Intracellular single-chain antibody inhibits integrin VLA-4 maturation and function

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A single-chain antibody construct was prepared containing the V_H and V_L regions of anti-(integrin α^4) antibody HP1/2, an interchain linker and a KDEL endoplasmic reticulum retention sequence. Intracellular expression of this single-chain antibody caused cell-surface expression of $\alpha^4\beta_1$ integrin to be decreased by 80% on selected RD cells and by 65–100% on selected Jurkat cells, relative to mock transfectants. Immunoprecipitation from single-chain-antibody-transfected cells showed that the single-chain antibody was complexed with the integrin α^4 and β_1 subunits, and the diminished sizes of α^4 and β_1 were consistent with impaired maturation. Furthermore, cell adhesion to $\alpha^4\beta_1$ ligands [VCAM-1 (vascular cell adhesion molecule-1), FN40 (40 kDa chymotryptic fragment of fibronectin) and CS1] was greatly impaired in both RD and Jurkat cells, and cell spreading on immobilized FN40 protein was almost completely eliminated. Thus we conclude that intracellular single-chain antibodies may be used to reduce or eliminate cell-surface expression of a specific integrin, with specific functional consequences. This approach should be generally applicable to other integrin subunits.

INTRODUCTION

The integrin family of adhesion receptors contains at least 22 distinct heterodimers [1–3], and several of these different integrins are present on nearly all cell lines and tissue cell types. The VLA-4 ($\alpha^4\beta_1$) integrin is expressed on several different circulating blood cell types, where it mediates cell attachment to vascular cell adhesion molecule-1 (VCAM-1) on activated endothelium [4]. The VLA-4–VCAM-1 interaction contributes to normal myogenesis [5] and haematopoiesis [6–8], and also plays an essential role during various types of inflammation [4,9]. VLA-4 binds to fibronectin through an alternatively spliced domain (CS1) within the HepII region. This interaction may be important during embryogenesis, haematopoiesis and thymocyte maturation, and for migration of T lymphocytes to antigenic sites [4,9]. The VLA-4–VCAM-1 and VLA-4–fibronectin interactions can provide co-stimulatory signals for T lymphocytes [10,11], regulate apoptosis [12,13], trigger specific gene expression [14,15] and induce tyrosine phosphorylation in lymphocytes [16].

Studies of specific integrin functions have been greatly facilitated by cDNA transfection experiments, in which the consequences of expressing a single integrin can be evaluated. For example, our studies of the integrin α^4 subunit have been assisted by transfections of α^4 cDNA into K562 erythroleukaemia cells, MIP101 carcinoma cells and CHO cells that do not ordinarily express α^4 [17,18]. The transfection of specific integrin cDNA into cells has become relatively commonplace but, in contrast, the removal of a specific integrin from its natural cellular environment has been difficult to achieve.

Gene targeting approaches in ES cells have been used to produce mice lacking α^4 [19], α^5 [20] and β_1 [21], but in each case loss of these integrin subunits caused embryonic lethality. Also, targeted inactivation of the β_1 gene was used to generate an integrin-deficient cell line *in vitro* [22]. In other approaches, an α^5-deficient CHO clone was selected by panning cells on anti-integrin antibody [23], and antisense RNA was used to repress expression of both β_1 [24,25] and α^2 [26] integrin subunits. We have made extensive efforts to select α^4-negative cell lines by monoclonal antibody (mAb) panning, or by whole-cell mutagenesis followed by panning, but have not been successful. In addition, our laboratory and many other laboratories have tried antisense RNA approaches without success. Thus it would be useful to have an alternative approach.

Active forms of mAb fragments have been expressed intracellularly in plants and animals [27–29]. Intracellular expression of such antibodies has been used to inhibit the processing of an HIV-1 envelope protein [30], alter the function of the HIV regulatory protein Rev [31], inhibit HIV reverse transcriptase activity [32], and prevent surface expression of a high-affinity interleukin-2 receptor [33] and of ErbB-2 receptor tyrosine kinase [34]. Antibodies targeted to the endoplasmic reticulum (ER) trap specific proteins inside the cell and retain them in the ER, thereby preventing their maturation and transport to the cell surface [30,35]. Thus far, the 'intrabody' approach has been directed at molecules that are monomeric, inducible and/or expressed at relatively low levels. In the present paper, we describe the use of a single-chain antibody to alter the cell-surface expression, maturation and function of $\alpha^4\beta_1$ integrin. This may be the first application of this technology towards modulating the expression of a cell-surface protein that is a heterodimer, is constitutively present at high levels and has a very slow turnover rate.

MATERIALS AND METHODS

Construction of single-chain variable-region fragment (sFv) coding sequences

From the mouse mAb HP1/2 [36] a humanized form (hHP1/2) was prepared (D. R. Leone, P. R. Tempest, P. White, R. B. Pepinsky, M. Rosa, B. Griffiths-Browning, R. J. Carr, W. M. Abraham, T. Papayannopoulou, B. Nakamoto and R. R. Lobb, unpublished work), and then used for construction of an sFv of HP1/2. Coding sequences for the V_H and V_L region of hHP1/2

Abbreviations used: mAb, monoclonal antibody; CS1-BSA, BSA derivatized with peptide CS1; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FN40 and FN120, 40 and 120 kDa chymotryptic fragments respectively of fibronectin; HBSA, heat-denatured BSA; sFv, single-chain variable-region fragment; VCAM-1, vascular cell adhesion molecule-1; VCAM-1-κ, VCAM-1–(mouse C kappa) fusion protein.

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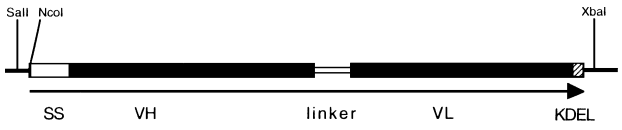

Figure 1 Schematic representation of the sFv-195 single-chain antibody

SS, heavy-chain signal sequence; VH, heavy-chain variable region; VL, light-chain variable region. See the text for further details.

were modified by PCR or by insertion of double-stranded synthetic oligonucleotides (i) to connect the two domains with a $(GGGGS)_3$ linker [38], (ii) to add a KDEL sequence to the C-terminus of the protein, and (iii) to introduce convenient restriction sites. The final sFv-195 construct contains the heavy-chain signal sequence and variable region followed by the $(GGGGS)_3$ linker and the light-chain variable region, followed by a KDEL sequence for ER retention [39]. A schematic diagram of sFv-195 is shown in Figure 1. All constructions were performed in pUC19 or its derivatives. Transformed bacterial strains were cultured in 2×YT medium to lessen the toxicity of the sFv construct. The sequence of sFv-195 was confirmed by DNA sequence analysis.

Antibodies and VLA-4 ligands

Antibodies utilized were anti-α^4 (B5G10) [40], anti-α^5 (A5-PUJ2) [41], anti-β_1 (A-1A5) [42] and the negative control antibody P3 [43]. Notably, B5G10 recognizes an epitope distinct from that recognized by the HP1/2 mAb [44] used to make the single-chain antibody. Polyclonal antiserum recognizing hHP1/2 sFv was generated by immunization of rabbits with murine HP1/2 using standard protocols. This serum recognizes only the antigen-binding region of the sFv derived from humanized HP1/2; all other regions present in murine HP1/2 were either modified or removed.

FN40 and FN120 (Gibco-BRL, Gaithersburg, MD, U.S.A.) are 40 and 120 kDa chymotryptic fragments respectively of fibronectin. The FN40 fragment contains the CS1 binding region, a 25-amino-acid sequence that includes a critical LDV site recognized by VLA-4. The FN120 fragment contains the RGD binding region recognized by VLA-5. BSA derivatized with the 25-amino-acid CS1 peptide (CS1-BSA) was obtained from Dr. Tadashi Shimo-Oka (Iwaki Glass Co., Tokyo, Japan). Purified VCAM–(mouse C kappa) fusion protein (VCAM-1-κ) was obtained from Dr. Philip Lake (Sandoz Co., East Hanover, NJ, U.S.A.). VCAM-1-κ is produced by Sf9 cells and contains all seven human VCAM domains, except that the transmembrane and cytoplasmic portions of domain 7 have been replaced by a 100-amino-acid mouse C kappa segment.

Transfection and selection of stable clones

Single-chain antibody cDNA sFv-195 was cloned into the mammalian expression vector pMHneo [45] using *Sal*I and *Xba*I sites. Then, RD rhabdomyosarcoma cells were transfected using the Lipofectin reagent (Gibco-BRL) following standard protocols. For one confluent 10 cm Petri dish, 30 μg of DNA was used, and pMHneo vector alone was used for a separate transfection. Single colonies selected for growth in 1.0 mg/ml G418 were further expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, and then analysed by flow cytometry.

Jurkat cells were transfected via electroporation at 960 μF and 270 mV using a gene pulser (Bio-Rad Laboratories, Cambridge, MA, U.S.A.). For 6×10^6 cells, 20 μg of sFv-195 or pMHneo vector was used. The Jurkat cells were then cloned and selected in 96-well flat-bottomed Falcon microtitre plates (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.) containing RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and G418 at 1.0 mg/ml. Resistant clones were expanded in 48-well plates and analysed by flow cytometry.

Flow cytometry and immunoprecipitation

Flow cytometry analyses were performed as described [46]. Briefly, cells were first incubated with mouse anti-human mAbs, washed and then stained with fluorescein isothiocyanate-conjugated goat anti-(mouse IgG) (Gibco-BRL), and then washed and analysed using a FACScan machine (Becton Dickinson, Oxnard, CA, U.S.A.).

For metabolic labelling, cells were preincubated (60 min) in methionine- and cysteine-free DMEM (for RD cells) or RPMI 1640 medium (for Jurkat cells) containing 10% dialysed fetal calf serum (JRH Biosciences, Lenexa, KS, U.S.A.). Then, cells were labelled with 1 mCi each of [^{35}S]methionine and [^{35}S]cysteine for 12 h. Cells were lysed in 2 ml of RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid and 0.1% SDS) containing PMSF (2 mM), aprotinin (0.1 mg/ml) and leupeptin (0.1 mg/ml). Immunoprecipitation and SDS/PAGE analysis (on 10% polyacrylamide gels) were carried out as previously described [47].

Adhesion and spreading assays

Cell attachment to FN40, VCAM-1-κ and CS1-BSA was carried out as previously described [48]. Briefly, FN40 and CS1-BSA were coated on to 96-well microtitre plates (Flow Laboratories, McLean, VA, U.S.A.) in 0.1 M $NaHCO_3$, pH 8.3, for 16 h at 4 °C, and then 0.1% heat-denatured BSA (HBSA) was added to block non-specific adhesion. For VCAM-1-κ assays, plates were precoated with goat anti-(mouse κ) (Gibco-BRL) in 0.1 M $NaHCO_3$ for 16 h, washed with PBS and then coated with VCAM-1-κ for 1 h at 4 °C, and were subsequently blocked with HBSA. Cells were labelled by incubation with the fluorescent dye 2′,6′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR, U.S.A.) for 30 min, and then 5×10^4 cells in RPMI containing 0.1% HBSA (assay medium) were added to each well of a ligand-coated microtitre plate and incubated for 20–25 min at 37 °C. After incubation, unbound cells were removed by washing the plate three times with RPMI medium. Cells remaining attached to the plate were analysed using a Fluorescence Analyser (CytoFluor 2300; Millipore Co., Bedford, MA, U.S.A.). After subtraction of background cell binding (assessed using BSA-coated wells), values for cells bound/mm^2 were calculated as described [48]. Background binding was typically less than 5% of the total. Assay results are reported as means ± S.D. of triplicate determinations.

For spreading assays, 12-well tissue culture plates (Costar Corp., Cambridge, MA, U.S.A.) were coated with FN40 or FN120 in 0.1 M $NaHCO_3$ for 16 h at 4 °C, and blocked with 0.1% HBSA for non-specific binding. Cells were plated at 10^6 cells/well in DMEM with 0.1% HBSA and incubated at 37 °C for 3 h. The numbers of spread cells relative to total cells were counted from three randomly chosen fields under phase contrast using a Zeiss microscope (Model Axiovert 135). Results were photographically recorded using Kodak film Tri-X PAN400.

RESULTS

Inhibition of α^4 surface expression in transfected RD and Jurkat cells

After selection, 25 neomycin-resistant sFv-195-transfected RD cell colonies and 32 vector control colonies were analysed. Also, 288 resistant sFv-195-transfected Jurkat clones and 288 vector control clones were analysed by flow cytometry. Among the sFv-195 transfectants, 13 RD cell clones and 23 Jurkat cell clones displayed reduced α^4 surface expression. Thus, of the total numbers of neomycin-resistant clones analysed, 52% of RD clones and 8% of Jurkat clones were altered (Table 1). Notably, no spontaneous decrease in α^4 expression was observed for any of the control clones of either cell type.

A summary of flow cytometry results for two sFv-195-transfected RD clones and two Jurkat clones, relative to control clones, is shown in Figure 2. As indicated, the mean fluorescence intensity levels for α^4 were reduced by $\sim 80\%$ on RD R-1 and R-2 cells and by 65–100% on Jurkat J-1 and J-2 cells relative to mock-transfected controls. In addition, β_1 expression was also partially reduced for all four sFv-195-transfected clones, with this reduction being especially obvious for clone J-2. The expression of α^5 was also reduced on clone J-2, but this was not a consistent finding, and α^5 levels were not markedly reduced on the three other clones (J-1, R-1 and R-2).

Table 1 Transfected RD and Jurkat clones showing G418 resistance and reduced α^4 expression

Reduced expression is defined as 50% or more reduced relative to untransfected cells, as determined by flow cytometry.

Cell type	G418-resistant clones	Clones with reduced α^4 expression
RD-mock	32	0
RD-sFV-195	25	13
Jurkat-mock	288	0
Jurkat-sFv-195	288	23

Impaired α^4 integrin maturation in RD and Jurkat cells

Analysis of α^4 integrins immunoprecipitated from sFv-195 transfectants revealed that integrin maturation was impaired. Using anti-α^4 antibody, the R-1 and R-2 clones yielded predominantly the smaller-sized precursor forms [49] of the α^4 and β_1 subunits (Figure 3, lanes e and f), rather than mature subunits such as obtained from RD-M cells (lane d). With negative control antibody, no labelled proteins were obtained from any of these cells (lanes a–c). Immunoprecipitations using the anti-β_1 mAb A-1A5 confirmed that there was a large proportion of immature β_1 and associated α^4 subunit in R-1 and R-2 cells (results not shown). Also, immunoprecipitations of α^4 and β_1 from Jurkat clones J-1 and J-2 similarly revealed an abundance of precursor proteins (results not shown).

The 28 kDa protein co-precipitated with α^4 and β_1 precursor proteins (Figure 3, lanes e and f), but not with mature α^4 and β_1 (lane d), corresponds to the expected size of the sFv-195 protein. Rabbit anti-(murine HP1/2) (anti-idiotype) antibody precipitated similar 28 kDa proteins from R-1 and R-2 (lanes k and l) but not R-M (lane j) cells, thus confirming the identity of this protein. Together these results show that, in the presence of intracellular sFv-195, α^4 can still dimerize with the β_1 chain, but maturation of α^4 and associated β_1 is impaired.

Impaired cell adhesion and spreading on VLA-4 ligands

To examine further the consequences of reduced integrin α^4 subunit expression, functional assays were carried out. Two Jurkat clones and two RD clones that were sFv-195-positive all showed markedly reduced adhesion to the VLA-4 ligand VCAM-

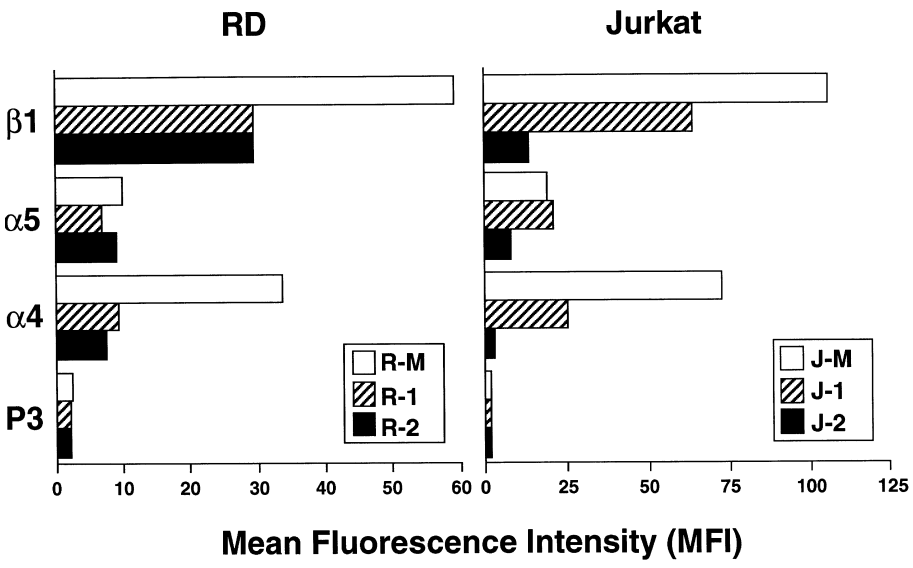

Figure 2 Flow cytometry analysis of α^4 surface expression

Cells were stained with control mAb P3, anti-α^4 mAb B5G10, anti-α^5 mAb A5-PUJ2 and anti-β_1 mAb A-1A5. For each cell, the results for two sFv-195 transfectants and a mock transfectant (J-M, R-M) are shown.

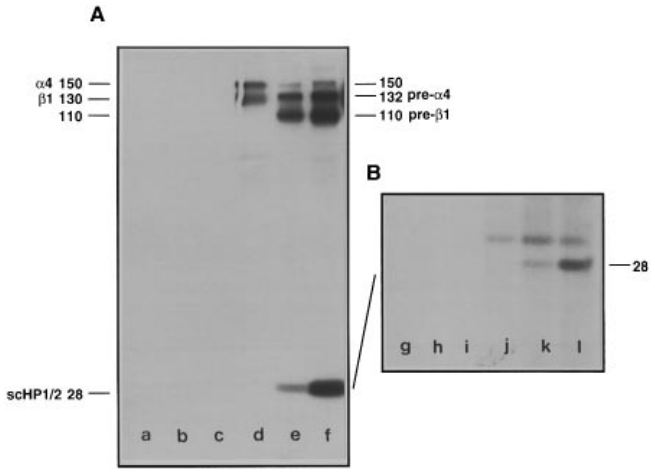

Figure 3 Immunoprecipitation of integrin and associated single-chain antibody

(**A**) Lysates from metabolically labelled RD cells were immunoprecipitated using negative control mAb P3 (lanes a–c) or anti-α^4 mAb B5G10 (lanes d–f). (**B**) Lysates were immunoprecipitated with preimmune rabbit serum (lanes g–i), and with rabbit anti-HP1/2 anti-idiotype antibody (lanes j–l). Results are from transfectants R-M (lanes a, d, g and j), R-1 (lanes b, e, h and k) and R-2 (lanes c, f, i and l). Molecular masses (kDa) are indicated. scHP1/2 is the single-chain antibody sFv-195 (28 kDa).

1, to FN40 and to the fibronectin-derived CS1 peptide (Figure 4). The nearly complete loss of adhesion for Jurkat clone J-2 is consistent with its almost complete loss of α^4 surface expression, and the partial loss of adhesion for the other clones (J-1, R-1 and R-2) is consistent with partially reduced α^4 expression. Notably, for clones J-1, R-1 and R-2 the most pronounced reduction of adhesion (relative to mock transfectants) was observed when adhesion was weaker (i.e. at lower VCAM-1 and FN40 ligand doses, or when the CS1 ligand was utilized).

In addition, cell spreading on FN40 was severely impaired in the sFv-195-transfected RD clones R-1 and R-2 compared with mock-transfected RD cells (Figure 5, left panels). Quantification of approx. 200 cells from each of three fields revealed that 90 % of mock-transfected cells were spread, whereas less than 1 % of either R-1 or R-2 cells were spread. In a control experiment, mock-transfected RD cells and clones R-1 and R-2 displayed comparably high levels of spreading on FN120 (Figure 5, right panels). In each case, 84–96 % cell spreading was observed.

DISCUSSION

In the present study we have used a novel approach to manipulate α^4 integrin cell-surface expression *in vitro*. The intracellular expression of a single-chain antibody against α^4 integrin markedly inhibited the maturation and surface expression of α^4, by trapping the α^4 integrin and presumably targeting it to the ER. Hence α^4-dependent functions were consequently impaired, as indicated by markedly diminished adhesion and spreading of cells on VLA-4 ligands. We are confident that the results are not simply due to random sFv-195 cDNA integration effects, because similar data were obtained from four different sFv-195-transfected clones in two different cell lines, and because α^4 but not α^5 integrin function was selectively modulated.

In previous studies, the KDEL sequence was required to achieve more complete and stable single-chain antibody in-

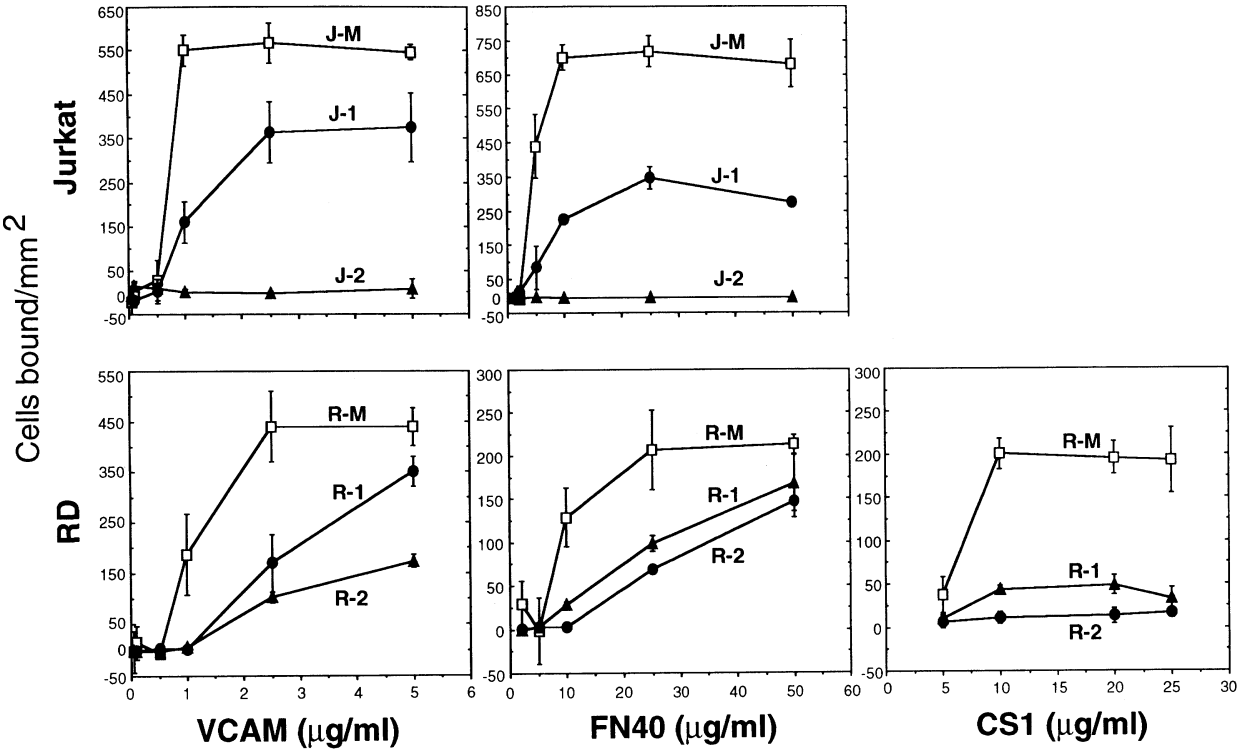

Figure 4 Adhesion of RD and Jurkat cells to VLA-4 ligands

Adhesion of Jurkat and RD cells to VCAM-1-κ, FN40 and CS1-BSA, coated at the indicated concentrations, was carried out as described in the Materials and methods section.

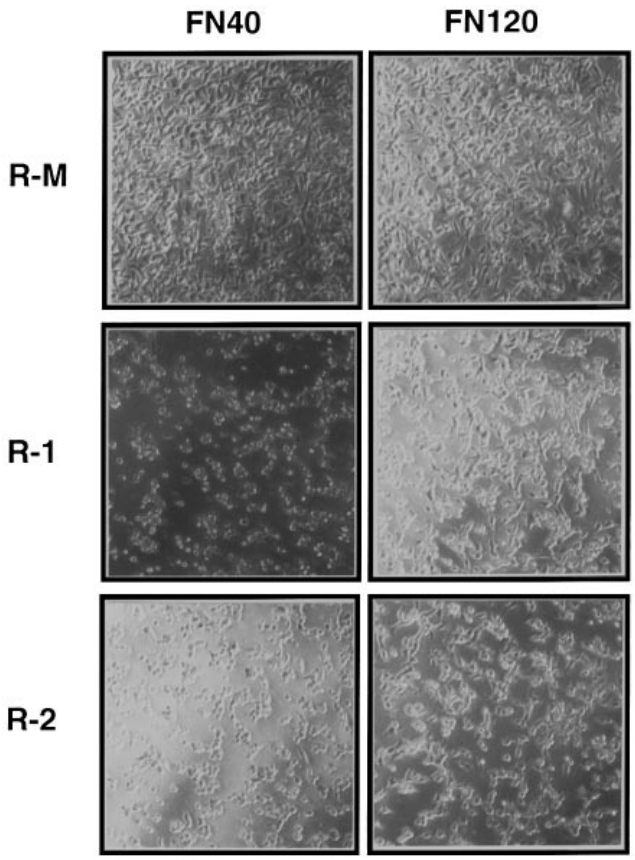

Figure 5 Spreading of RD transfectants on FN40 and FN120 ligands

Plastic surfaces were coated with 10 μg/ml FN40 (left panels) or FN120 (right panels). Photographs were taken 3 h after cells were plated. Magnification $\times$ 70.

hibition of interleukin-2 receptor expression [33] and to inhibit ErbB-2 expression and function [34]. In our study, if KDEL was omitted from the sFv-195 protein, the latter was no longer effective in reducing the cell-surface expression of $\alpha^4\beta_1$ or the maturation of the α^4 and β_1 subunits in Jurkat cells (results not shown). Furthermore, anti-α^4 and anti-β_1 antibodies co-precipitated a markedly smaller amount of sFv-195 KDEL-minus protein; the majority was present in the cell culture supernatant, as detected by rabbit anti-HP1/2 antibody (results not shown). Thus the tetrapeptide KDEL ER-retention sequence may be necessary for the single-chain antibody protein to cause diminished α^4 expression.

Antibody engineering has been utilized previously to prepare optimally active integrin 'ligands' [50]. However, as far as we know, the present study represents the first use of intracellular single-chain antibodies to inhibit integrin expression. Other *in vitro* techniques have been used in this regard, including selection of mutants deficient for α^5 integrin expression [23], anti-sense RNA targeting of β_1 and α^2 integrins [24–26] and genetic knockout of the β_1 subunit [22]. However, these approaches have been attempted many times and are often unsuccessful. Our success in inhibiting α^4 integrin expression and function suggests that the single-chain antibody approach is a very useful alternative. In contrast to previous protein targets of intracellular single-chain antibodies [30–33], the $\alpha^4\beta_1$ integrin is a heterodimer, is constitutively expressed on cell surfaces and has a relatively low turnover rate [51,52]. Nonetheless, marked decreases in cell-surface VLA-4 levels were achieved, and then maintained in sFv-195-transfected Jurkat cells for 3–4 weeks and in transfected RD cells for longer than 3 months. Not only did we completely eliminate integrin surface expression, but we generated a series of cell lines which express a given integrin at variable levels (Figure 2, and results not shown). This aspect may be particularly useful for correlating cell functions and signalling pathways with integrin expression levels.

Whereas we exclusively analysed $\alpha^4\beta_1$ in the present study, the same sFv-195 antibody should be equally active in cells expressing the $\alpha^4\beta_7$ integrin. Furthermore, we anticipate that any other of the 16 integrin α and eight integrin β subunits should be similarly amenable to single-chain antibody targeting. Obviously, mAbs unable to recognize immature forms of integrins or other cell-surface proteins would probably not be useful in this regard. Also, mAbs must be chosen in which the Fab fragment has a potency comparable with that of the bivalent mAb itself. In this regard, HP1/2 is an ideal choice for sFv targeting because the potency of the murine HP1/2 Fab fragment both *in vivo* and *in vitro* is very similar to that of intact murine HP1/2 [53].

The single-chain antibody approach also should have potential for *in vivo* functional studies. Transgenic animals expressing a single-chain antibody would be likely to show reduced expression of a particular integrin. Technically, this approach would probably be simpler than conventional methods for gene knockout. Also, by placing the single-chain antibody construct under the control of a cell-specific promoter, this approach would achieve inhibition of integrin expression in a lineage-specific fashion. Furthermore, under the control of an inducible promoter, inhibition of integrin expression could perhaps be achieved at a particular time point, such as during development.

In summary, we have used the targeted intracellular expression of an anti-α^4 sFv as a novel means of modulating integrin expression. This approach has obvious potential utility for the study of α^4 integrin functions both *in vitro* and *in vivo*. Also, it should be feasible to modulate the expression of other integrin subunits by an approach similar to that described here.

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