification of $\alpha 7\beta 1$ (lower panel). (B) The X1 and X2 splice variants of integrin $\alpha 7$ bind equally well to laminin columns. CHO cells stably transfected with either integrin $\alpha 7X1B$ or $\alpha 7X2B$ were grown in six-well plates. Cells harvested from one well were loaded on laminin columns, as in (A). The lysates (2% of samples) and column eluates (17% of samples) were analysed by SDS/PAGE and Western blotting with anti- $\alpha 7B$ antibody (upper panel). The intensities of the bands were determined by densitometry, and the amounts of integrin $\alpha 7$ bound to the columns were calculated (lower panel; results are means $\pm$ S.E.M. for three determinations).

The effect of ADP-ribosylation on the binding of integrin $\alpha 7\beta 1$ to laminin *in vitro* in the presence of Mn^{2+}

First, we examined the effect of ADP-ribosylation of integrin $\alpha 7$ on the affinity of $\alpha 7\beta 1$ dimer for laminin in the presence

Figure 3 Effects of ADP-ribosylation on the binding of integrin $\alpha 7\beta 1$ to laminin in the presence of Mn^{2+}

(A) Differentiated C2C12 cells (4 days in differentiation medium; two wells in a six-well plate) were incubated for 30 min in DPBS containing indicated concentrations of [^{32}P]NAD⁺ (specific activity, 0.4 Ci/mmol in each case). Membrane proteins were extracted with 2 ml of buffer containing 1 mM Mn^{2+} and loaded on laminin columns (0.1 ml columns, 3 mg of laminin/ml of bed volume), columns were washed and eluted with 0.2 ml of elution buffer containing 20 mM EDTA. Volumes of 10 μl of cell lysates and column eluates (i.e. 0.5 and 5% of the total volumes respectively) were analysed by SDS/PAGE and autoradiography (top panel) and Western blotting with anti-integrin $\alpha 7A$ antibody (middle panel); 2 μl of cell lysates and column eluates (i.e. 0.1% and 1% of the total volumes respectively) were analysed by Western blotting with anti-integrin $\alpha 7B$ antibody (lower panel). In the autoradiogram, the 100-kDa heavy chain of $\alpha 7$ is shown; in Western blots, the light chains of $\alpha 7$ are visualized. (B) The intensities of the bands in (A) were determined by densitometry; the percentages of ^{32}P -labelled heavy chain of $\alpha 7$ (linked to either A or B light chain) and the A and B light chains (linked to either ^{32}P -labelled or non-labelled heavy chain) bound to the columns were calculated (means $\pm$ S.E.M. for three experiments).

of Mn^{2+} . Differentiated C2C12 cells were incubated for 30 min with NAD⁺ concentrations ranging from 0 to 100 μM , followed by laminin-column-binding assay (used in the linear range, as determined in Figure 2A). Integrin $\alpha 7\beta 1$ retained on the column was eluted with elution buffer containing EDTA. The amount of [^{32}P]ADP-ribosylated integrin $\alpha 7$ in the lysates and in laminin column eluates was evaluated by SDS/PAGE and autoradiography (Figure 3A). The amount of total integrin $\alpha 7$ (both ADP-ribosylated and non-ADP-ribosylated) in the eluate and in the flow-through fraction was determined by Western blotting using anti- $\alpha 7A$ and anti- $\alpha 7B$ antibodies. As shown in Figure 3(B), the eluate contained ~ 2 –8% of the total integrin $\alpha 7$ loaded on the columns. In contrast, up to 38% of the ^{32}P -labelled heavy chain that was ADP-ribosylated at 5 or 10 μM NAD⁺ was retained on the columns. When higher NAD⁺ concentrations were

Figure 4 The amount of integrin $\alpha 7$ heavy chain retained on laminin columns in the presence of Mn^{2+} is modulated by ADP-ribosylation

(A) Differentiated C2C12 cells were incubated with the indicated concentrations of NAD^+ , integrin $\alpha 7$ was purified on laminin-affinity columns in the presence of 1 mM Mn^{2+} , as in Figure 3. The amounts of the 100-kDa heavy chain of $\alpha 7$ in the eluates were evaluated by SDS/PAGE and silver staining. A representative silver-stained gel is shown. (B) The intensities of the bands in (A) were determined by densitometry, and the relative amounts of integrin $\alpha 7$ heavy chain were calculated; the amount of integrin $\alpha 7$ eluted from the column after incubation of cells without NAD^+ corresponds to 1.0. Results are means $\pm$ S.E.M. for three experiments.

used in the ADP-ribosylation reactions, the amount of ^{32}P -labeled $\alpha 7$ retained on laminin columns decreased, and at 100 μM NAD^+ , it represented only $\sim 5\%$ of the input (Figure 3B). This suggested that integrin $\alpha 7$ that was ADP-ribosylated at NAD^+ concentrations $\leq 10 \mu\text{M}$ (when ADP-ribosylation was restricted to the central 37-kDa fragment; see Figures 1A and 1B) bound more readily to laminin columns than $\alpha 7$ that was not modified or than $\alpha 7$ that was ADP-ribosylated at higher NAD^+ concentrations (with ADP-ribosylation involving the N-terminal 63-kDa fragment; Figures 1A and 1B). Direct visualization of the integrin $\alpha 7$ heavy chain present in laminin column eluates by SDS/PAGE and silver-staining of the gel confirmed that the eluates obtained after ADP-ribosylation at 5 or 10 μM NAD^+ contained the highest amounts of $\alpha 7$ (Figure 4).

We next measured the stoichiometry of ADP-ribosylation of integrin $\alpha 7$ before and after binding to laminin columns. When calculations were performed for $\alpha 7$ in total cell lysates, the stoichiometry of modification was strongly dependent on NAD^+ concentration and rose from ~ 0.05 mol of ADP-ribose/mol of $\alpha 7$ at 5–10 μM NAD^+ to ~ 0.4 mol of ADP-ribose/mol of $\alpha 7$ at 100 μM NAD^+ (Figure 5). In contrast, integrin $\alpha 7$ eluted from laminin columns contained ~ 0.4 mol of ADP-ribose/mol of $\alpha 7$ at all NAD^+ concentrations tested. Thus, after ADP-ribosylation at 5–10 μM NAD^+ , the stoichiometry of modification of integrin $\alpha 7$ eluted from laminin columns was significantly higher than the stoichiometry of modification of integrin $\alpha 7$ in total cell lysates. Since the ADP-ribosylation reaction was terminated when lysis buffer was added to cells and no further ADP-ribosylation occurred during purification on laminin columns, we conclude that

Figure 5 Stoichiometry of ADP-ribosylation of integrin $\alpha 7$ before and after binding to laminin columns

Differentiated C2C12 cells were incubated with the indicated concentrations of ^{32}P - NAD^+ , and integrin $\alpha 7$ was purified on laminin columns in the presence of 1 mM Mn^{2+} , as shown in Figures 3 and 4. The amounts of integrin $\alpha 7$ heavy chain present in the column eluates were determined after silver staining of the gels, using BSA as a standard. The amounts of ^{32}P -ADP-ribose incorporated into $\alpha 7$ and the abundance of integrin $\alpha 7$ in cell lysate before loading on laminin columns were measured as described in the Materials and methods section. Results are means $\pm$ S.E.M. for three determinations.

laminin-affinity chromatography in the presence of Mn^{2+} resulted in the enrichment of integrin $\alpha 7$ that was ADP-ribosylated in the 37-kDa middle region, but not in the 63-kDa N-terminal fragment. Collectively, the results in Figures 3–5 suggest that ADP-ribosylation in the 37-kDa region of $\alpha 7$ increases the affinity of $\alpha 7\beta 1$ for laminin, whereas ADP-ribosylation of the 63-kDa fragment inhibits laminin binding.

The effect of ADP-ribosylation on the binding of integrin $\alpha 7\beta 1$ to laminin *in vitro* in the presence of Mg^{2+}

The experiments shown in Figures 3–5, in which ADP-ribosylation of the 37-kDa central fragment of integrin $\alpha 7$ appeared to enhance $\alpha 7\beta 1$ binding to laminin, were performed in the presence of Mn^{2+} . Since Mn^{2+} itself is a partial integrin activator, we asked whether ADP-ribosylation of the 37-kDa $\alpha 7$ fragment is sufficient to increase the affinity of $\alpha 7\beta 1$ for laminin in the absence of Mn^{2+} . As expected, when Mn^{2+} in the cell lysis buffer was replaced with Mg^{2+} , the amount of $\alpha 7$ retained on laminin columns was much lower than in the presence of Mn^{2+} (Figure 6). Importantly, incubation of cells with 5–10 μM NAD^+ leading to ADP-ribosylation of the 37-kDa fragment of $\alpha 7$ did not increase the subsequent binding of $\alpha 7\beta 1$ to laminin columns (Figure 6B, compare with Figure 3B). This suggests that ADP-ribosylation of the 37-kDa fragment of $\alpha 7$ alone is not sufficient to increase the affinity of $\alpha 7\beta 1$ for laminin *in vitro*. Instead, ADP-ribosylation of this region in $\alpha 7$ works in concert with Mn^{2+} to induce an active conformation of integrin $\alpha 7\beta 1$ *in vitro*, with a high affinity for laminin.

The effect of ADP-ribosylation on the conformation of integrin $\alpha 7\beta 1$ in intact myotubes

We next examined whether ADP-ribosylation of integrin $\alpha 7$ results in a change of conformation of integrin $\alpha 7\beta 1$ in intact cells, and whether such an effect would require the presence of Mn^{2+} . Since ADP-ribosylation of integrin $\alpha 7$ occurs in myotubes and not in non-differentiated myoblasts (Figure 1C), we decided to probe the conformation of the $\alpha 7\beta 1$ integrin by staining differentiating adherent C2C12 cultures with mAb 9EG7. This

Figure 6 Effects of ADP-ribosylation on the binding of integrin $\alpha 7\beta 1$ to laminin in the presence of Mg^{2+}

(A) Differentiated C2C12 cells (4 days in differentiation medium; two wells in a six-well plate) were incubated for 30 min in DPBS with the indicated concentrations of [³²P]NAD⁺ (specific radioactivity, 0.5 Ci/mmol in each case). Membrane proteins were extracted with 2 ml of buffer containing 1 mM Mg^{2+} and loaded on laminin columns (0.1 ml columns, 3 mg of laminin/ml of bed volume), columns were washed and eluted with 0.2 ml of elution buffer containing 20 mM EDTA. A volume of 4 μ l of cell lysates and 60 μ l of column eluates (i.e. 0.2% and 30% of the total volumes respectively) were analysed by SDS/PAGE and autoradiography (top panel), 2 μ l of cell lysates and 60 μ l of column eluates (i.e. 0.1% and 30% of the total volumes respectively) were analysed by Western blotting with anti-integrin $\alpha 7A$ antibody (middle panel); 2 μ l of cell lysates and 40 μ l of column eluates (i.e. 0.1% and 20% of the total volumes respectively) were subjected to Western blotting with anti-(integrin $\alpha 7B$) antibody (bottom panel). In the autoradiogram, the 100-kDa heavy chain of $\alpha 7$ is shown; in Western blots, the light chains of $\alpha 7$ are visualized. (B) The intensities of the bands in (A) were determined by densitometry; the percentages of ³²P-labelled heavy chain of $\alpha 7$ (linked to either A or B light chain) and the A and B light chains (linked to either ³²P-labelled or non-labelled heavy chain) bound to the columns were calculated. Results are means $\pm$ S.E.M. for three determinations.

antibody recognizes an 'activation epitope' in the mouse integrin $\beta 1$ subunit located between residues 495 and 602 and induced in the presence of Mn^{2+} or ligand binding [30,31].

When a confluent layer of differentiating C2C12 cells was incubated with mAb 9EG7 in the absence of NAD⁺ and Mn^{2+} , relatively little binding of the antibody was detected (Figures 7A and 7B). In this case, most of the activated integrins were most likely to be located beneath the cells, where they were engaged in the interactions with ECM ligands (derived from serum or from cells) on the plates and thus were poorly accessible to the antibody. While pre-incubation of cells with 10 μ M NAD⁺ only slightly increased the subsequent binding of mAb 9EG7 (Figures 7C and 7D), pre-incubation with 100 μ M NAD⁺ in

Figure 7 Effects of ADP-ribosylation of integrin $\alpha 7$ on the expression of the 9EG7 epitope in intact differentiated C2C12 cells

Confluent C2C12 cells (4 days in differentiation medium) were incubated for 30 min in DPBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM ADP-ribose (A and B), or the same buffer supplemented with 10 μ M NAD⁺ (C and D) or 100 μ M NAD⁺ (E and F). Cells were then washed and incubated for 30 min in Tyrode's buffer containing 1 mM ADP-ribose and mAb 9EG7, followed by incubation with rhodamine-conjugated anti-rat IgG antibody. In (G) and (H), cells were incubated for 30 min in Tyrode's buffer containing 1 mM ADP-ribose and 1 mM $MnCl_2$, then for 30 min in the same buffer supplemented with mAb 9EG7, followed by incubation with the rhodamine-conjugated secondary antibody. Cells were then analysed by fluorescence microscopy to evaluate mAb 9EG7 immunostaining (B, D, F and H). (A), (C), (E) and (G) represent the phase-contrast images of the fields shown in (B), (D), (F) and (H) respectively. Representative microscopic fields are shown. Notice that the immunostaining of differentiated myotubes is more pronounced in (F) than in (B) and (D).

the absence of Mn^{2+} resulted in a striking enhancement of the immunostaining (Figures 7E and 7F). Importantly, the activation epitopes recognized by the 9EG7 antibody were present exclusively on differentiated myotubes and not on surrounding non-differentiated myoblasts (Figures 7E and 7F). Interestingly, the immunostaining of differentiated myotubes with mAb 9EG7 after pre-incubation with 100 μ M NAD⁺ was more pronounced and more clearly restricted to myotubes than the staining observed in the presence of Mn^{2+} (Figures 7G and 7H). As the strength of Mn^{2+} -induced epitopes detected by 9EG7 is highly divergent among different $\beta 1$ integrins [31], the relatively low level of

Mn²⁺-induced 9EG7 epitopes is most likely to be determined by the set of integrins expressed in C2C12 cells.

We propose the following interpretation of the results shown in Figure 7. In the presence of 10 μ M NAD⁺, ADP-ribosylation occurring exclusively at the central 37-kDa fragment of $\alpha 7$ might have induced an active conformation of integrin $\alpha 7 \beta 1$, but the number of modified integrin molecules ($\sim 5\%$, see Figure 5) was probably too low to detect the activation epitopes by immunostaining. In the presence of 100 μ M NAD⁺, the overall number of integrin $\alpha 7$ molecules undergoing ADP-ribosylation increased, and the 63-kDa N-terminal domain, in addition to the 37-kDa fragment, was modified. As the stoichiometry of modification reached ~ 0.4 mol of ADP-ribose/mol of $\alpha 7$ (see Figure 5), the number of ADP-ribosylated $\alpha 7$ molecules was at least 20% (assuming that each of the $\alpha 7$ molecules modified at the 37-kDa fragment contained also a modification at the 63-kDa fragment). Since pre-incubation with 100 μ M NAD⁺ strongly enhanced the binding of mAb 9EG7 to myotubes (Figures 7E and 7F), we presume that the number of activated integrin $\alpha 7 \beta 1$ molecules (containing the ADP-ribosylated $\alpha 7$ subunit) was now detectable by immunostaining. Interestingly, although ADP-ribosylation of the 63-kDa domain of integrin $\alpha 7$ prevented the binding of integrin $\alpha 7 \beta 1$ to laminin *in vitro* in the presence of Mn²⁺ (Figures 3–5), modification of the 63-kDa apparently did not interfere with the expression of the activation epitope detected by 9EG7. Collectively, the results shown in Figure 7 suggest that ADP-ribosylation of integrin $\alpha 7$ modulates the activation status of integrin $\alpha 7 \beta 1$ (defined by the expression of the 9EG7 epitope) in intact myotubes and, in contrast with the conditions present *in vitro*, this modulation does not require Mn²⁺ ions.

DISCUSSION

Eukaryotic arginine-specific ARTs represent an intriguing class of ecto-enzymes that utilize NAD⁺, a typical intracellular metabolite, to exert a post-translational modification of cell-surface or secreted proteins. Despite of a potential role of extracellular ADP-ribosylation in tissue damage and/or regeneration, very little is known about the effect of this post-translational modification on the structure and function of ADP-ribosylated proteins. Integrin $\alpha 7 \beta 1$ in differentiated mouse myoblasts [13,14], integrin $\alpha L \beta 2$ (LFA-1), CD8, CD27, CD43, CD44 and CD45 in mouse T-cells [21,22], and defensin-1 in bronchoalveolar lavage fluid [32], have been reported to undergo ecto-ADP-ribosylation. In the case of defensin, it appears that ADP-ribosylation decreases its antimicrobial and cytotoxic activities [32]. The effect of ADP-ribosylation on the function of integrins or other cell-surface molecules has not been explored. In the present paper, we report that ADP-ribosylation of integrin $\alpha 7$ modulates the binding of integrin $\alpha 7 \beta 1$ to laminin. Modification in the central 37-kDa domain increases the binding of the $\alpha 7 \beta 1$ dimer to laminin *in vitro* in the presence of Mn²⁺, whereas additional ADP-ribosylation of the 67-kDa N-terminal domain of $\alpha 7$ leads to inhibition of laminin binding. Moreover, ADP-ribosylation of integrin $\alpha 7$ increases the level of 9EG7 epitopes in intact myotubes, and this effect does not require Mn²⁺ ions.

How can ADP-ribosylation of the $\alpha 7$ subunit modulate the activation status of integrin $\alpha 7 \beta 1$ and its affinity for laminin? The answer to this question may be postulated based on recent structural studies of several integrin dimers and current models of integrin activation [23–25]. Extracellular domains of integrins are composed of a globular N-terminal head domain containing the ligand-binding site and two long, flexible stalks that connect

to the transmembrane and cytoplasmic domains. Integrins can exist in several conformational states with different ligand binding affinities. An 'inactive' conformation, with the stalks of the heterodimer bent in the middle and the headpiece facing towards the membrane, has the lowest ligand-binding affinity. An 'active' conformation, with straightened integrin stalks and an open headpiece, has the highest ligand-binding affinity. Several intermediate conformations may exist that have intermediate ligand-binding affinities [23–25]. Integrin activation is achieved by shifting the equilibrium from the inactive state towards more active conformations. An inherent feature of this model is that induction of the high-affinity state and straightening of the integrin stalks is intrinsically coupled to the separation of the stalks, transmembrane domains and cytoplasmic tails. A transition from the bent conformer to the extended conformers is usually initiated by ligand-binding events in the extracellular domains ('outside-in' signalling) or by protein–protein interactions mediated by the cytoplasmic tails ('inside-out' signalling).

Although the structure of integrin $\alpha 7 \beta 1$ is not known, the activation of $\alpha 7 \beta 1$ probably includes similar structural rearrangements. At low NAD⁺ concentrations (≤ 10 μ M), ADP-ribosylation involves an arginine residue located in the 37-kDa central region of $\alpha 7$ corresponding to the integrin stalk. If this residue normally participates in the electrostatic interaction with a negatively charged residue juxtaposed on the $\beta 1$ integrin chain, ADP-ribosylation, which introduces a negative charge at the arginine site, could lead to repulsion of the $\alpha 7$ and $\beta 1$ stalks and could induce an extended, high-affinity conformer. Increasing NAD⁺ concentration leads, however, to ADP-ribosylation of additional site(s) located in the 63-kDa N-terminal fragment of integrin $\alpha 7$. Since the 63-kDa fragment comprises the integrin headpiece where the ligand-binding site is located, ADP-ribosylation of arginine residue(s) in this region may result in steric hindrance and direct inhibition of ligand binding (seen in Figures 3–5), but may not abolish the activation epitope recognized by mAb 9EG7 (Figure 7).

We observed that ADP-ribosylation of the 37-kDa middle region of integrin $\alpha 7$ resulted in increased *in vitro* binding of the $\alpha 7 \beta 1$ dimer to laminin only in the presence of Mn²⁺. When Mn²⁺ was replaced with Mg²⁺, ADP-ribosylation of the 37-kDa $\alpha 7$ fragment did not have any effect on laminin binding. Mn²⁺ is an integrin 'activator' which shifts the equilibrium from the inactive towards more active conformations. In contrast, Ca²⁺, and, to a lesser extent, Mg²⁺, stabilizes the bent, inactive conformation [24,25,33]. It has to be stressed, however, that, although Mn²⁺ clearly causes conformational rearrangements in the extracellular integrin domains, Mn²⁺ alone does not seem to be sufficient for a full separation of integrin stalks, transmembrane segments and cytoplasmic domains [24,25,34]. Usually, the presence of integrin ligands or activating antibodies, in addition to Mn²⁺, are required for induction of the fully active conformer *in vitro*. Similarly, ADP-ribosylation in the integrin $\alpha 7$ stalk region may co-operate with the rearrangements caused by Mn²⁺ in the induction of the high-ligand-binding-affinity state. Notably, induction of the 9EG7 epitope by ADP-ribosylation in intact myotubes (Figure 7) occurred in the absence of Mn²⁺, which may highlight the physiological significance of ADP-ribosylation *in vivo*.

Increased binding to laminin after ADP-ribosylation of integrin $\alpha 7$ in the stalk region is consistent with recent studies showing that manipulations that move apart the C-terminal regions of the extracellular domains lead to activation of integrin $\alpha 5 \beta 1$ [35], $\alpha M \beta 2$ [36] and $\alpha X \beta 2$ [37]. Similarly, mutations leading to the separation of transmembrane segments of integrin $\beta 3$ cause increased activation of integrin $\alpha II \beta 3$ [38]. ADP-ribosylation, which represents a post-translational modification by an enzyme

that is endogenously expressed in muscle, may provide a natural mechanism for modulating the conformation of integrin stalks. In line with our findings on the role of ADP-ribosylation, several other studies have suggested recently that post-translational modification of integrin subunits by N-linked glycosylation may represent an important regulatory mechanism of integrin activation [39–41].

Since NAD⁺ serves as an obligatory substrate in ADP-ribosylation reactions, this mechanism may play a role in the regulation of integrin $\alpha 7 \beta 1$ function in a situation where tissue damage has occurred and NAD⁺ has leaked out from the cytosol to the extracellular compartment. Interestingly, integrin $\alpha 7 \beta 1$ can partially substitute for the dystroglycan–dystrophin complex in linking muscle fibres to basal lamina [42–46]. Thus up-regulation of integrin $\alpha 7 \beta 1$ binding to laminin by ADP-ribosylation of the $\alpha 7$ chain in the stalk region may contribute to stabilization of myofibres when the function of the dystrophin–dystroglycan complex is compromised in conditions such as muscular dystrophy [47]. The most important questions that need to be addressed in the future are whether the loss of myofibre integrity in diseased muscle leads to ADP-ribosylation of integrin $\alpha 7$ *in vivo*, and whether such a modification is robust enough to account for a noticeably increased ligand-binding affinity of $\alpha 7 \beta 1$.

This work was supported by the NIH (National Institutes of Health) COBRE (Centers of Biomedical Research Excellence) Award P20 RR17708 and matching support from the State of Kansas. This is contribution 04-322J from the Kansas Agricultural Experiment Station.

REFERENCES

- 1 Corda, D. and Di Girolamo, M. (2003) Functional aspects of protein mono-ADP-ribosylation. *EMBO J.* **22**, 1953–1958
- 2 Corda, D. and Di Girolamo, M. (2002) Mono-ADP-ribosylation: a tool for modulating immune response and cell signaling. *Sci STKE* 2002, PE53
- 3 Barbieri, J. T. (2000) *Pseudomonas aeruginosa* exoenzyme S, a bifunctional type-III secreted cytotoxin. *Int. J. Med. Microbiol.* **290**, 381–387
- 4 Lerm, M., Schmidt, G. and Aktories, K. (2000) Bacterial protein toxins targeting rho GTPases. *FEMS Microbiol. Lett.* **188**, 1–6
- 5 Lesnick, M. L. and Guiney, D. G. (2001) The best defense is a good offense – *Salmonella* deploys an ADP-ribosylating toxin. *Trends Microbiol.* **9**, 2–4
- 6 Zolkiewska, A., Nightingale, M. S. and Moss, J. (1992) Molecular characterization of NAD:arginine ADP-ribosyltransferase from rabbit skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11352–11356
- 7 Okazaki, I. J., Zolkiewska, A., Nightingale, M. S. and Moss, J. (1994) Immunological and structural conservation of mammalian skeletal muscle glycosylphosphatidylinositol-linked ADP-ribosyltransferases. *Biochemistry* **33**, 12828–12836
- 8 Koch-Nolte, F., Haag, F., Braren, R., Kuhl, M., Hoovers, J., Balasubramanian, S., Bazan, F. and Thiele, H. G. (1997) Two novel human members of an emerging mammalian gene family related to mono-ADP-ribosylating bacterial toxins. *Genomics* **39**, 370–376
- 9 Koch-Nolte, F. and Haag, F. (1997) Mono(ADP-ribosyl)transferases and related enzymes in animal tissues: emerging gene families. *Adv. Exp. Med. Biol.* **419**, 1–13
- 10 Glowacki, G., Braren, R., Cetkovic-Cvrlje, M., Leiter, E. H., Haag, F. and Koch-Nolte, F. (2001) Structure, chromosomal localization, and expression of the gene for mouse ecto-mono(ADP-ribosyl)transferase ART5. *Gene* **275**, 267–277
- 11 Glowacki, G., Braren, R., Firner, K., Nissen, M., Kuhl, M., Reche, P., Bazan, F., Cetkovic-Cvrlje, M., Leiter, E., Haag, F. and Koch-Nolte, F. (2002) The family of toxin-related ecto-ADP-ribosyltransferases in humans and the mouse. *Protein Sci.* **11**, 1657–1670
- 12 Okazaki, I. J. and Moss, J. (1999) Characterization of glycosylphosphatidylinositol-anchored, secreted, and intracellular vertebrate mono-ADP-ribosyltransferases. *Annu. Rev. Nutr.* **19**, 485–509
- 13 Zolkiewska, A. and Moss, J. (1993) Integrin $\alpha 7$ as substrate for a glycosylphosphatidylinositol-anchored ADP-ribosyltransferase on the surface of skeletal muscle cells. *J. Biol. Chem.* **268**, 25273–25276
- 14 Zolkiewska, A. and Moss, J. (1995) Processing of ADP-ribosylated integrin $\alpha 7$ in skeletal muscle myotubes. *J. Biol. Chem.* **270**, 9227–9233
- 15 Burkin, D. J. and Kaufman, S. J. (1999) The $\alpha 7 \beta 1$ integrin in muscle development and disease. *Cell Tissue Res.* **296**, 183–190
- 16 Song, W. K., Wang, W., Sato, H., Bielser, D. A. and Kaufman, S. J. (1993) Expression of $\alpha 7$ integrin cytoplasmic domains during skeletal muscle development: alternate forms, conformational change, and homologies with serine/threonine kinases and tyrosine phosphatases. *J. Cell Sci.* **106**, 1139–1152
- 17 Ziober, B. L., Vu, M. P., Waleh, N., Crawford, J., Lin, C. S. and Kramer, R. H. (1993) Alternative extracellular and cytoplasmic domains of the integrin $\alpha 7$ subunit are differentially expressed during development. *J. Biol. Chem.* **268**, 26773–26783
- 18 Collo, G., Starr, L. and Quaranta, V. (1993) A new isoform of the laminin receptor integrin $\alpha 7 \beta 1$ is developmentally regulated in skeletal muscle. *J. Biol. Chem.* **268**, 19019–19024
- 19 von der Mark, H., Dürr, J., Sonnenberg, A., von der Mark, K., Deutzmann, R. and Goodman, S. L. (1991) Skeletal myoblasts utilize a novel $\beta 1$ -series integrin and not $\alpha 6 \beta 1$ for binding to the E8 and T8 fragments of laminin. *J. Biol. Chem.* **266**, 23593–23601
- 20 Song, W. K., Wang, W., Foster, R. F., Bielser, D. A. and Kaufman, S. J. (1992) H36- $\alpha 7$ is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. *J. Cell Biol.* **117**, 643–657
- 21 Nemoto, E., Yu, Y. and Dennert, G. (1996) Cell surface ADP-ribosyltransferase regulates lymphocyte function-associated molecule-1 (LFA-1) function in T cells. *J. Immunol.* **157**, 3341–3349
- 22 Okamoto, S., Azhipa, O., Yu, Y., Russo, E. and Dennert, G. (1998) Expression of ADP-ribosyltransferase on normal T lymphocytes and effects of nicotinamide adenine dinucleotide on their function. *J. Immunol.* **160**, 4190–4198
- 23 Liddington, R. C. and Ginsberg, M. H. (2002) Integrin activation takes shape. *J. Cell Biol.* **158**, 833–839
- 24 Shimaoka, M., Takagi, J. and Springer, T. A. (2002) Conformational regulation of integrin structure and function. *Annu. Rev. Biophys. Biomol. Struct.* **31**, 485–516
- 25 Humphries, M. J., McEwan, P. A., Barton, S. J., Buckley, P. A., Bella, J. and Mould, P. A. (2003) Integrin structure: heady advances in ligand binding, but activation still makes the knees wobble. *Trends Biochem. Sci.* **28**, 313–320
- 26 Crawley, S., Farrell, E. M., Wang, W., Gu, M., Huang, H.-Y., Huynh, V., Hodges, B. L., Cooper, D. N. W. and Kaufman, S. J. (1997) The $\alpha 7 \beta 1$ integrin mediates adhesion and migration of skeletal myoblasts on laminin. *Exp. Cell Res.* **235**, 274–286
- 27 Carnac, G., Fajas, L., L'honore, A., Sardet, C., Lamb, N. J. and Fernandez, A. (2000) The retinoblastoma-like protein p130 is involved in the determination of reserve cells in differentiating myoblasts. *Curr. Biol.* **10**, 543–546
- 28 Schöber, S., Mielenz, D., Echtermeyer, F., Hapke, S., Pöschl, E., von der Mark, H., Moch, H. and von der Mark, K. (2000) The role of extracellular and cytoplasmic splice domains of $\alpha 7$ -integrin in cell adhesion and migration on laminins. *Exp. Cell Res.* **255**, 303–313
- 29 von der Mark, H., Williams, I., Wendler, O., Sorokin, L., von der Mark, K. and Pöschl, E. (2002) Alternative splice variants of $\alpha 7 \beta 1$ integrin selectively recognize different laminin isoforms. *J. Biol. Chem.* **277**, 6012–6016
- 30 Bazzoni, G., Shih, D. T., Buck, C. A. and Hemler, M. E. (1995) Monoclonal antibody 9EG7 defines a novel $\beta 1$ integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. *J. Biol. Chem.* **270**, 25570–25577
- 31 Bazzoni, G., Ma, L., Blue, M. L. and Hemler, M. E. (1998) Divalent cations and ligands induce conformational changes that are highly divergent among $\beta 1$ integrins. *J. Biol. Chem.* **273**, 6670–6678
- 32 Paone, G., Wada, A., Stevens, L. A., Matin, A., Hirayama, T., Levine, R. L. and Moss, J. (2002) ADP ribosylation of human neutrophil peptide-1 regulates its biological properties. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 8231–8235
- 33 Plow, E. F., Haas, T. A., Zhang, L., Loftus, J. and Smith, J. W. (2000) Ligand binding to integrins. *J. Biol. Chem.* **275**, 21785–21788
- 34 Kim, M., Carman, C. V. and Springer, T. A. (2003) Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* **301**, 1720–1725
- 35 Takagi, J., Erickson, H. P. and Springer, T. A. (2001) C-terminal opening mimics 'inside-out' activation of integrin $\alpha 5 \beta 1$. *Nat. Struct. Biol.* **8**, 412–416
- 36 Xiong, Y. M., Chen, J. and Zhang, L. (2003) Modulation of CD11b/CD18 adhesive activity by its extracellular, membrane-proximal regions. *J. Immunol.* **171**, 1042–1050
- 37 Zang, Q. and Springer, T. A. (2001) Amino acid residues in the PSI domain and cysteine-rich repeats of the integrin $\beta 2$ subunit that restrain activation of the integrin $\alpha X \beta 2$. *J. Biol. Chem.* **276**, 6922–6929
- 38 Li, R., Mitra, N., Gratkowski, H., Vilaire, G., Litvinov, R., Nagasami, C., Weisel, J. W., Lear, J. D., DeGrado, W. F. and Bennett, J. S. (2003) Activation of integrin $\alpha IIb \beta 3$ by modulation of transmembrane helix associations. *Science* **300**, 795–798
- 39 Luo, B. H., Springer, T. A. and Takagi, J. (2003) Stabilizing the open conformation of the integrin headpiece with a glycan wedge increases affinity for ligand. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2403–2408

-
- 40 Isaji, T., Gu, J., Nishiuchi, R., Zhao, Y., Takahashi, M., Miyoshi, E., Honke, K., Sekiguchi, K. and Taniguchi, N. (2004) Introduction of bisecting GlcNAc into integrin $\alpha 5 \beta 1$ reduces ligand binding and down-regulates cell adhesion and cell migration. *J. Biol. Chem.* **279**, 19747–19754
- 41 Bellis, S. L. (2004) Variant glycosylation: an underappreciated regulatory mechanism for $\beta 1$ integrins. *Biochim. Biophys. Acta* **1663**, 52–60
- 42 Kaariainen, M., Kaariainen, J., Jarvinen, T. L., Nissinen, L., Heino, J., Jarvinen, M. and Kalimo, H. (2000) Integrin and dystrophin associated adhesion protein complexes during regeneration of shearing-type muscle injury. *Neuromuscular Disord.* **10**, 121–132
- 43 Kaariainen, M., Liljamo, T., Pelto-Huikko, M., Heino, J., Jarvinen, M. and Kalimo, H. (2001) Regulation of $\alpha 7$ integrin by mechanical stress during skeletal muscle regeneration. *Neuromuscular Disord.* **11**, 360–369
- 44 Kaariainen, M., Nissinen, L., Kaufman, S., Sonnenberg, A., Jarvinen, M., Heino, J. and Kalimo, H. (2002) Expression of $\alpha 7 \beta 1$ integrin splicing variants during skeletal muscle regeneration. *Am. J. Pathol.* **161**, 1023–1031
- 45 Hodges, B. L., Hayashi, Y. K., Nonaka, I., Wang, W., Arahata, K. and Kaufman, S. J. (1997) Altered expression of the $\alpha 7 \beta 1$ integrin in human and murine muscular dystrophies. *J. Cell Sci.* **110**, 2873–2881
- 46 Burkin, D. J., Wallace, G. Q., Nicol, K. J., Kaufman, D. J. and Kaufman, S. J. (2001) Enhanced expression of the $\alpha 7 \beta 1$ integrin reduces muscular dystrophy and restores viability in dystrophic mice. *J. Cell Biol.* **152**, 1207–1218
- 47 Straub, V., Rafael, J. A., Chamberlain, J. S. and Campbell, K. P. (1997) Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. *J. Cell Biol.* **139**, 375–385

Received 8 April 2004/17 August 2004; accepted 13 September 2004

Published as BJ Immediate Publication 13 September 2004, DOI 10.1042/BJ20040590