Evidence for the presence of a low-mass β1 integrin on the cell surface

Xiaobo Meng[1,2], Keding Cheng[1,2], Oleg Krohkin[1,3], A. Paul Mould[4], Martin J. Humphries[4], Werner Ens[1,3], Kenneth Standing[1,3] and John A. Wilkins[1,2,*]

[1]Manitoba Centre for Proteomics and Systems Biology and [2]Rheumatic Diseases Research Laboratory, Department of Internal Medicine, University of Manitoba, 715 McDermot Avenue, Winnipeg, MB, Canada R3E 3P4
[3]Time of Flight Laboratory, Department of Physics and Astronomy, University of Manitoba, Winnipeg, MB, Canada R3T 2N2
[4]The Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK
*Author for correspondence (e-mail: jwilkin@cc.umanitoba.ca)

Accepted 6 June 2005
Journal of Cell Science 118, 4009-4016 Published by The Company of Biologists 2005
doi:10.1242/jcs.02520

Summary

Although the cell line K562 reportedly expresses a single species of β1 integrin, α5β1, surface staining with monoclonal antibodies JB1A, 12G10 and B3B11 to the β1 chain clearly demonstrated differences in the expression levels of the epitopes detected by these antibodies. The present studies were initiated to determine the basis for this molecular heterogeneity in the integrins. Cross-linking of surface integrins with B3B11 caused their selective aggregation. This distribution was similar to that observed for the α5 chain. In contrast, cross-linking the β1 chains with 12G10 did not cause codistribution of α5, suggesting that these two species were not associated on the cell surface. Immunoprecipitates of the surface integrins of K562 cells indicated the presence of 120 and 140 kDa forms of the β1 chain which were detected by 12G10 and B3B11, respectively. Immunological, biochemical and mass spectrometric analysis of K562 surface integrins also failed to demonstrate the presence of any α chain in association with the 120 kDa species of β1 of K562 cells. Treatment of the two forms of β1 with PGNase reduced their masses to ~90 kDa, suggesting that *N*-glycosylation was responsible for the mass differences. Collectively, these results provide evidence for a novel species of β1 on the cell surface, which does not appear to be associated with any α chain. The data also suggest that differences in glycosylation may be involved in defining the association between the integrin α and β chains and the functional properties of these integrins.

Key words: β1 integrin, 12G10, Glycosylation, Monomer, Mass spectrometry

Introduction

Integrins constitute a large family of proteins composed of noncovalently associated α and β heterodimers which play a role in cell signalling and adhesion (Giancotti, 2003; Humphries et al., 2003; Hynes, 2002). The αβ combination defines the binding specificity and signalling properties of the integrin complex. To date, 24 distinct integrins deriving from a combination of 18 α and 8 β subunits have been described. Integrins are often expressed on the cell surface in a latent form that can be activated by external or intracellular stimuli (Schwartz, 2001). Changes in conformation, protrusion from the membrane and aggregation have been reported to occur in association with activation (Blystone, 2004). Implying that there are multiple changes associated with transition to the high affinity state.

The interactions between α and β cytoplasmic domains have been implicated in the regulation of integrin activity. Deletion of the cytoplasmic domain of the α chains results in a constitutively active integrin (O'Toole et al., 1994). Engineered constraints have demonstrated the ability of the cytoplasmic tails of the integrins to regulate integrin function (Lu and Springer, 1997; Luo et al., 2004). More recently, it has been suggested that homotypic molecular interactions between α or β chains may play a role in the aggregation of the heterodimers on the cell surface as a component step in acquiring a fully adhesive state (Gottschalk and Kessler, 2004).

Monoclonal antibodies have been used extensively to determine integrin expression on the cell surface and it has been suggested that some antibodies may report on functional status or ligand occupancy (Frelinger, III et al., 1991; Mould et al., 1995; Wilkins et al., 1996; Bazzoni and Hemler, 1998; Bazzoni et al., 1998). However, the distinctive patterns of reactivity of such antibodies, particularly on cell types expressing a single integrin species, suggests that there may be considerable heterogeneity in the integrins on a given cell. This could be attributed to epitope accessibility as a result of different protein associations or because of differences in conformation of the integrins. The present studies were undertaken to determine the basis for integrin microheterogeneity on the K562 line, a cell type reported to express a single species of integrin. Evidence is presented for the existence of two species of integrin β1 chains on the cell surface with distinctive interaction properties with integrin α5 chain.

Materials and Methods

Materials

Anti-β1 integrin monoclonal antibodies B3B11 (Wilkins, 1998; Li et al., 2001; Ni et al., 1998; Stupack et al., 1991; Shen, 1996), JB1A (Ni and Wilkins, 1998), and anti-α5 integrin monoclonal antibody JBS5 were produced in this laboratory. Anti-β1 monoclonal antibody 12G10 has been described previously (Mould et al., 1995). Rabbit polyclonal anti-integrin α5 (H-104) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to α1, α2, α3, α4, α5, α6, and αV were purchased from Chemicon. Horseradish peroxide-conjugated goat anti-mouse immunoglobulin, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and FITC-conjugated goat anti-mouse immunoglobulin were purchased from Sigma-Aldrich (St Louis, MI, USA). Cy3-conjugated goat anti-mouse immunoglobulin was the product of Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Oregon Green 488-conjugated goat anti-rabbit immunoglobulin was bought from Molecular Probes (Eugene, OR, USA). Purified human fibronectin alpha-chymotryptic fragment 120 kDa was purchased from Chemicon (Temecula, CA, USA). Protease inhibitor cocktail and endoglycosidase F were from Roche (Mannheim, Germany). Protein G Sepharose 4 fast flow beads was from Amersham Biosciences (Piscataway, NJ, USA).

Cell culture

The human erytholeukemia cell line K562 (ATCC, Rockville, MD, USA) was maintained in RPMI 1640 medium with 10% fetal bovine serum. The cells were washed and cultured in AIM-V serum-free medium for 12 hours prior to analysis.

The cell lines, Jurkat, HT1080 and MDA, were purchased from ATCC and maintained in RPMI 1640 medium with 10% fetal bovine serum. These cells were not cultured in serum-free medium prior to analysis.

Replication

All experiments were performed at least three times with the exception of the mass spectrometry which was performed twice.

Flow cytometry and immunocytochemistry

K562 cells were incubated with primary anti-integrin antibody (10 μg/ml) at room temperature. FITC-conjugated secondary antibody was applied and the fluorescence was analysed using a BD FACScaliber. To examine the colocalization of the integrin α5 and β subunits, antibody-mediated cross-linking experiments were performed. K562 cells were incubated with the primary anti-β1 antibody at room temperature for 30 minutes. A Cy3-conjugated secondary antibody was applied to cross-link the first antibody and the associated integrins. After fixing with freshly prepared 4% paraformaldehyde, rabbit anti-α5 integrin polyclonal antibody and Oregon Green 488-conjugated goat anti-rabbit secondary antibodies were used to detect the integrin α subunit.

12G10 was biotinylated with Sulfo-NHS-LC-Biotin (Pierce Chemicals) according to the manufacturer's instructions.

Immunofluorescence was analysed with an Olympus BX60 microscope equipped with a xenon lamp and a light pipe. Images where captured using a Cooke SensiCam with either a 60× oil immersion lens with a 1.25 NA or a 100× oil immersion lens with a 1.30 NA and processed with Image Pro software.

Immunoprecipitation

The serum-free-cultured cells were incubated with the indicated anti-integrin antibody for 30 minutes at room temperature. The ratio of antibody to cells was adjusted to ensure that the antibody on the cell surface was saturated. Cells were washed three times with PBS to remove unbound antibody. Cell lysates were prepared by adding lysis buffer (1% Triton X-100 in PBS, protease inhibitor cocktail, 1 mM Na_3VO_4, 10 mM NaF) for 30 minutes. The supernatants were collected after centrifugation at 12,000 ***g*** for 10 minutes. The integrins were immunoprecipitated by mixing with protein G Sepharose beads for 2 hours, and then washed. The samples were subjected to 8% SDS-PAGE and proteins were transferred to nitrocellulose membranes. Immunoblotting was carried out with the specified primary antibody and HRP or alkaline phosphatase-conjugated second antibodies.

Deglycosylation

The protein complexes isolated by immunoprecipitation were treated with *N*-glycosidase F (Roche). The samples were boiled in 20 μl of denaturing buffer (1% SDS, 1% 2-mercaptoethanol) for 2 minutes. The samples were incubated for 2 hours at 37°C with 1 unit *N*-glycosidase F (in 18 μl 0.5% Triton X-100) after which the reaction was terminated by adding SDS-PAGE sample buffer. The samples were subjected to 8% SDS-PAGE, and analysed with the indicated antibodies by western blotting.

In-gel trypsin digestion

Immunoprecipitates were separated using 8% SDS-PAGE and stained with GelCode blue stain reagent (Pierce, Rockford IL, USA). In-gel trypsin digestion was performed as previously described (Dasuri et al., 2004). Briefly the protein bands were excised, minced and shrunk by dehydration in acetonitrile. The gel was treated with 10 mM DTT in 100 mM NH_4HCO_3 for 1 hour at 56°C and the DTT solution was replaced with 100 mM iodoacetamide in the same buffer. The alkylation was carried out at room temperature for 45 minutes in the dark. The gel pieces were washed with NH_4HCO_3 and dehydrated with acetonitrile. The gel pieces were rehydrated in digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, 12.5 μg/ml trypsin and incubated overnight at 37°C. The peptides were extracted and vacuum dried.

Mass spectrometry analysis

The peptides was dissolved in 0.1% TFA (trifluoroacetic acid) and analysed by matrix assisted laser desorption ionization (MALDI) on a QqTOF mass spectrometry (Shevchenko et al., 2000). Spectra were analysed using the Knexus automation client and the Profound search engine as previously described (Weiler et al., 2003) against the NCBI nonredundant mammalian database. Search parameters included modification of cysteines with iodoacetamide, partial methionine oxidation and one missed trypsin cleavage with a monoisotopic mass accuracy of 20 p.p.m. On-line nano liquid-chromatography-coupled electrospray ionization (LC ESI) was performed with a Qstar (Applied Biosystems) equipped with an LC Packings nano LC.

Results

Characterization of β1 integrin species on the surface of K562 cells

Analysis of the binding patterns of a panel of monoclonal antibodies to the β1 chain (Fig. 1A) revealed marked differences in the relative levels of staining with these antibodies. The epitope recognized by JB1A appears to be accessible on the greatest number of integrins while those detected by 12G10 and B3B11 are present at 33.9% and 83.9% of the levels detected by JB1A. For these studies the antibodies were used at saturating concentrations to ensure maximal

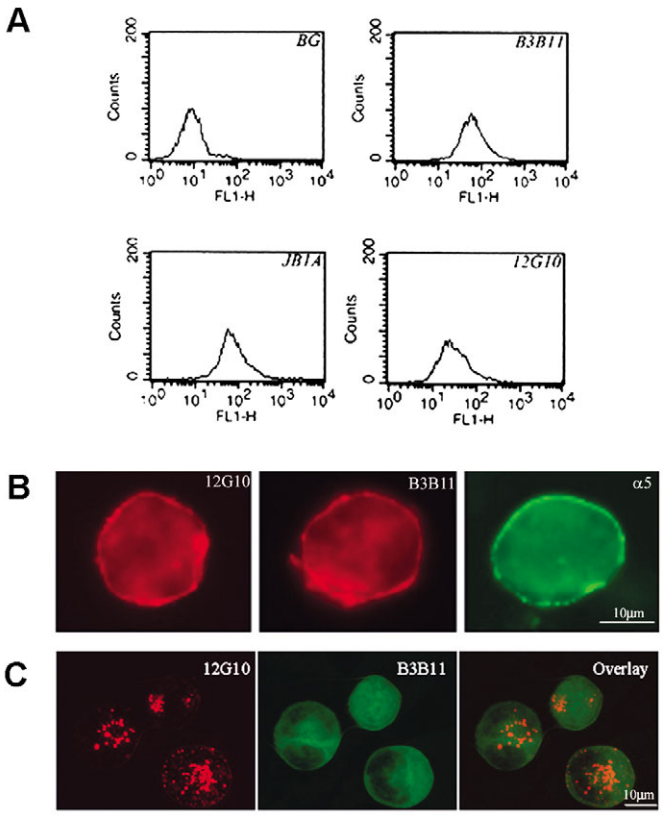

Fig. 1. (A) Differential expression of β1 integrin epitopes on K562 Cells. Cells were grown in serum-free medium and either stained with JB1A, B3B11 or 12G10, or reacted with only the secondary antibody (BG). The cells were analysed by flow cytometry. The geometric means of the expression values for each category were BG 8. 27, 12G10 29. 97, B3B11 61. 95 and JB1A 72. 27. (B) Distribution of β1 integrin species identified by B3B11 or 12G10. K562 cells were fixed in 4% paraformaldehyde, stained with the indicated antibodies and visualized with Cy3-conjugated goat anti-mouse immunoglobulin. The cells were stained with a polyclonal antibody α5 and stained with an Oregon Green goat anti-rabbit immunoglobulin. (C) 12G10-reactive β1 does not codistribute with B3B11 reactive β1. Cells were reacted with 12G10 and the bound integrins were cross-linked with Cy3-coupled goat anti-mouse immunoglobulin. The cells were fixed and blocked with mouse immunoglobulin to saturate the combining sites on the anti-mouse immunoglobulin. The cells were then reacted with biotin-labelled B3B11 and the bound antibody was detected with an FITC-labelled Avidin conjugate. 12G10 labelled integrins were clustered; in contrast B3B1ll integrins were diffusely distributed. The overlay of the two images shows that there was little or no colocalization of the integrins detected by these two antibodies.

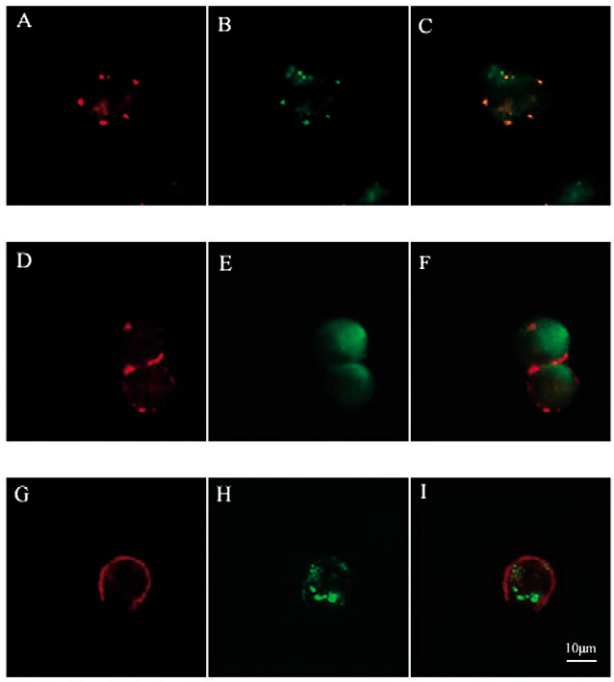

Fig. 2. Differential distribution of the integrin α5 chain and 12G10 reactive β1 chains on the cell surface. K562 cells were incubated with B3B11 antibody (A-C) or 12G10 (D-F), washed, and a Cy3 (red)-conjugated anti-mouse immunoglobulin second antibody was applied to trigger aggregation of the antibody-reactive β1 species. The cells were fixed and stained with a polyclonal anti-α5, followed by Oregon Green-conjugated second antibody (A-F). A representative double stained cell was selected and photographed. (A) B3B11, (B) α5, (C) overlay of A and B. (D) 12G10, (E) α5, (F) overlay of D and E. (G-I) α5 integrins of K562 cells were cross-linked with polyclonal anti-α5 and Oregon Green 488-conjugated secondary antibody. The cells were fixed and stained with 12G10 plus Cy3-conjugated second antibody. (G) 12G10, (H) α5, (I) overlay of G and I.

staining of the cells. These results suggested that there was microheterogeneity in the β1 integrin pool on these cells as defined by antibodies 12G10 and B3B11.

As K562 cells reportedly express only α5β1 integrins (Faull et al., 1993; Hemler et al., 1987), a microscopic analysis of the distribution of integrins detected by B3B11, 12G10 and anti-α5 was performed. The staining patterns for 12G10, B3B11 and anti-α5 were very similar with diffuse distribution on the cell surface (Fig. 1B). However, following cross-linking with 12G10, the integrins recognized by the two antibodies had completely different distributions. Those stained with 12G10 were clustered (Fig. 1C), while the B3B11 reactive integrins were diffusely distributed on the cell surface with no evidence of colocalization to the regions of 12G10 staining. These results suggested that the 12G10 and B3B11 epitopes were present in different sets of integrins on the surface of these cells. Furthermore, it suggested that the interaction with 12G10 did not induce the exposure of the B3B11 epitope.

The relationships between α5 and these β1 chain populations were also examined. In this case, the integrin species recognized by either 12G10 or B3B11 were cross-linked with a Cy3-labelled secondary antibody to mouse IgG. The cells were then fixed and stained with a polyclonal rabbit antibody to detect the α5 subunit. Cross linking with B3B11 resulted in extensive aggregation of the β1 subunits which colocalised with the distribution of α5 (Fig. 2A-C). Similarly cross-linking with 12G10 resulted in extensive aggregation of β1 on the cell surface (Fig. 2D). However, the α5 chains on these cells did not codistribute with 12G10-reactive integrins (Fig. 2E-F).

The converse experiment was performed in which the integrins on the cell surface were cross-linked with a

polyclonal antibody to α5. The cells were subsequently fixed, reacted with 12G10 and the distribution of the staining was determined. The 12G10 staining was diffuse (Fig. 2G), while the α5 chains were fully aggregated (Fig. 2H). There was little evidence of co-distribution of the 12G10-reactive β1 integrins and the α5 chains (Fig. 2I).

These results demonstrated that independent of the order of the cross-linking step (i.e. β1 or α5), there was no correlation between the staining patterns of 12G10-reactive surface β1 and α5. Thus 12G10 identifies a distinct subset of β1 integrins on the surface of K562 cells that does not co-distribute with α5. These results raised the possibility of the presence of another α chain associated with the 12G10-reactive integrins. However, analysis of the cells by FACS for the presence of α1, α2, α3, α4, α5, α6 and αV failed to reveal the presence of α chains other than α5 (Fig. 3).

Biochemical characterization of β1 integrins on K562 cells

The above results suggested that there were two species of β1 chain on the surface of K562 cells as defined by reactivity with 12G10 and B3B11. In order to examine these molecules, a method was developed to selectively capture cell surface integrins. The cells were exposed to subsaturating concentrations of antibodies to β1 (JB1A, 12G10 and B3B11) such that all of the antibody-combining sites were saturated with surface antigen. The cells were washed extensively to remove unbound immunoglobulin and subsequently lysed. The isolated integrins were then analysed by SDS-PAGE. As a direct test of the method, cells were preloaded with the antibodies, washed, lysed and biotinylated purified β1 integrin was added to the lysates. If there were free combining sites or if there was exchange of integrins with the antibodies during the isolation procedure then the biotinylated species should be captured and detectable in the immunoprecipitates by blotting with avidin–alkaline phosphatase conjugates. Using this approach there was no evidence of any capture of free integrin suggesting that only surface integrins were being captured (data not shown).

The separated integrins from the precipitates with JB1A, 12G110 and B3B11 were examined by western blot using JB1A. This antibody recognizes an epitope that appears to be accessible on all β1 chains, independent of the integrin activation state, thus providing a reporter for all β1 species (Ni and Wilkins, 1998). The JB1A precipitates of surface labelled

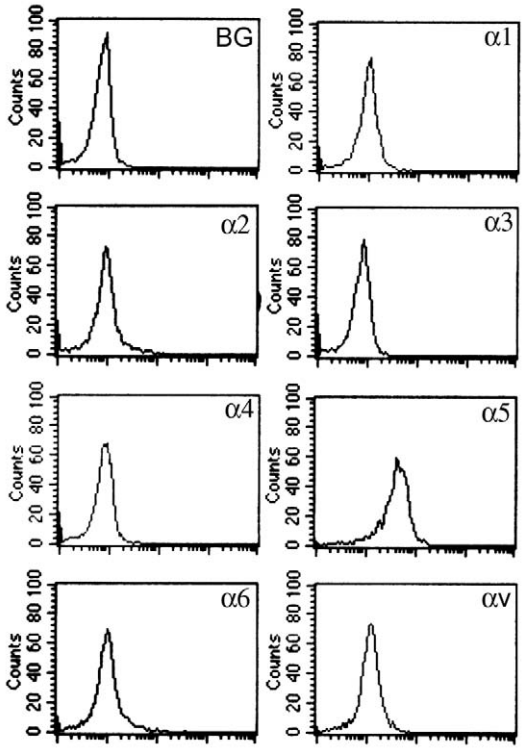

Fig. 3. The α chain expression pattern of K562 cells. Cells were surface labelled with the indicated α chain-specific antibodies or with secondary antibody only (BG) and analysed by flow cytometry. α5 was the only one of the integrins detected in significant levels on these cells. The activities of each of these antibodies were confirmed on control cell lines that expressed the corresponding integrins.

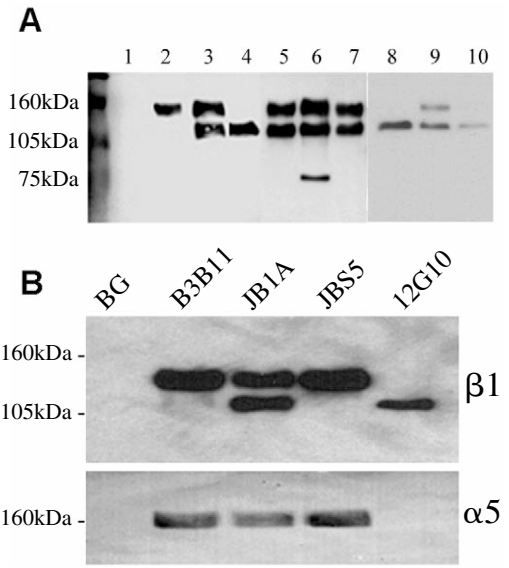

Fig. 4. (A) Distinct species of β1 chain on the cell surface. Lanes 1-4: K562 were surface labelled with different antibodies to β1 integrin (lane 1, control; 2, B3B11; 3, JB1A; 4, 12G10) and washed to remove free antibodies. The cells were lysed and the antibody-associated integrins were immunoprecipitated, separated by SDS-PAGE and immunoblotted with JB1A to detect total β1. Lanes 5-7: total cell lysates from untreated cells were prepared and incubated with antibodies for immunoprecipitation (lanes 5, B3B11; 6, JB1A; 7, 12G10). The samples were analysed as described above. Note that the patterns of capture are different for the surface-labelled and the cell lysate-derived integrins demonstrating the specificity of the cell surface capture. Lanes 8-10: cells were grown in serum-free medium and surface labelled with 12G10, lane 8; 12G10 in the presence of 120 kDa fragment of fibronectin, lane 9; or surface labelled with 12G10, washed and exposed to the 120 kDa fragment of fibronectin, lane 10. The bound integrins were isolated by immunoprecipitation and processed as described above. (B) Lack of association between α5 and the 12G10 reactive β1 subunits. Cell surface integrins were immunoprecipitated with antibodies to α5 with JBS5 or to β1, with B3B11, JB1A or 12G10. The precipitates were separated by SDS-PAGE and probed by western blot analysis with anti-β1, JB1A (upper panel) or a polyclonal anti-α5 (lower panel).

cells contained two species of β1 with molecular masses of 120 and 140 kDa (Fig. 4A, lane 3). Although B3B11 appeared to exclusively detect the 140 kDa form (Fig. 4A, lane 2), the 12G10 precipitates only contained the 120 kDa species (Fig. 4A, lane 4).

In contrast to the above situation, when cells were lysed and the antibodies were then added to the lysates the resulting immunoprecipitates with any of the antibodies contained both species of integrins (Fig. 4A, lanes 5-7). These results indicated that under some conditions both forms of integrin could express the epitopes recognized by each of these antibodies. Equally significantly, the differences in the patterns of integrins isolated from cell surface labelling as compared to the total lysates further supported the conclusion that the cell surface protocol was not capturing intracellular integrins.

Ligand binding can induce the 12G10 epitope and it was questioned whether this would result in capture of the 140 kDa species. Cells were cultured under serum-free conditions and surface labelled with 12G10 in the absence (Fig. 4A, lane 8) or presence (Fig. 4A, lane 9) of the 120 kDa fragment of fibronectin. Examination of the bound integrins indicated that in the presence of fibronectin the 140 kDa species was induced to express the 12G10 epitope. However, if the cells were surface labelled with 12G10 and washed before exposure to fibronectin only the 120 kDa species was observed (Fig. 4A, lane 10). These results demonstrated that the ligand binding induced 12G10 epitope accessibility on the 140 kDa species but binding of the ligand did not result in the conversion of the 120 kDa species to the 140 kDa form of β1.

The results of the microscope studies in the previous section suggested that 12G10 was not associated with α5. As a direct test of this possibility, surface integrins were captured with JB1A, 12G10, B3B11, or JBS5, an anti-α5 monoclonal (Stupack et al., 1991). The precipitates were then probed with either JB1A to determine the species of β1 or a polyclonal anti-α5 to detect α5. The JB1A and B3B11 precipitates contained the previously observed species of β1 and α5 chains (Fig. 4B). In the case of 12G10, the 120 kDa form of β1 was present but there was no evidence of an α5 chain. Once again these results suggested that this form of surface integrin was not associated with α5.

The FACS analysis had indicated that the K562 cell did not express significant levels of the α chains examined except for α5. However, these observations could not exclude the presence of other species of α chain. Cell surface integrins were isolated by immunoprecipitation with B3B11 or 12G10, separated by SDS-PAGE under reducing conditions and stained with Coomassie Blue. All visible bands were cut and digested in gel with trypsin followed by MALDI QqTOF analysis by both single (MS) and tandem (MSMS) mass spectrometry.

The B3B11 precipitates contained a single major band of ~140 kDa made up of the β1 and the heavy chain fragment of α5. There were also 50 kDa and 23-25 kDa bands that were the heavy and light chains of the B3B11 antibody (Fig. 5, lane 2). In the case of the 12G10, there was a single 120 kDa species that was identified as β1(Fig. 5, lane 3). There was no evidence of α5 or any other integrin species. The remaining bands were all identified as IgG components of 12G10. In another series of experiments the Coomassie Blue-stained protein bands from the SDS-PAGE-separated B3B11 and 12G10 isolates were in

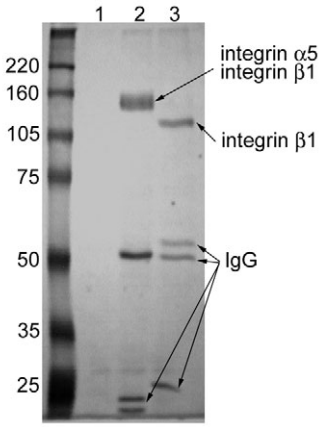

Fig. 5. Mass spectrometric analysis of β1 integrin-associated proteins on the cell surface. Cells were surface labelled with antibody (lane 1, control; lane 2, B3B11; lane 3, 12G10). The protein complexes were isolated and separated in 8% SDS-PAGE gels under reducing conditions and stained with Coomassie Blue. The visible protein bands were cut out for in-gel digestion and mass spectrometric analysis. The identified proteins are labelled and marked with arrows.

gel digested and the peptides analysed by nano-LC ESI MSMS. Once again, β1 was the only species of protein identified in the 12G10 precipitates (data not shown). These results suggested that the cell surface integrin β1, isolated by 12G10, was not associated with another unidentified protein.

The basis for the mass differences in the β1 chains was examined, as this appeared to correlate with the association properties of the α5 chain. Glycosylation was viewed as a potential mechanism that could account for the large mass differences observed for the β1 species. Thus the effects of deglycosylation on integrin size were examined.

Surface-associated integrins were purified from K562 cells with B3B11 or 12G10 and treated with PGNase F. The deglycosylated samples were separated by SDS-PAGE and analysed by western blotting using JB1A to detect the integrin chains (Fig. 6). The masses of both 12G10 and B3B11 β1 isolates decreased to ~90 kDa after deglycosylation. These

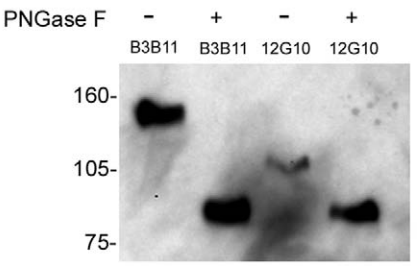

Fig. 6. (A) *N*-link glycosylation contributed to the mass loss. The surface integrins isolated with either B3B11 or 12G10 were either analysed directly (–) or treated (+) with PGNase F for 2 hours at 37°C. The integrins were then separated and analysed by western blotting with JB1A. The glycosylation resulted in a decrease in the molecular masses of the 12G10- and B3B11-reactive integrins to 90 kDa, indicating that the mass differences are related to *N*-glycosylation levels or patterns on the two integrin species.

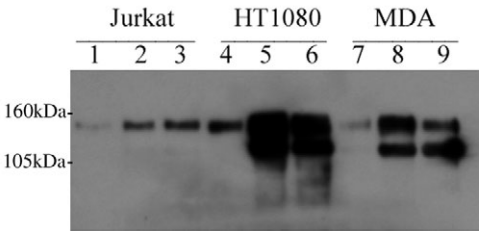

Fig. 7. Expression patterns of β1 integrin chains on different cell lineages. Human cell lines of different lineages (Jurkat, T lymphoma; HT1080, fibrosarcoma; MDA-MB-231 breast cancer) were surface labelled with anti-integrin β1 antibody B3B11 (lanes 1, 4, 7), JB1A (lanes 2, 5, 8) or 12G10 (lanes 3, 6, 9) and the immunoprecipitates were immunoblotted with JB1A. The 120 kDa integrin β1 were identified in HT1080 and MDA cells by JB1A and 12G10, but not in Jurkat cells.

results suggested that differences in glycosylation patterns were responsible for the altered masses and epitope expression of the β1 species on the K562 surface.

Surface expression of integrin species on other cell types

Three other human cell lines of different lineages (Jurkat, HT1080, MDA) were examined to determine the generality of the above observations. Each of the cell lines was surface labelled with JB1A, B3B11 or 12G10 and the captured integrins were visualized by western blotting with JB1A (Fig. 7). Jurkat cells only expressed the 140 kDa species of β1 and this was detected by all three antibodies. In contrast, MDA and HT1080 expressed both the 120 kDa and the 140 kDa forms of β1. In the latter two cell types B3B11 detected the higher molecular mass species, while JB1A and 12G10 reacted with both forms of β1. These results indicated that the surface expression of multiple forms of β1 was not a unique property of K562 cells. However, the staining patterns detected by the antibodies were significantly different depending on the cell type.

Discussion

The antibodies 12G10 and B3B11 identified distinct species of integrins on K562 cells, as defined by their relative intensities of staining and their distribution following cross-linking. The aim of the present study was to determine the basis for this microheterogeneity. Two species of β1 integrin with different molecular masses (i.e. 120 kDa and 140 kDa) were identified. These differences in molecular mass were attributable to the levels of integrin *N*-glycosylation. The 120 kDa species on K562 cells did not appear to be associated with any species of α chain or any other protein. These results suggested that the heterogeneity in these cells was related to the glycosylation status of the β1 chain, which may also influence α chain association.

There have been numerous FACS-based studies demonstrating marked heterogeneity of integrins on the surface of various cell types (Bazzoni and Hemler, 1998); Frelinger, III et al., 1991); Mould et al., 1995; Ni and Wilkins, 1998); Wilkins et al., 1996). Various interpretations of these observations have been made based on the premise that subsets of integrins on the cell surface are in different functional or conformational states. The antibodies, B3B11, 12G10 and JB1A, used in our studies, are well characterized with defined epitopes and functional properties, thus providing a useful set of reporters of integrin heterogeneity. B3B11 recognizes a linear epitope (residues 677-696) in the membrane-proximal extracellular region of the β1 chain (Wilkins et al., 1996). 12G10 recognizes a conformational epitope that appears to include residues K218, R154 and R155 (Mould et al., 2002). The interaction of cells with either 12G10 or B3B11 has been shown to increase cellular adherence. Accessibility to the 12G10 epitope is upregulated by Mn^{2+} or ligand, while the B3B11 epitope is enhanced by exposure to bifunctional reducing agents such as dithiothreitol (Ni et al., 1998). JB1A recognizes a linear epitope near the N terminus of β1 (residues 92-97) and it blocks cell adhesion. The epitope of this antibody recognizes integrins independent of their functional status which provides a useful reporter for total β1 integrin (Ni and Wilkins, 1998).

K562 cells were selected for the present studies because they reportedly express a single species of integrin (i.e. α5β1). This avoids the potentially confounding effects of different α chain pairings on β chain conformation. Also the analyses were performed under serum-free conditions to minimize the risk of ligand-induced changes in integrin conformation. Under these conditions it was noted that the epitope recognized by B3B11 was expressed at approximately 2.5-fold greater level than that of 12G10. The levels of staining by these antibodies were markedly lower than those observed with JB1A. These results indicated that the epitopes recognized by these antibodies were not equally accessible or expressed on all cell surface integrins.

Although microscopic analysis failed to reveal any differences in the distribution patterns of the integrins detected by 12G10 and B3B11 on untreated K562 cells, cross-linking of the b1 integrins with either of these antibodies induced independent redistributions of their corresponding target populations. Furthermore, there were differences in the association patterns with a5. There was a clear colocalization of B3B11-reactive surface integrins with a5. In contrast, 12G10-bearing integrins were distributed independently of the a5 and B3B11-reactive b1. This differential distribution was also observed when a5 chains were cross-linked on the cell surface. In this case, 12G10 and a5 staining did not overlap, implying a lack of association between the two on intact cells.

The possibility that there was an alternate α chain associated with the 12G10 reactive β1 species was examined using biochemical methods and flow cytometry. There was no evidence of a1, α2, α3, α4, α6 or αV on the cell surface or any of these chains associated with b1 integrins purified from K562 cells (X.M., J.A.W., unpublished data). A detailed mass spectrometric analysis of the total integrins isolated with 12G10 also failed to identify any proteins other than b1 in the immunoprecipitates. This contrasted with the B3B11 isolates in which both a5 and b1 were readily detected. Thus it appears that under the conditions employed there are no other species of integrin a chain or unknown proteins that co-purify with the b1 chains from K562. These observations suggest that 120 kDa species are not associated with an a chain on the cell surface. There is still the possibility that these b1 chains are expressed as homodimers of b1 chains. There is precedent for this

suggestion as others have described the presence of b1 homodimers in total integrins isolated from human smooth muscle cells (Belkin et al., 1996). They reported that up to 30% of the total integrins were found as disulphide bonded b1 dimers. All of our efforts to demonstrate the presence of such species were unsuccessful in the present study. Given that there have been reports demonstrating the formation of homo-oligomers involving integrin a or b chains, the possibility of some form of weak non-covalent association cannot be ruled out at this point (Li et al., 2001).

As there have been descriptions of low molecular mass intracellular β1 precursors, an obvious concern was the possibility that the immunoprecipitation conditions were also isolating an intracellular form (Akiyama et al., 1989). However, the present experiments were designed to minimize this possibility. The cells were treated with subsaturating amounts of antibodies to reduce the possibility of unoccupied antibody combining sites on the cell surface. The fact that both 12G10 and B3B11 capture both species of β1 in total cell lysates yet each antibody was selective for only one form when surface capture was performed, argues against an intracellular origin of the 120 kDa species. If there had been any free antibody combining sites in those cases where the cells were surface preloaded with antibodies, both species of integrins would be expected in the precipitates of both antibodies. Furthermore the failure of surface loaded cells to capture exogenous biotinylated integrin suggests that the antibody binding sites were fully occupied. Collectively these results indicate that the surface labelling with antibody was selective.

The expression of the 12G10 epitope warrants discussion as it can clearly report on distinct integrin species. The cells used in these studies were grown in serum-free medium to minimize the possibility of fibronectin-induced 12G10 epitope expression. However, this form was not an artefact of the culture conditions as the 120 kDa species was also observed on the surface of cells cultured in serum-containing media. Thus it appears that the 12G10 epitope was constitutively expressed on the 120 kDa species. In contrast, the expression of the 12G10 epitope on the 140 kDa species was ligand dependent and reversible, suggesting that the glycosylation patterns or the α5 chain association influenced epitope induction or accessibility.

It has recently been reported that the cellular responses to 12G10 interactions with the β1 chain can be differentially influenced by the associated α chain (Humphries et al., 2005). Thus on a single cell type, 12G10 binding to β1 results in an increased α5β1-dependent cellular adhesion to fibronectin, while binding to β1 associated with α4 disrupts cytoskeletal organisation and inhibits α4β1-mediated cell adhesion to fibronectin. The β-propeller domain from the ligand-binding pocket of the associated α4 chain was responsible for conferring these differences in signalling responses. These effects were not dependent on the cytoplasmic domains of the associated α chains as tailless mutants of α4 were equally active in mediating this process. However, there was an absolute requirement for the cytoplasmic domain of the β1 in transmitting these effects to the intracellular compartment.

There were notable differences in the surface expression patterns of β1 in different cell lines. Jurkat cells expressed only the higher molecular mass form and this was detected by all three antibodies (B3B11, JB1A and 12G10), although the B3B11 reactivity was much lower than that of the other two antibodies. In the cases of HT1080 and MDA cells, B3B11 detected only the higher mass β1 while JB1A and 12G10 reacted with both species. For these two cell types it may be that constitutive production of integrin ligands results in occupancy of the higher mass species. However, the possibility that both species spontaneously display the 12G10 epitope cannot be ruled out at this time. These observations highlight some of the potential caveats of assuming that any given antibody is necessarily reporting on the same process in different cell types.

The biochemical basis for the mass difference in the two species of surface β1 chains appeared to be totally related to the levels of glycosylation. PGNase-F-mediated deglycosylation of integrins isolated with 12G10 or B3B11 resulted in species with molecular masses of approximately 90 kDa. These results indicate that both the 120 kDa and 140 kDa forms are differentially *N*-glycosylated. Alterations in cellular glycosylation patterns have been shown to influence cellular adherence properties in several systems (Akiyama et al.,1989; Bellis et al., 1999; Guo et al., 2002; Guo et al., 2003; Pochec et al., 2003; Symington et al., 1989; Veiga et al., 1995). Although inhibitors of *N*-linked oligosaccharide processing inhibit α5β1-mediated binding to fibronectin, they did not influence their surface expression levels or their ability to form αβ dimers. Over expression of *N*-acetylglucosaminyl transferase V (GnT-V), an enzyme involved in the processing of multiantennary asparagine-linked glycans, results in decreased spreading and adhesion of HT1080 cells (Guo et al., 2002). The β1 but not the α5 integrin chains of these cells had altered glycosylation patterns, with an increase in β1,6 branching. These integrin changes did not alter cell surface expression levels of the integrins but a decrease in capacity for integrin clustering was noted on fibronectin-adherent cells. These results were consistent with glycosylation playing a role in the distributional and functional properties of integrins. Treatment of intact cells with inhibitors of glycosylation or PGNase also reduced cellular adherence. It was also observed that enzyme treatment resulted in a loss of stable association between α5 and β1 in cellular and purified integrins (Zheng et al., 1994). These results further indicate that glycosylation can influence integrin chain association and function.

The present results demonstrate that a low molecular 12G10 reactive species of β1 with no apparent α chain association appears to be present on the surface of K562 cells. This expression does not appear to be a unique property of these cells implying that this may be more widely expressed. Preliminary studies suggest that this β1 is competent to bind ligand (X.M., J.A.W., unpublished data). These observations raise questions about the functional properties and biological significance of this species of β1.

This research was supported by grants from the Canadian Institutes for Health Research (J.A.W.), National Science and Engineering Research Council of Canada (W.E.) and National Institutes of Health (K.S.).

References

Akiyama, S. K., Yamada, S. S. and Yamada, K. M. (1989). Analysis of the role of glycosylation of the human fibronectin receptor. *J. Biol. Chem.* **264**, 18011-18018.

Bazzoni, G. and Hemler, M. E. (1998). Are changes in integrin affinity and conformation overemphasized? *Trends Biochem. Sci.* **23**, 30-34.

Bazzoni, G., Ma, L., Blue, M. L. and Hemler, M. E. (1998). Divalent cations and ligands induce conformational changes that are highly divergent among beta1 integrins. *J. Biol. Chem.* **273**, 6670-6678.

Belkin, V. M., Kozlova, N. I., Bychkova, V. V. and Shekhonin, B. V. (1996). Beta 1 integrin subunit dimerization *via* disulfide bonds. *Biochem. Mol. Biol. Int.* **40**, 53-60.

Bellis, S. L., Newman, E. and Friedman, E. A. (1999). Steps in integrin beta1-chain glycosylation mediated by TGFbeta1 signaling through Ras. *J. Cell Physiol* **181**, 33-44.

Blystone, S. D. (2004). Integrating an integrin: a direct route to actin. *Biochim. Biophys. Acta* **1692**, 47-54.

Dasuri, K., Antonovici, M., Chen, K., Wong, K., Standing, K., Ens, W., El Gabalawy, H. and Wilkins, J. A. (2004). The synovial proteome: analysis of fibroblast-like synoviocytes. *Arthritis Res. Ther.* **6**, R161-R168.

Faull, R. J., Kovach, N. L., Harlan, J. M. and Ginsberg, M. H. (1993). Affinity modulation of integrin alpha 5 beta **1**, regulation of the functional response by soluble fibronectin. *J. Cell Biol.* **121**, 155-162.

Frelinger, A. L., III, Du, X. P., Plow, E. F. and Ginsberg, M. H. (1991). Monoclonal antibodies to ligand-occupied conformers of integrin alpha IIb beta 3 (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function. *J. Biol. Chem.* **266**, 17106-17111.

Giancotti, F. G. (2003). A structural view of integrin activation and signaling. *Dev. Cell* **4**, 149-151.

Gottschalk, K. E. and Kessler, H. (2004). A computational model of transmembrane integrin clustering. *Structure (Camb.)* **12**, 1109-1116.

Guo, H. B., Lee, I., Kamar, M., Akiyama, S. K. and Pierce, M. (2002). Aberrant N-glycosylation of beta1 integrin causes reduced alpha5beta1 integrin clustering and stimulates cell migration. *Cancer Res.* **62**, 6837-6845.

Guo, P., Zhang, Y., Zhao, J. H., Wang, L. Y., Guo, H. B., Zhang, X. Y. and Chen, H. L. (2003). Regulation on the expression and N-glycosylation of integrins by N-acetylglucosaminyltransferase V. *Biochem. Biophys. Res. Commun.* **310**, 619-626.

Hemler, M. E., Huang, C. and Schwarz, L. (1987). The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight beta subunit. *J. Biol. Chem.* **262**, 3300-3309.

Humphries, J. D., Schofield, N. R., Mostafavi-Pour, Z., Green, L. J., Garratt, A. N., Mould, A. P. and Humphries, M. J. (2005). Dual functionality of the anti-beta1 integrin antibody, 12G10, exemplifies agonistic signalling from the ligand binding pocket of integrin adhesion receptors. *J. Biol. Chem.* **280**, 10234-10243.

Humphries, M. J., McEwan, P. A., Barton, S. J., Buckley, P. A., Bella, J. and Paul, M. A. (2003). Integrin structure: heady advances in ligand binding, but activation still makes the knees wobble. *Trends Biochem. Sci.* **28**, 313-320.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687.

Li, R., Babu, C. R., Lear, J. D., Wand, A. J., Bennett, J. S. and DeGrado, W. F. (2001). Oligomerization of the integrin alphaIIbbeta3: roles of the transmembrane and cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* **98**, 12462-12467.

Lu, C. F. and Springer, T. A. (1997). The alpha subunit cytoplasmic domain regulates the assembly and adhesiveness of integrin lymphocyte function-associated antigen-1. *J. Immunol.* **159**, 268-278.

Luo, B. H., Springer, T. A. and Takagi, J. (2004). A specific interface between integrin transmembrane helices and affinity for ligand. *PLoS Biol.* **2**, 776-786.

Mould, A. P., Garratt, A. N., Askari, J. A., Akiyama, S. K. and Humphries, M. J. (1995). Identification of a novel anti-integrin monoclonal antibody that recognises a ligand-induced binding site epitope on the beta 1 subunit. *FEBS Lett.* **363**, 118-122.

Mould, A. P., Askari, J. A., Barton, S., Kline, A. D., McEwan, P. A., Craig, S. E. and Humphries, M. J. (2002). Integrin activation involves a conformational change in the alpha 1 helix of the beta subunit A-domain. *J. Biol. Chem.* **277**, 19800-19805.

Ni, H. and Wilkins, J. A. (1998). Localisation of a novel adhesion blocking epitope on the human beta 1 integrin chain. *Cell Adhes. Commun.* **5**, 257-271.

Ni, H., Li, A., Simonsen, N. and Wilkins, J. A. (1998). Integrin activation by dithiothreitol or Mn2+ induces a ligand-occupied conformation and exposure of a novel NH2-terminal regulatory site on the beta1 integrin chain. *J. Biol. Chem.* **273**, 7981-7987.

O'Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Loftus, J. C., Shattil, S. J. and Ginsberg, M. H. (1994). Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* **124**, 1047-1059.

Pochec, E., Litynska, A., Amoresano, A. and Casbarra, A. (2003). Glycosylation profile of integrin alpha 3 beta 1 changes with melanoma progression. *Biochim. Biophys. Acta* **1643**, 113-123.

Schwartz, M. A. (2001). Integrin signaling revisited. *Trends Cell Biol.* **11**, 466-470.

Shevchenko, A., Loboda, A., Shevchenko, A., Ens, W. and Standing, K. G. (2000). MALDI quadrupole time-of-flight mass spectrometry: a powerful tool for proteomic research. *Anal. Chem.* **72**, 2132-2141.

Stupack, D. G., Stewart, S., Carter, W. G., Wayner, E. A. and Wilkins, J. A. (1991). B lymphocyte fibronectin receptors: expression and utilization. *Scand. J. Immunol.* **34**, 761-769.

Symington, B. E., Symington, F. W. and Rohrschneider, L. R. (1989). Phorbol ester induces increased expression, altered glycosylation, and reduced adhesion of K562 erythroleukemia cell fibronectin receptors. *J. Biol. Chem.* **264**, 13258-13266.

Veiga, S. S., Chammas, R., Cella, N. and Brentani, R. R. (1995). Glycosylation of beta-1 integrins in B16-F10 mouse melanoma cells as determinant of differential binding and acquisition of biological activity. *Int. J. Cancer* **61**, 420-424.

Weiler, T., Sauder, P., Cheng, K., Ens, W., Standing, K. and Wilkins, J. A. (2003). A proteomics-based approach for monoclonal antibody characterization. *Anal. Biochem.* **321**, 217-225.

Wilkins, J. A., Li, A., Ni, H., Stupack, D. G. and Shen, C. (1996b). Control of beta1 integrin function. Localization of stimulatory epitopes. *J. Biol. Chem.* **271**, 3046-3051.

Zheng, M., Fang, H. and Hakomori, S. (1994). Functional role of N-glycosylation in alpha 5 beta 1 integrin receptor. De-N-glycosylation induces dissociation or altered association of alpha 5 and beta 1 subunits and concomitant loss of fibronectin binding activity. *J. Biol. Chem.* **269**, 12325-12331.