Molecular Biology of the Cell
Vol. 5, 203–215, February 1994

Distinct and Overlapping Ligand Specificities of the α3Aβ1 and α6Aβ1 Integrins: Recognition of Laminin Isoforms

Gepke O. Delwel,* Annemieke A. de Melker,* Frans Hogervorst,* Lies H. Jaspars,† Danielle L.A. Fles,* Ingrid Kuikman,* Anders Lindblom,‡ Mats Paulsson,‡ Rupert Timpl,§ and Arnoud Sonnenberg*

*Division of Cell Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam; †Department of Pathology, Free University Hospital, 1007 MB Amsterdam, The Netherlands; ‡M.E. Müller Institute for Biomechanics, University of Bern, CH-3010 Bern, Switzerland; and §Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany

Submitted October 4, 1993; Accepted December 23, 1993
Monitoring Editor: Richard Hynes

The ligand specificity of the α3Aβ1 integrin was analyzed using K562 cells transfected with full-length α3A cDNA and was compared with that of α6Aβ1 in similarly transfected K562 cells. Clones were obtained that showed comparable surface expression of either α3Aβ1 or α6Aβ1 integrins. Those expressing α3Aβ1 attached to and spread on immunopurified human kalinin and cellular matrices containing human kalinin, which is a particular isoform of laminin. In addition, α3A transfectants adhered to bovine kidney laminins possessing a novel A chain variant. Binding to kalinin was blocked by a monoclonal antibody against the A chain constituent of kalinin and adhesion to both kalinin and kidney laminins by anti-α3 and β1 monoclonal antibodies. The α3A transfected cells bound more strongly to kalinin and bovine kidney laminins after treatment with the β1 stimulatory antibody TS2/16. A distinctly weaker and activation-dependent adhesion of α3A transfectants was observed on human placental laminins possessing the Am chain variant (merosin), and no adhesion occurred on bovine heart laminins and murine EHS tumor laminin. Further inactive substrates were fibronectin, nidogen, and collagen types IV and VI, indicating that the α3Aβ1 integrin is a much less promiscuous receptor than thought before. By contrast, α6A transfected cells adhered to all laminin isoforms when stimulated with TS2/16. Adhesion also occurred only on bovine kidney laminins in the absence of TS2/16. These results demonstrate that both α3Aβ1 and α6Aβ1 integrins are typical laminin receptors but that their affinity and activation dependence for binding to various laminin isoforms differ considerably.

INTRODUCTION

Integrins are members of a large family of cell adhesion receptors that are involved in cell-extracellular matrix (ECM) and cell–cell interactions. Besides acting as simple receptors, integrins are signaling molecules that can be involved in both outside/in and inside/out cell signaling pathways (Hynes, 1992). The α3β1 integrin initially has been described as a receptor for the ECM proteins fibronectin, collagen, and laminin in studies using affinity chromatography and cell adhesion assays (Wayner and Carter, 1987; Wayner *et al.*, 1988; Gehlsen *et al.*, 1988, 1989; Takada *et al.*, 1988; Tomaselli *et al.*, 1990). Although the α3β1 integrin is a promiscuous receptor, the specificity of its activity can be masked by other integrins present on the same cell, e.g., α2β1, α5β1, and α6β1, which are receptors for collagen (Wayner and Carter, 1987), fibronectin (Pytela *et al.*, 1985), and laminin (Sonnenberg *et al.*, 1988), respectively. Furthermore, it

has been suggested that the avidity of $\alpha 3\beta 1$ for these ligands is lower than that of the other integrins (Elices *et al.*, 1991). Indeed, there are numerous studies in which $\alpha 3\beta 1$ could not be identified as a receptor for fibronectin, collagen, or laminin (Kramer *et al.*, 1990; Lotz *et al.*, 1990; Brown and Goodman, 1991; Carter *et al.*, 1991; Sonnenberg *et al.*, 1991; Dedhar *et al.*, 1992; Pfaff *et al.*, 1993; Weitzman *et al.*, 1993). In addition to the abovementioned ligands, the $\alpha 3\beta 1$ integrin was shown to bind to the ECM protein nidogen, a small glycoprotein associated with laminin (Dedhar *et al.*, 1992). Also, $\alpha 3\beta 1$ has been implicated as a receptor for the bacterial protein invasin (Isberg and Leong, 1990). Recently, Carter *et al.* (1991) showed that epiligrin, a new adhesive epithelium-specific glycoprotein secreted by human keratinocytes, is a ligand for $\alpha 3\beta 1$.

Laminins are multidomain heterotrimeric proteins found in basement membranes in various species. Murine EHS tumor laminin is composed of three disulphide-linked polypeptide chains: Ae (400 kDa), B1e (215 kDa), and B2e (200 kDa) (Timpl, 1989; Beck *et al.*, 1990). In addition, several laminin isoforms have been identified with a different tissue distribution. 1) Merosin, in which the Ae chain is replaced by a homologous Am chain (300 + 80 kDa). Am-containing laminins were first purified from human placenta and are homologous to murine and bovine laminins purified from heart (Paulsson and Saladin, 1989; Ehrig *et al.*, 1990; Paulsson *et al.*, 1991). They may also contain the 190-kDa B1s chain isoform. 2) B1s complexed to the Ae and B2e chains forms s-laminin (Green *et al.*, 1992). 3) A further laminin isoform containing a 375-kDa A chain variant combined with B1e or B1s and B2e chains recently has been purified from bovine kidney (Lindblom *et al.*, 1994). 4) The epithelium-specific K-laminin that contains a variant A chain (190 kDa) complexed to the B1e and B2e chains (Marinkovich *et al.*, 1992b). 5) Kalinin, another isoform that initially has been detected in skin epithelium, is immunologically distinct from EHS tumor laminin and is composed of three disulphide-linked polypeptide chains of 165, 155, and 140 kDa (Rousselle *et al.*, 1991). Its A chain variant (165 kDa) is immunologically related to the A chain of K-laminin (Marinkovich *et al.*, 1992b), and its B2 chain variant (155 kDa) is identical to the truncated laminin B2t chain described by Kallunki *et al.* (1992). 6) Epiligrin, purified from keratinocyte extracellular matrix, which is composed of three disulphide-bonded polypeptide chains of 170, 145, and 135 kDa that may be bound to a copurified laminin-like 200-kDa molecule (Carter *et al.*, 1991). Epiligrin probably represents a complex of kalinin and K-laminin molecules (Domloge-Hultsch *et al.*, 1992).

Binding of $\alpha 3\beta 1$ to laminin has been described and, in particular, binding to the carboxy terminal region of the long arm of laminin (Gehlsen *et al.*, 1988, 1989; Tomaselli *et al.*, 1990), but our results do not fully support that conclusion. Using affinity chromatography on the major cell adhesive E8 fragment of EHS tumor laminin, we isolated $\alpha 6A\beta 1$ but not $\alpha 3A\beta 1$, although both integrins were isolated on a large pepsin fragment of human placental laminin (Sonnenberg *et al.*, 1991). Thus, it was concluded that $\alpha 3A\beta 1$ and $\alpha 6A\beta 1$ may be receptors for human laminin but that only $\alpha 6A\beta 1$ is a receptor for murine EHS tumor laminin. These discrepant results may be due to the existence of several laminin isoforms and/or to the presence of multiple laminin receptors on the same cell, which were not completely identified.

Recently, we showed that in addition to EHS tumor laminin, bovine heart laminins possessing the Am chain (merosin) and kalinin are ligands for the $\alpha 6A\beta 1$ integrins by using $\alpha 6A$ and $\alpha 6B$ transfected K562 cells (Delwel *et al.*, 1993). In the present study we used K562 cells for transfection with full-length $\alpha 3A$ cDNA to study the ligand specificity of the $\alpha 3A\beta 1$ integrin and to compare it with that of the $\alpha 6A\beta 1$ integrin on K562 transfectants. Our data present the first evidence that the $\alpha 3A\beta 1$ integrin on K562 cells is a kalinin receptor. Notably, Weitzman *et al.* (1993) recently published a study in which $\alpha 3$ transfected K562 cells bound to ECM deposited by epidermal and carcinoma cell lines. The ligand was assumed to be kalinin/epiligrin but not identified as such. Furthermore, we show that $\alpha 3A\beta 1$ reacts with bovine kidney laminins, human placental laminins, but not with other laminin isoforms, in contrast to the $\alpha 6A\beta 1$ integrin, which is a receptor for all of them.

MATERIALS AND METHODS

Cell Culture, Antibodies, and Matrix Proteins

The human erythroleukemic cell line K562 was grown in RPMI-1640 supplemented with 10% fetal calf serum, penicillin, and streptomycin. K562 transfectants were grown in the same medium supplemented with 1 mg/ml Geneticin (G-418 sulfate, GIBCO-BRL, Grand Island, NY). The human squamous cell carcinoma cell line UMSCC-22B (kindly provided by Dr. T.E. Carey, University of Michigan, Ann Arbor, MI) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Antibodies directed against integrin subunits were the rat monoclonal antibody (mAb) GoH3 to $\alpha 6$ (Sonnenberg *et al.*, 1987); the mouse anti-$\alpha 6$ mAb 450-33D (Kennel *et al.*, 1990), kindly provided by Dr. S.J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN); the rabbit polyclonal antibody directed against the carboxy-terminal sequence of $\alpha 6A$ (Delwel *et al.*, 1993); the mouse mAb 1A10 to the cytoplasmic domain of $\alpha 6A$ (Hogervorst *et al.*, 1993). The mouse mAbs P1B5 (Telios Pharmaceuticals, La Jolla, CA) and J143 (Kantor *et al.*, 1987), a kind gift from Dr. A. Albino (Memorial Sloan-Kettering Cancer Center, New York, NY), are directed against the integrin $\alpha 3$ subunit. The mouse mAb Sam-1 to $\alpha 5$ (Keizer *et al.*, 1987) was a gift from Dr. C.G. Figdor (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The mouse mAb TS2/16 (Hemler *et al.*, 1984) and rat mAb AIIB2 (Werb *et al.*, 1989), directed against the common integrin $\beta 1$ subunit, were kindly provided by Drs. F. Sánchez-Madrid (Hospital de lao Princesa, Madrid, Spain) and C.H. Damsky (University of California, San Francisco, CA), respectively. The mouse mAb K20 to $\beta 1$ (Amiot *et al.*, 1986) was submitted for the 4th International Workshop on Leukocytes.

The following antibodies to extracellular matrix proteins were used in this study: polyclonal antibodies directed against murine EHS lam-

inin and its proteolytic fragments E3 and E8 (Ott *et al.*, 1982; Paulsson *et al.*, 1985). Rabbit antisera directed against recombinant human nidogen were prepared as previously described (Fox *et al.*, 1991). A guinea pig antiserum to recombinant rat laminin B1s chain (Sanes *et al.*, 1990) was obtained from Dr. J.R. Sanes (Washington University School of Medicine, St. Louis, MI). Rabbit antisera directed against human collagen IV, human placental laminins (merosin), and the C-terminal 80-kDa fragment of the Am chain, comprising repeats four and five of the C-terminal domain (Leivo and Engvall, 1988), were provided by Dr. E. Engvall (La Jolla Cancer Research Foundation, La Jolla, CA). An antiserum directed against human kalinin (Marinkovich *et al.*, 1992a) and the mAb BM165 directed against the human kalinin A chain (Rousselle *et al.*, 1991) were kindly provided by Dr. R.E. Burgeson (Harvard Medical School, Boston, MA). MAb 4C7 reacts with human kalinin (Jaspars *et al.*, 1993).

Human kalinin–containing matrices were prepared from the UMSCC-22B cell line as previously described (Delwel *et al.*, 1993). Immunopurified human kalinin was isolated from UMSCC-22B culture medium. Proteins in the culture medium were precipitated with 50% ammonium sulphate (2 h, 4°C). The precipitate was dissolved, dialyzed against phosphate-buffered saline (PBS), and applied onto a column of anti-human kalinin mAb (4C7) coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was washed with 10 mM phosphate buffer (pH 8) containing 1 M NaCl, whereafter bound proteins were eluted with 100 mM triethylamine (pH 11.5). The eluted fractions were immediately neutralized with one-sixth of a volume of 1 M phosphate buffer (pH 6.8). Human kalinin–containing fractions were pooled, dialyzed against PBS, and stored at −70°C. Human merosin was purchased from Telios Pharmaceuticals. This sample was subjected to Western blot analysis using specific antisera to the various laminin variant chains, nidogen, kalinin, fibronectin, and collagen type IV, all described above, and shown to contain a mixture of two laminin isoforms: mainly AmB1sB2e and little AmB1eB2e complexed to nidogen. This human merosin preparation will be referred to as human placental laminins. Human plasma fibronectin was purchased from Sigma Chemical Co. (St. Louis, MO) and murine collagen type IV from GIBCO-BRL (Gaithersburg, MD). Laminin–nidogen complex was prepared from the murine EHS tumor (Timpl *et al.*, 1987), and collagen type VI was prepared from pepsin-solubilized human placenta (Odermatt *et al.*, 1983). Recombinant murine nidogen has been described (Fox *et al.*, 1991). Bovine heart and kidney laminins were isolated and purified as described previously (Paulsson *et al.*, 1991; Lindblom *et al.*, 1994).

Generation of Hybridomas to α3A

A 2g amino acid–long peptide corresponding to the cytoplasmic domain of the integrin subunit α3A, CRTRALYEAKRQKAEMKSQPSETERLTDDY, was synthesized and coupled to keyhole limpet hemocyanin (Sigma Chemical Co.) using the extra NH_2-terminal cysteine residue (underlined). Both free and coupled peptides were used for immunization of Balb/c mice. After four immunizations the mice were killed for fusion. The fusions were carried out with polyethylene glycol 4000 using isolated spleen cells and Sp2/0 mouse myeloma cells at a ratio of 4:1. The cells were plated at 2×10^5 cells/well on mouse macrophage feeder cells in 96-well tissue culture plates in selection medium (hypoxanthine/aminopterin; Sigma Chemical Co.). Ten to 14 days after fusion the supernatants were collected and screened for antibodies in an enzyme-linked immunosorbent assay (ELISA) as previously described (Hogervorst *et al.*, 1993). The supernatants of hybridomas positive in ELISA were further screened by immunoprecipitation of lysates of ^{125}I surface–labeled HBL-100 cells, an α3Aβ1 positive human mammary epithelial cell line, and by immunoperoxidase staining on human skin and kidney cryosections. The results of these experiments will be described elsewhere. Selected hybridomas were cloned by limiting dilution.

Isolation and Construction of α3 cDNA

A lambda gt11 cDNA library prepared from poly(A)$^+$RNA isolated from human keratinocytes (Clontech Laboratories, Palo Alto, CA) was screened with the α3 cDNA clone 3.24, a kind gift of Dr. Y. Takada (Takada *et al.*, 1991). Forty positive plaques were identified and subjected to the polymerase chain reaction (PCR) with unique lambda gt11 primers and an α3 specific antisense primer for positions 988-1005 (5′-CTGCCAAAATAGGCGCCC-3′). Primer positions for α3 cDNA correspond to the sequence published by Takada *et al.* (1991). PCR conditions were as previously described (Hogervorst *et al.*, 1991). Only from one phage (D17) was a PCR product obtained. Phage D17 was plaque-purified and phage DNA was isolated. The cDNA insert was excised with *Eco*RI yielding two products of ~2.3 and 1.7 kb that were subcloned into the *Eco*RI site of pUC18. The α3 cDNA ranged from 199 to 4136 bp and encoded the α3A variant. However, the α3A N-terminus was not encoded by the isolated α3 cDNA and was also absent in a large number of cDNA libraries that were screened by PCR.

We subsequently constructed this 5′ cDNA stretch by site-directed mutagenesis using the PCR (Higuchi *et al.*, 1988). The 5′ untranslated region (UT), the α3 leader peptide sequence, and the sequence encoding the first 8 amino acids (FNLDTRFL) of the α3 mature protein were missing from D17-derived α3 cDNA as compared with the published α3 cDNA sequence by Takada *et al.* (1991). Because the first six amino acids (FNLDTR) of the α6 mature protein are identical to α3, we used α6 cDNA to construct α6/α3 cDNA that encoded the mature α3 protein and had an α6-derived 5′UT and leader sequence. The missing amino acids of α3 (F, L) were encoded in the primer. Site-directed mutagenesis was performed as follows: a first-round PCR on α3 cDNA was performed with an α3-specific antisense primer for positions 322–336 (5′-TTGGTGTAGCCATCG-3′), containing nine additional nucleotides encoding an *Eco*RI site, and a sense primer containing an α6 specific sequence (218–233), 6 basepairs encoding FL (underlined), and an α3 specific sequence (199–217) (5′-CAACTTGGACAATCGGTCCCTGGTAGTGAAGGAGGCCGGG-3′). A first-round PCR on α6 cDNA was performed with a sense primer (117–137) (5′-GTCCCCGCTCCCCTCCCCGTG-3′), containing a *Sph* I site, and an antisense primer (354–373) (5′-CTCTGCAGTGGAAGCGCTTC-3′). Primer positions for α6 cDNA correspond to the published sequence by Tamura *et al.* (1990). DNA amplification was performed using Pfu DNA polymerase (Stratagene, La Jolla, CA); after 35 cycles (1 min 94°C, 2 min 50°C, 2 min 72°C) the PCR products were isolated from agarose gel. Both products were mixed and used in a second-round PCR using the sense α6 primer (117–137) and the antisense α3 primer (322–336). The PCR conditions were similar as for first-round PCRs. The resulting PCR product was isolated from agarose gel, digested with *Sph* I and *Eco*RI, cloned into *Sph* I/*Eco*RI digested pUC18, and sequenced to check for correctness of the construct.

Full-length α3A cDNA was generated by ligation of the PCR derived α3 N terminus to the phage D17-derived α3 cDNA. This full-length α3A cDNA was subcloned into the *Hin*dIII/*Xba* I sites of the pRc/CMV expression vector (Invitrogen, San Diego, CA).

Transfections and Flow Cytometry

α3A plasmid DNA (10 μg) was transfected into K562 cells (5×10^6) by electroporation using the Bio-Rad (Richmond, CA) Gene Pulser set at 230 V and 960 μFD, as has been described for transfection of the α6A and α6B cDNAs (Delwel *et al.*, 1993). After 2 d, cells were selected with 1 mg/ml Geneticin (G418 sulfate) in RPMI medium. Stable transfectant bulk populations, which contained only a few α3A expressing cells, were sorted on the fluorescence-activated cell sorter (FACS) three times to enrich for α3A expressing cells. Transfectants were washed in PBS and incubated with a combination of mAbs to α3 (J143 and P1B5) (final concentration ascitic fluid 1:1000) for 45 min on ice. After being washed in PBS, cells were treated with fluorescein isothiocyanate labeled goat–anti-mouse IgG (Nordic, Tilburg, The Netherlands) for 45 min on ice, washed in PBS, and analyzed and sorted using a FACScan (Becton Dickinson, Mountain View, CA). Bulk populations strongly expressing α3A were obtained, of which several clones were generated by limiting dilution. FACS analysis of

the transfectants was performed as described above with mAbs 450-33D, P1B5, Sam-1, and K20.

Immunoprecipitation of ^{125}I-Labeled Cells

Transfectants were surface labeled with ^{125}I by the lactoperoxidase/hydrogen peroxide method as previously described (Sonnenberg *et al.*, 1987) and washed and solubilized in lysis buffer containing 1% (vol/vol) Nonidet P-40, 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin. Lysates were clarified at 14 000 rpm and precleared by incubation with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology). Precleared cell lysates were then added to protein A-Sepharose beads previously incubated with rabbit-anti-mouse IgG or rabbit-anti-rat IgG (Nordic) and the precipitating mAb. After incubation for 1 h at room temperature, the beads carrying the immune complexes were washed and treated with sodium dodecyl sulfate (SDS) sample buffer. Precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 5% polyacrylamide gel under nonreducing conditions according to Laemmli (1970).

Western Blot Analysis

EHS tumor laminin, immunopurified human kalinin (obtained as described above), and bovine heart and kidney laminins were dissolved in SDS-sample buffer and separated on a 5% polyacrylamide gel under reducing conditions according to Laemmli (1970). Gels were stained with the Bio-Rad silver stain kit or blotted to nitrocellulose following standard procedures. Blots were blocked in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and 1% (wt/vol) bovine serum albumin (BSA), and washed and incubated with antibodies (1:400 in TBS/Tween) for 60 min at room temperature. After washing, the blots were incubated with anti-rabbit IgG/Fc alkaline phosphatase conjugate (Promega Corp., Madison, WI) diluted 1:7500 in TBS/Tween (30 min, room temperature). After extensive washing of the blot, the color reaction was performed in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at room temperature.

Cell Adhesion, Inhibition, and Spreading Assays

Microtiter plates (96 well, Greiner GmBH, Trickenhausen, Germany) were coated with matrix components at a protein concentration of 20 μg/ml in PBS for 12–16 h at 4°C. The 96-well plates containing UMSCC-22B cell matrices (human matrix kalinin) were also used for adhesion assays. Plates were washed with PBS and incubated with 1% (wt/vol) BSA for 60 min at room temperature to block nonspecific adhesion.

Cells from exponentially growing cultures were washed in PBS and labeled with 400 μl Na_2 $^{51}CrO_4$ (1 mCi/ml) for 30 min at room temperature. After labeling, the cells were washed in Iscove's modified Dulbecco's medium containing 0.35% BSA (wt/vol) and resuspended at 1×10^6 cells/ml. In most assays, cells were stimulated by TS2/16 (1:5 hybridoma supernatant = 5 μg/ml) for 10 min at 37°C. One $\times 10^5$ cells/well were plated in triplicate in 96-well microtiter plates and incubated for 30 min at 37°C. Nonadherent cells were removed by inverting the microtiter plates onto tissue paper and adsorption of the medium. Plates were washed five times with Iscove's/0.35% BSA, whereafter the cells were lysed in 2% SDS. Radioactivity of lysed cells was measured in a γ-counter. In inhibition assays, activated cells were mixed with appropriate dilutions of antibodies for 10 min before plating onto coated plates for a 30-min adhesion assay.

The same procedures were used for cell spreading analysis on 24-well tissue culture plates. After 1 h incubation at 37°C, cells were washed in PBS, fixed with 3% formaldehyde for 15 min, and examined by a lightmicroscope (Zeiss, Germany). Photographs were taken with a Contax 167MT (Kyocera, Japan) camera using Tmax-100 films (Kodak, Rochester, NY).

RESULTS

Generation and Characterization of α3A and α6A Transfectants

To determine the ligand specificities of the α3Aβ1 and α6Aβ1 integrins, we transfected cDNAs encoding the α3A or α6A subunits into K562 cells, which only express α5β1, the fibronectin receptor. FACS analysis of two representative α3A and α6A transfectants showed the presence of the transfected α3 and α6 subunits, as well as that of the endogenous α5 and β1 subunits (Figure 1).

To establish whether the transfected cells express the A variants of α3 and α6, lysates of ^{125}I-surface labeled cells were immunoprecipitated with A-variant–specific mAbs. The anti-α3 mAbs J143, P1B5, and the anti-α3A specific mAb 7A3 all precipitated the α3β1 complex from α3A transfectants, demonstrating the presence of transfected α3A and its association with endogenous β1 (Figure 2, lanes 1, 2, and 4). Likewise, the anti-α6 mAb GoH3, polyclonal anti-α6A serum, and the anti-α6A specific mAb 1A10 precipitated the α6Aβ1 complex

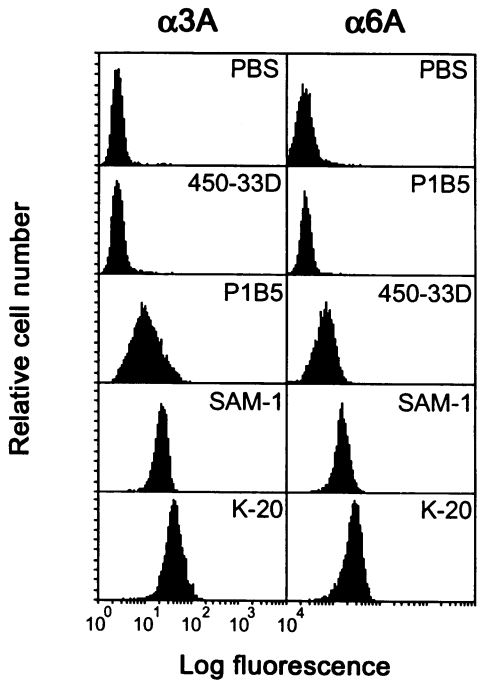

Figure 1. Cell surface expression of α3, α6, α5, and β1 integrin subunits on α3A and α6A transfectants. Flow cytometry of the transfectants was carried out with the following mAbs: P1B5 (anti-α3), 450-33D (anti-α6), Sam-1 (anti-α5), K20 (anti-β1), followed by incubation with FITC-labeled goat anti-mouse IgG. Negative controls are PBS, GoH3 for the α3A transfectants, and P1B5 for the α6A transfectants.

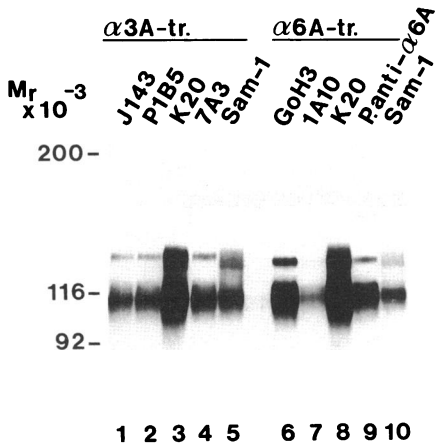

Figure 2. Immunoprecipitation analysis of α3A and α6A transfectants. ^{125}I-labeled cell lysates of α3A and α6A transfectants were immunoprecipitated with either anti-α3 mAbs J143, P1B5, and 7A3 (lanes 1, 2, and 4) or anti-α6 mAbs GoH3, 1A10, P.anti-α6A (lanes 6, 7, and 9) and with mAbs against β1 K20 (lanes 3 and 8) and α5 Sam-1 (lanes 5 and 10). Precipitates were analyzed by SDS-PAGE on a 5% nonreduced gel.

from α6A transfected cells, showing the association of transfected α6A with β1 (Figure 2, lanes 6, 7, and 9). K20 (anti-β1) precipitated α5β1 complexes in combination with either α3β1 or α6β1, and Sam-1 (anti-α5) exclusively precipitated α5β1 (Figure 2, lanes 3, 5, 8, and 10).

Biochemical Analyses of Immunopurified Human Kalinin and Bovine Kidney Laminins

We previously have shown that matrices deposited by UMSCC-22B cells contain human kalinin (Delwel *et al.*, 1993). Here, we purified human kalinin from UMSCC-22B culture medium by affinity chromatography using the anti-human kalinin mAb 4C7. Reduced immunopurified human kalinin and EHS tumor laminin (for comparison) were separated by SDS-PAGE and silver stained. The silver-stained gel showed three major protein bands of 160, 140, and 105 kDa, which were all recognized by a polyclonal anti-human kalinin antibody and which represent the kalinin A and B1 chains and a processed form of the B2 chain (Rousselle *et al.*, 1991; Marinkovich *et al.*, 1992a) (Figure 3A). The same antiserum did not react with EHS tumor laminin. There was also no blot reaction between the human kalinin preparation and an antiserum to EHS tumor laminin. This excludes the presence of both the laminin Ae, B1e, and B2e chains and of K-laminin, which shares the B1e and B2e chains in the kalinin preparation (Marinkovich *et al.*, 1992b), because the antiserum reacts with all those EHS laminin chains and with nidogen in EHS tumor laminin (Figure 3A). Furthermore, polyclonal antisera to the 80-kDa fragment of the merosin Am chain, s-laminin, nidogen, fibronectin, and collagen type IV did not react with the human kalinin preparation. Thus, human kalinin is the only protein present in the immunopurified preparation.

Similarly, reduced bovine kidney laminins and bovine heart laminins (for comparison) were also separated by SDS-PAGE and silver stained. The silver-stained bovine kidney preparation showed seven major protein bands of 400, 375, 290, 215, 200, 190, and 150 kDa, whereas in bovine heart laminins the Am (300 kDa) chain and its 80-kDa C-terminal fragment, the B1e (215 kDa), B2e (200 kDa), and B1s (190 kDa) chains and nidogen (150 kDa) were visualized (Paulsson *et al.*, 1991) (Figure 3B). Antisera to EHS tumor laminin and merosin reacted with the B1e (215 kDa) and B2e (200 kDa) chains in bovine kidney laminins. No Ae or Am chains were detected by these antisera in the bovine kidney preparation. This was confirmed by a negative reaction with polyclonal antibodies to the E3 fragment of the Ae chain and to the 80-kDa fragment of the Am chain. Thus, there are no Ae and Am chains in the bovine kidney laminin preparation. Kalinin chains were not present in

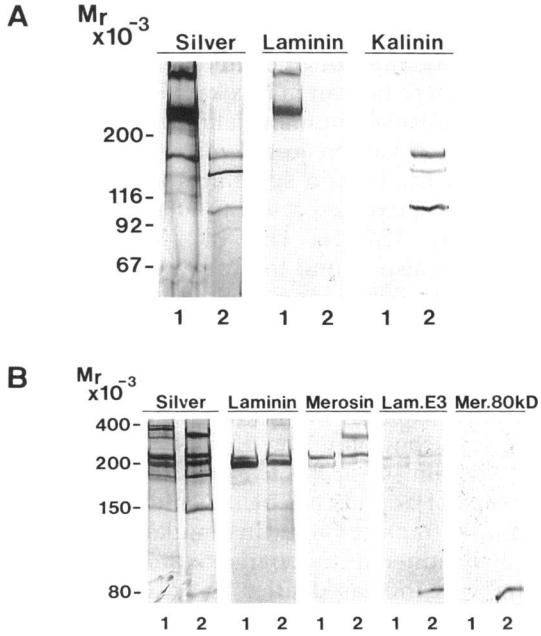

Figure 3. Immunopurified human kalinin consists of a single protein species and bovine kidney laminins possess a novel A chain. Samples were run on 5% reduced polyacrylamide gels, silver stained and blotted to nitrocellulose. (A) Silver-stained kalinin (lane 2) shows three major products of 160, 140, and 105 kDa. All products were detected by a polyclonal anti-human kalinin serum, whereas an antiserum to EHS tumor laminin did not react with any protein. Lane 1 contains EHS tumor laminin. (B) Silver-stained bovine kidney laminins (lane 1) shows seven major products of 400, 375, 290, 215, 200, 190, and 150 kDa. Lane 2 contains bovine heart laminins. Antisera to EHS tumor laminin and merosin detected the B1e (215 kDa) and B2e (200 kDa) chains. They did not detect Ae or Am chains; neither did antisera to the E3 fragment of the Ae chain and to the 80-kDa fragment of the Am chain. The 375-kDa protein band represents the novel A chain (Lindblom *et al.*, 1994).

this preparation either because an antiserum to human kalinin did not reveal any protein bands. Antisera to s-laminin and nidogen reacted with the B1s (190 kDa) chain and nidogen (150 kDa). The three protein bands of 400, 375, and 290 kDa, which do not react with any of the antisera used, may represent a single variant A chain and processed protein forms of this chain or they may be due to heterogeneity of the A chains, in which case all three would be new A chain variants. The most prominent band of 375 kDa has been referred to as a novel A chain by Lindblom *et al.* (1994).

Adhesion of α3A and α6A Transfectants to Different Extracellular Matrix Proteins

The ligand specificity of the α3Aβ1 integrin was compared with that of the α6Aβ1 integrin in adhesion assays with different ECM substrates. The two different clones used expressed the novel integrins at equivalent levels and were used either without prior activation or after treatment with TS2/16, which is a stimulatory anti-β1 mAb (Arroyo *et al.*, 1992; Van de Wiel-van Kemenade *et al.*, 1992).

Unstimulated α3A transfectants adhered to immunopurified human kalinin, cellular matrices containing human kalinin, and bovine kidney laminins (Figure 4). The adhesion to human matrix kalinin was weaker than to immunopurified human kalinin, probably because the amount of kalinin in the matrices is smaller. The adhesion to the kalinin substrates and bovine kidney laminins was increased after stimulation of α3A transfectants with TS2/16. The TS2/16-stimulated α3A transfectants also bound to human placental laminins. No binding to EHS tumor laminin, bovine heart laminins, collagen types IV and VI, or nidogen was detected.

Unstimulated α6A transfectants did not adhere to immunopurified human kalinin and only weakly to human matrix kalinin, in contrast to α3A transfectants (Figure 4). Strong adhesion of α6A transfectants to the human kalinin substrates was induced by TS2/16, the percentage of bound cells being similar as that of stimulated α3A transfectants. Unstimulated α6A transfectants also adhered to bovine kidney laminins, and binding was increased after TS2/16 treatment. Furthermore, TS2/16-stimulated α6A transfectants bound to EHS tumor laminin and bovine heart laminins consistent with our previous data (Delwel *et al.*, 1993) and to human placental laminins. As for α3A transfectants, collagen types IV and VI and nidogen were not adhesive for α6A transfected K562 cells. Neither of the substrates tested were adhesive for unstimulated or TS2/16-stimulated parental K562 cells. In conclusion, both α3A and α6A transfectants bound to human kalinin, bovine kidney laminins, and human placental laminins, whereas α6A transfected cells also adhered to murine EHS tumor laminin and bovine heart laminins.

Obviously, the α3A transfectants share their adhesive substrates with α6A transfectants; however, the binding

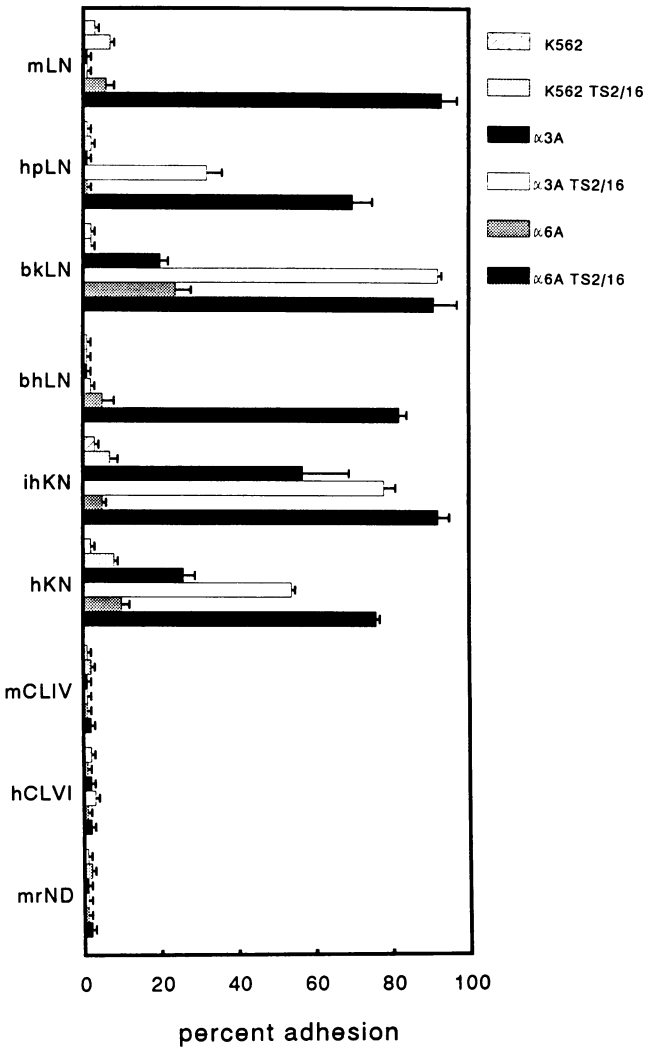

Figure 4. Adhesion of K562 cells and α3A and α6A transfectants to different ECM components. The cells were labeled with ^{51}Cr. Both unstimulated and TS2/16- (5 μg/ml) stimulated transfected cells were added to the substrates: EHS tumor laminin (mLN), human placental laminins (hpLN), bovine kidney laminins (bkLN), bovine heart laminins (bhLN), immunopurified human kalinin (ihKN), human kalinin deposited by UMSCC-22B cells (hmKN), murine collagen type IV (mCL), human collagen type VI (hCL), and murine recombinant nidogen (mrND). Error bars, SDs of three determinations within a representative of three experiments. Binding percentages are expressed as percentage of the total input per well (100%).

percentages of α3A and α6A transfectants to these substrates were not identical (Figure 4). To study the differences in adhesion in more detail, we performed adhesion assays using different concentrations of immunopurified human kalinin, bovine kidney laminins, and human placental laminins. The α3A transfectants adhered strongly to immunopurified human kalinin in all concentrations tested, whereas α6A transfectants did not bind (Figure 5A). After stimulation by TS2/16, both α3A and α6A transfectants adhered equally well (Figure

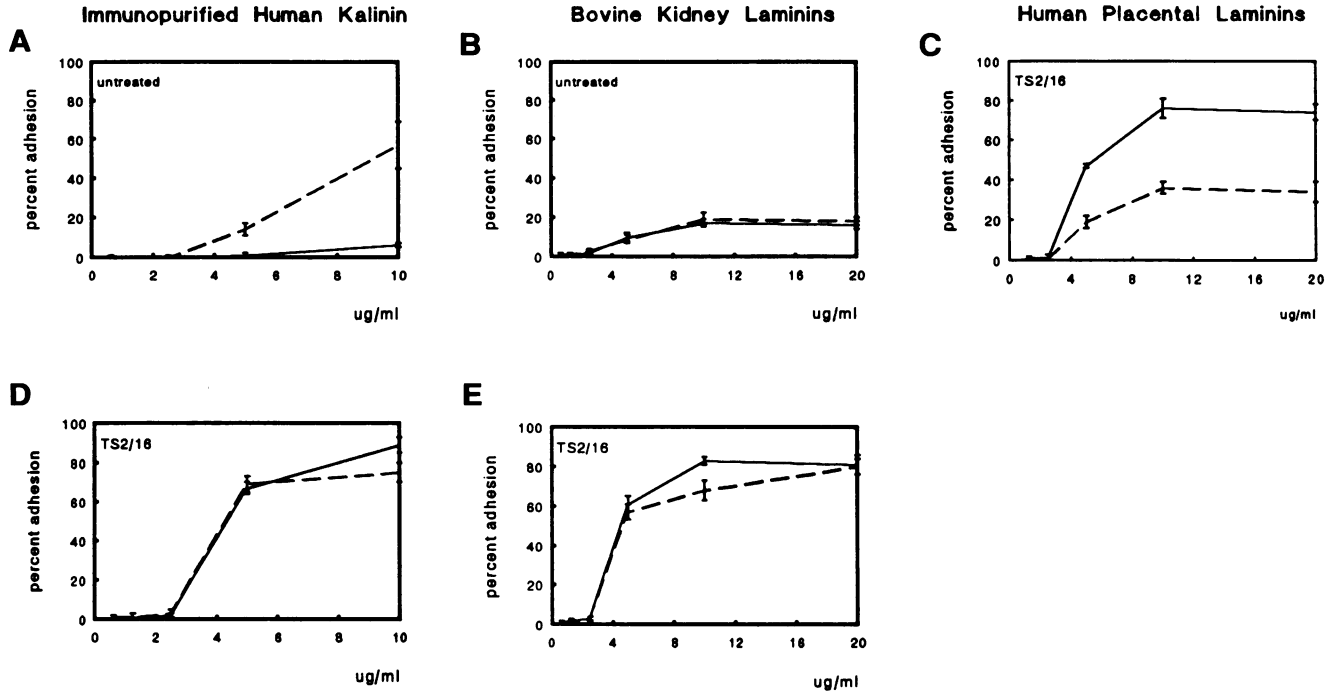

Figure 5. Adhesion of α3A and α6A transfectants to human kalinin, bovine kidney laminins, and human placental laminins. (A) Unstimulated α3A transfectants (– – –) adhered to immunopurified human kalinin; unstimulated α6A transfectants (——) failed to bind. (B) Both unstimulated transfectants adhered similarly to bovine kidney laminins. (C) TS2/16- (5 μg/ml) stimulated α6A transfectants bound better to human placental laminins than stimulated α3A transfectants. Both TS2/16-stimulated transfectants adhered to (D) immunopurified human kalinin and to (E) bovine kidney laminins. Error bars, SDs of three determinations within a representative of two experiments. Binding percentages are expressed as percentage of the total input per well (100%).

5D). Unstimulated and TS2/16-stimulated α3A and α6A transfectants adhered to a similar extent to bovine kidney laminins (Figure 5, B and E), whereas TS2/16-stimulated α6A transfectants adhered to human placental laminins in all concentrations tested better than the α3A transfected cells (Figure 5C). Thus, in transfected K562 cells the α3Aβ1 and α6Aβ1 integrins were present in a partially active state that enabled α3A transfectants to bind to kalinin and both transfectants to bovine kidney laminins. The activity of these integrins was also subjected to regulation, as kalinin binding by the α6A transfectants and binding to human placental laminins by the α3A and α6A transfectants required stimulation.

Antibody Inhibitions Demonstrate the Specificity of α3Aβ1 and α6Aβ1 Adhesions

The binding to human kalinin, bovine kidney laminins, and human placental laminins mediated by the α3Aβ1 and α6Aβ1 integrins was further studied in inhibition assays with specific antibodies to integrin subunits and matrix components.

Because the binding of the TS2/16-stimulated α3A transfectants to human placental laminins was rather weak; inhibition assays with these cells were not feasible and therefore we used a different α3A transfectant (clone Kα3A.6; see below) that expressed α3Aβ1 much more strongly. After stimulation with TS2/16, these cells bound more strongly to human placental laminins (60% of the total cells added bound) but still more weakly than the α6A transfectants. For all the other substrates, α3A and α6A transfectants expressing equivalent levels of α3Aβ1 and α6Aβ1 were used.

As shown in Figure 6 the adhesion of unstimulated and TS2/16-stimulated α3A and α6A transfectants to the various ECM components could be specifically blocked by the anti-α3 mAb, P1B5, or the anti-α6 mAb, GoH3, and was not affected by the anti-α5 mAb, Sam-1. The binding of unstimulated transfected cells was also completely blocked by the anti-β1 mAb, AIIB2, whereas binding of the TS2/16-stimulated transfectants was not affected by AIIB2, which is probably due to masking of the corresponding epitope by bound TS2/16 (Delwel *et al.*, 1993). These data demonstrate the involvement of the α3Aβ1 and α6Aβ1 integrins in adhesion to human kalinin, bovine kidney laminins, and human placental laminins.

The anti-human kalinin A chain mAb BM165 inhibited binding of the transfectants to human matrix kalinin and immunopurified human kalinin (Figure 6, A, B, E, and F), but it did not affect adhesion to the other sub-

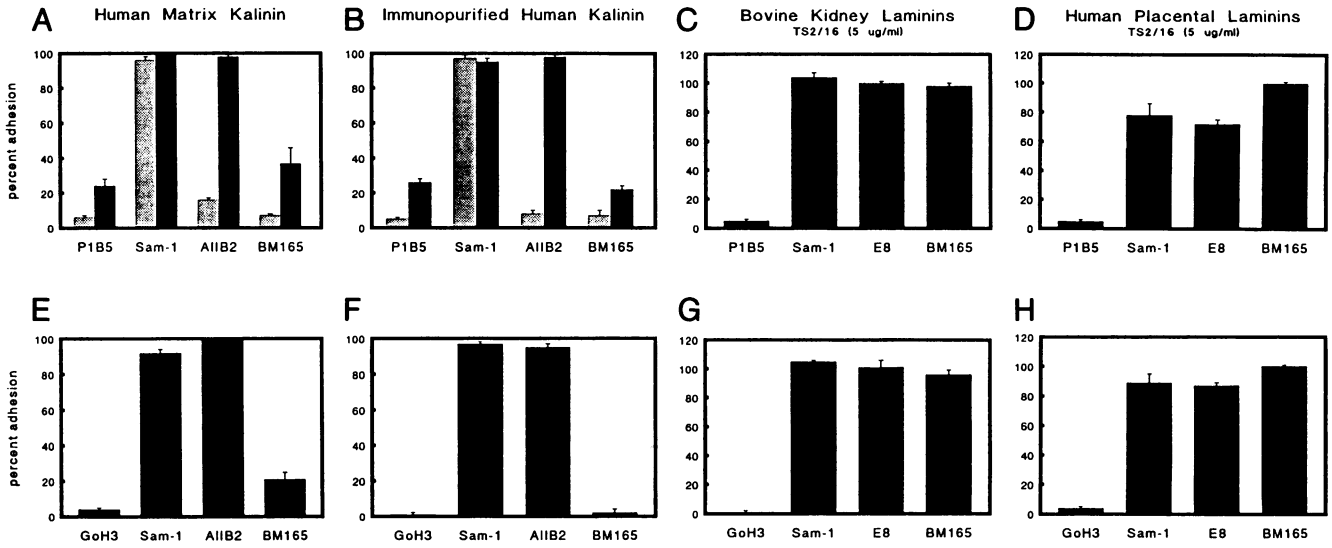

Figure 6. Inhibition of cell attachment to human kalinin, bovine kidney laminins, and human placental laminins by antibodies against integrin subunits and extracellular matrix components. Cells were labeled with ^{51}Cr. Unstimulated (▨) or TS2/16- (5 μg/ml) stimulated (■) cells were preincubated with or without antibody and added to the coated substrates. Antibodies used were P1B5 (anti-α3), GoH3 (anti-α6), Sam-1 (anti-α5), AIIB2 (anti-β1), BM165 (anti-human kalinin A chain), and E8 (anti-laminin proteolytic fragment E8). (A and B) Adhesion of α3A transfectants to human kalinin is mediated by the α3Aβ1 integrin. (E and F) The α6Aβ1 integrin mediates binding to human kalinin. α3Aβ1 also binds to bovine kidney laminins (C) and human placental laminins (D), as does α6Aβ1 (G and H). Total cells bound to the coated substrates in the absence of antibody is indicated as 100%. Error bars, SDs of three independent determinations within a representative of two experiments.

strates. The inhibitory effect of BM165 was less strong for the TS2/16-stimulated α3A transfectants, which may indicate that TS2/16 treatment increases the affinity of α3Aβ1 for human kalinin so much that it cannot be as efficiently blocked. The adhesion of the α3A and α6A transfectants to human placental laminins was weakly inhibited by an antiserum to EHS tumor laminin fragment E8 (Figure 6, D and H), whereas the adhesion to human kalinin and bovine kidney laminins was not affected. Presumably, the anti-E8 antibodies inhibit by binding to the B1e and/or B2e chains that are in close contact with the cell-binding site.

In conclusion, the α3Aβ1 and α6Aβ1 integrins are receptors for human kalinin, human placental laminins, and bovine kidney laminins. Furthermore, the α6Aβ1 integrin mediates cell adhesion to murine EHS tumor laminin and bovine heart laminins consistent with results of our previous work (Delwel *et al.*, 1993).

The α3Aβ1 Integrin is not a Fibronectin Receptor

The α3A transfectants adhered to fibronectin, and their adhesion could be increased by stimulation of the cells with TS2/16 (Figure 7B). Both the endogenous α5β1 integrin and the α3Aβ1 integrin might be involved in fibronectin binding. For studying these possibilities we used two α3A clones, Kα3A.8 and Kα3A.6, on which α3 was moderately or strongly expressed. Furthermore, the expression of the α5 subunit was slightly downregulated on the Kα3A.6 cells, whereas the expression of the β1 subunit was increased as compared with Kα3A.8 (Figure 7A) and K562 cells (not shown). Binding of these clones to fibronectin shows that Kα3A.8 cells bound better than Kα3A.6 cells, whereas TS2/16-treated cells showed no difference in adhesive activity (Figure 7B). Blocking studies with both TS2/16-stimulated clones revealed a complete inhibition of adhesion to fibronectin by Sam-1 (anti-α5), whereas no effect of P1B5 (anti-α3) was observed (Figure 7C). Taken together, these results demonstrate that the α3Aβ1 integrin is not a fibronectin receptor in these transfected clones. Similar data were previously obtained with α6A transfectants (Delwel *et al.*, 1993).

Unstimulated α3A Transfectants Spread on Kalinin

Finally, we examined whether α3A and α6A transfectants could spread when bound to their ligand human kalinin. Unstimulated α3A transfectants spread on human matrix kalinin within 1 h. TS2/16-stimulated cells showed enhanced binding, but the number of spread cells was similar (Figure 8, A and B). α6A transfectants only spread on human matrix kalinin after activation with TS2/16, but considerably fewer cells spread than in the case of α3A transfectants (Figure 8C).

DISCUSSION

In this study we used α3A and α6A transfected K562 cells to elucidate the ligand specificity of the α3Aβ1 integrin and to compare it with that of the homologous α6Aβ1 integrin. We show for the first time that the

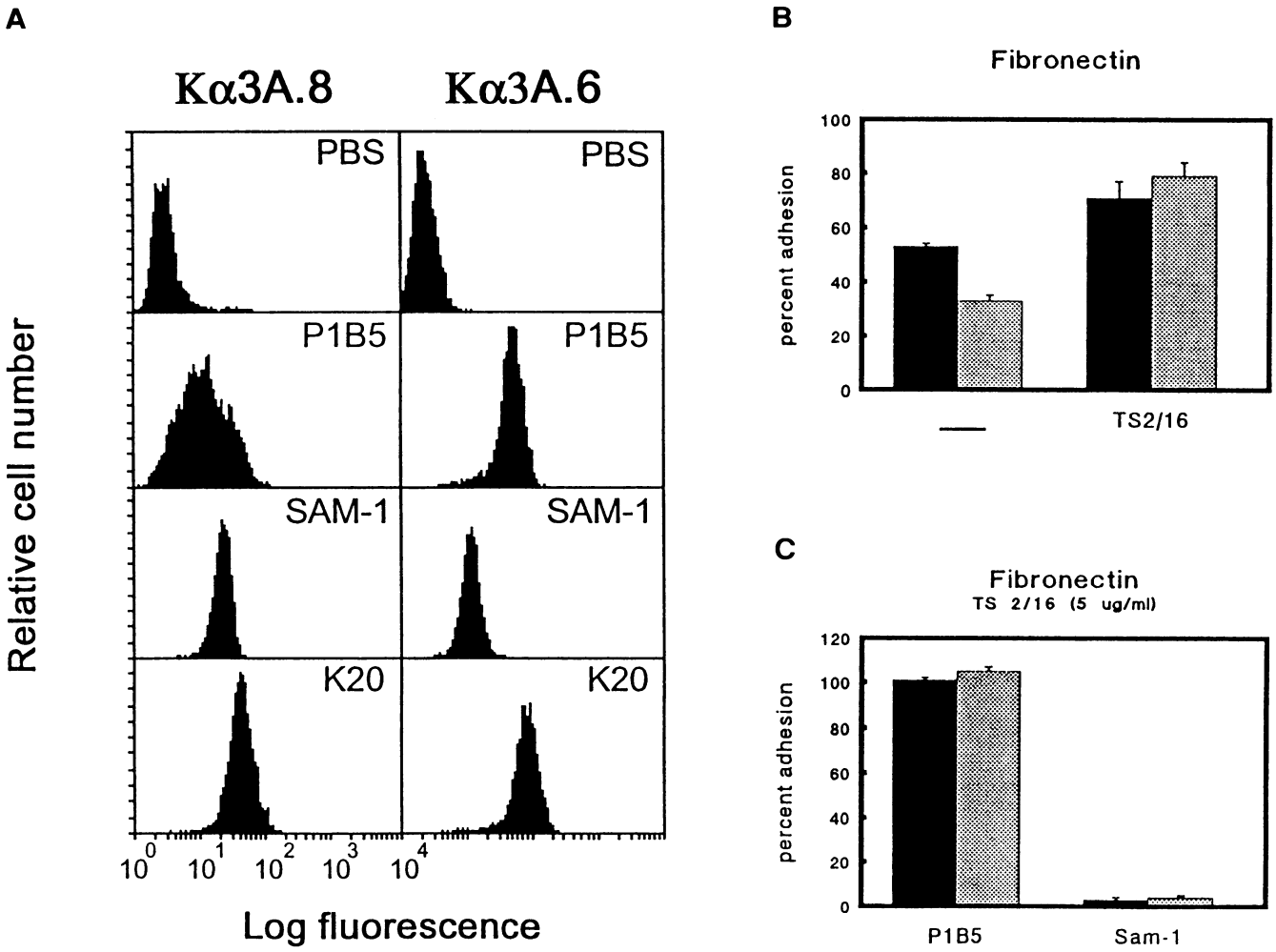

Figure 7. The α5β1 integrin, and not α3Aβ1, is the fibronectin receptor. (A) Cell surface expression of the α3, α5, and β1 subunits on α3A.8 and α3A.6 clones. (B) Unstimulated and TS2/16 (5 μg/ml) stimulated α3A.8 (■) and α3A.6 (▩) transfectants adhere to fibronectin. (C) Adhesion of TS2/16 stimulated (5 μg/ml) α3A transfectants to fibronectin is mediated by the α5β1 integrin.

α3Aβ1 integrin is a kalinin receptor. This conclusion is based on the following data: (1) α3A transfectants bound to immunopurified human kalinin; (2) α3A transfected cells also adhered to ECM deposited by the UMSCC-22B cell line, which previously has been shown to contain human kalinin (Delwel *et al.*, 1993); and (3) adhesion to human kalinin was completely blocked by a mAb to the human kalinin A chain (BM165) and binding to all kalinin substrates was blocked by an anti-α3 mAb (P1B5) or an anti-β1 mAb (AIIB2).

Carter and co-workers (1991) recently have identified epiligrin as a ligand for the α3β1 integrin (Wayner *et al.*, 1993). The epiligrin used in these studies consisted of four polypeptides of 200, 170, 145, and 135 kDa, respectively. The latter three polypeptides were described as epiligrin and the 200-kDa product as a laminin-like molecule coprecipitating with it. This product could either correspond to a novel A chain or to the B1e/B2e chains of K-laminin (Marinkovich *et al.*, 1992a,b). Thus, although epiligrin has been described as a ligand for α3β1, the laminin-like molecule could also be involved. Weitzman *et al.* (1993) also proposed that the α3β1 integrin, when expressed on transfected K562 cells, is an epiligrin/kalinin receptor. Because the adhesive substrates used in this latter study were prepared according to Carter *et al.* (1990), they probably contained epiligrin and the laminin-like protein. The kalinin substrate used in our study was shown not to be contaminated with other laminin-like proteins. We therefore attribute the observed adhesion entirely to kalinin.

We recently showed that the α6Aβ1 and α6Bβ1 integrin variants are kalinin receptors using kalinin containing matrices of human UMSCC-22B and RAC-11P/SD cells (Delwel *et al.*, 1993). Here, these observations were extended and confirmed for the α6Aβ1 integrin

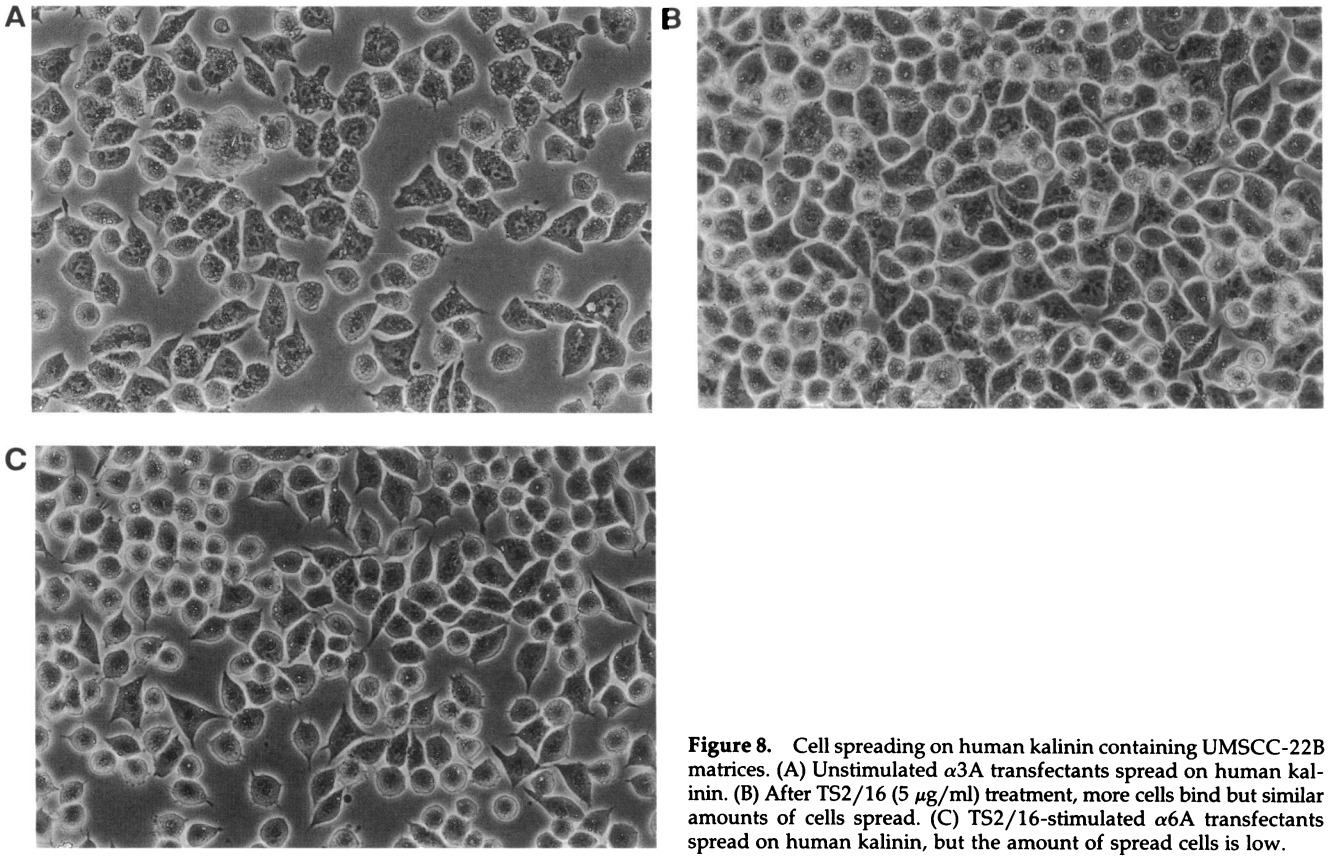

Figure 8. Cell spreading on human kalinin containing UMSCC-22B matrices. (A) Unstimulated α3A transfectants spread on human kalinin. (B) After TS2/16 (5 μg/ml) treatment, more cells bind but similar amounts of cells spread. (C) TS2/16-stimulated α6A transfectants spread on human kalinin, but the amount of spread cells is low.

by using immunopurified human kalinin. However, we observed substantial differences in the kalinin-binding properties of unstimulated α3A and α6A transfectants. Whereas unstimulated α3A transfectants adhered strongly to immunopurified human kalinin, unstimulated α6A transfectants did not bind at all. Furthermore, unstimulated α3A transfectants adhered quite strongly to and spread on human kalinin containing matrices, whereas unstimulated α6A transfectants adhered weakly. By contrast, stimulated α3A and α6A transfectants bound equally well to human kalinin, although α3A transfectants always spread more extensively. Thus, both α3A and α6A transfectants can bind to kalinin but apparently with different affinities. The activity of the α6Aβ1 integrin on K562 cells was subject to regulation and required activation, whereas the α3Aβ1 integrin is already partially active. Recently, Weitzman *et al.* (1993) also observed that α3β1 on K562 cells was constitutively active, in contrast to the collagen receptor α2β1 and the fibronectin/VCAM-1 receptor α4β1 on transfected K562 cells that, like α6Aβ1, required activation before they could bind to their ligands (Chan and Hemler, 1993; Masumoto and Hemler, 1993).

Other ligands for the α3Aβ1 and α6Aβ1 integrins identified in this study are bovine kidney laminins. That α3Aβ1 and α6Aβ1 may have a physiological role in the kidney is shown by the expression of the α3β1 integrin in the glomeruli and distal tubules and of the α6β1 integrin in all tubules (Korhonen *et al.*, 1990). Laminins purified from bovine kidney do not contain Ae and Am chains but contain a new variant A chain of 375 kDa (Lindblom *et al.*, 1994). The true identity of this 375-kDa protein, however, awaits further confirmation by sequence analysis and studies using monoclonal antibodies directed against this laminin A chain. The 400-kDa protein band in bovine kidney laminin may be a precursor of the 375-kDa protein and the 290 kDa a further processed form of the 375-kDa protein. Alternatively, these two protein bands may be different A chain variants.

Human placental laminins that possess the Am chain are also ligands for activated α3Aβ1 and α6Aβ1 integrins. After stimulation with TS2/16, α6A transfectants adhered more strongly to human placental laminins, whereas α3A transfectants only bound weakly. After stimulation, α6A but not α3A transfectants bound to bovine heart laminins, which like human placental laminins consist of a mixture of two laminin isoforms possessing the Am chain. Apparently, the affinity of α3Aβ1 for bovine heart laminins is lower than for human placental laminins and too low to mediate cell adhesion to this substrate. Lack of α3Aβ1 binding to

bovine heart laminins may be due to species specificity. Thus, it seems that after activation, α6Aβ1 becomes a high affinity receptor for laminins possessing the Am chain, whereas α3Aβ1 is a low affinity receptor. This is in line with the finding of Dedhar *et al.* (1992) that α6β1 but not α3β1 could be isolated by affinity chromatography on a human placental laminin column.

Using affinity chromatography on pepsin-digested human placental laminins, we previously have shown that α3β1 dominates over α6β1, that is, we could only isolate α6β1 after we had depleted the lysates of α3β1 (Sonnenberg *et al.*, 1991). These data seem to be in contrast with the higher affinity of α6β1 for laminins possessing the Am chain than of α3β1, but may be explained by either the exposure of cryptic binding sites on human placental laminins that are specifically recognized by α3β1 or to the presence of other laminin isoforms in the pepsin digested material that are not present in the placental laminins isolated by EDTA extraction. The possibility of unmasked cryptic binding sites by proteolysis is not without precedent because a cryptic binding site exposed on EHS tumor laminin, digested with pepsin, is recognized by αvβ3 (Nurcombe *et al.*, 1989; Sonnenberg *et al.*, 1990).

α3Aβ1 is not a receptor for EHS tumor laminin, which is consistent with previous studies (Kramer *et al.*, 1990; Lotz *et al.*, 1990; Carter *et al.*, 1991; Sonnenberg *et al.*, 1991; Dedhar *et al.*, 1992; Weitzman *et al.*, 1993). Like Weitzman (1993), who also used α3 transfected K562 cells, we have obtained no evidence that the α3Aβ1 integrin is a receptor for fibronectin. Furthermore, we could not detect binding of the α3A transfectants to nidogen nor to collagen types IV and VI, although nidogen and collagen type VI have been reported as ligands for α3β1 (Wayner and Carter, 1987; Takada *et al.*, 1988; Dedhar *et al.*, 1992). At present, it cannot be excluded that the α3Aβ1 integrin is a cell type–specific receptor like the collagen receptor α2β1 that on some cells also is a laminin receptor (Elices and Hemler, 1989; Languino *et al.*, 1989).

In summary, in transfected K652 cells, the α3Aβ1 integrin is a kalinin receptor and an activation-dependent receptor for bovine kidney laminins and human placental laminins. The α6Aβ1 integrin is an activation-dependent receptor for kalinin, human placental laminins, bovine kidney and heart laminins, and for EHS tumor laminin. Conclusively, both α3Aβ1 and α6Aβ1 are receptors for laminin isoforms, and, from the results of this study, it appears that α6Aβ1 is a more promiscuous receptor than α3Aβ1.

Note added in proof. Recently, Ziober *et al.* have shown alternative mRNA splicing in the extracellular domain of the α7 and α6 subunits, resulting in X1 or X2 variant forms. (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 α7 subunit are differentially expressed during development. J. Biol. Chem. *268*, 26773–26783). The α3A and α6A cDNAs used in the present study are of the X2 and X1 type, respectively.

ACKNOWLEDGMENTS

We gratefully acknowledge the many colleagues for providing the various reagents used in this study. We thank C.P. Engelfriet for critical reading of the manuscript. This work was supported by grants from the Dutch Cancer Society (NKI 91-260) and the "Nierstichting" (The Dutch Kidney Foundation; C91.1179). M.P. is supported by the Swiss National Science Foundation.

REFERENCES

Amiot, M., Bernard, A., Tran, H.C., Leca, G., Kanellopoulos, J.M., and Boumsell, L. (1986). The human cell surface glycoprotein complex (gp120,200) recognized by monoclonal antibody K20 is a component binding to phytohaemagglutinin on T cells. Scand. J. Immunol. *23*, 109–118.

Arroyo, A.G., Sánchez-Mateos, P., Campanero, M.R., Martin-Padura, I., Dejana, E., and Sánchez-Madrid, F. (1992). Regulation of the VLA integrin-ligand interactions through the β1 subunit. J. Cell Biol. *117*, 659–670.

Beck, K., Hunter, I., and Engel, J. (1990). Structure and function of laminin: anatomy of a multidomain glycoprotein. FASEB J. *4*, 148–160.

Brown, J.C., and Goodman, S.L. (1991). Different receptors for human placental laminin and murine EHS laminin. FEBS Lett. *282*, 5–8.

Carter, W.G., Ryan, M.C., and Gahr, P.J. (1991). Epiligrin, a new cell adhesion ligand for integrin α3β1 in epithelial basement membranes. Cell *65*, 599–610.

Carter, W.G., Wayner, E.A., Bouchard, T.S., and Kaur, P. (1990). The role of integrins α2β1 and α3β1 in cell-cell and cell-substrate adhesion of human epidermal cells. J. Cell Biol. *110*, 1387–1404.

Chan, B.M.C., and Hemler, M.E. (1993). Multiple functional forms of the integrin VLA-2 can be derived from a single α2 cDNA clone: interconversion of forms induced by an anti-β1 antibody. J. Cell Biol. *120*, 537–543.

Dedhar, S., Jewell, K., Rojiani, M., and Gray, V. (1992). The receptor for the basement membrane glycoprotein entactin is the integrin α3β1. J. Biol. Chem. *267*, 18908–18914.

Delwel, G.O., Hogervorst, F., Kuikman, I., Paulsson, M., Timpl, R., and Sonnenberg, A. (1993). Expression and function of the cytoplasmic variants of the integrin α6 subunit in transfected K562 cells: activation-dependent adhesion and interaction with isoforms of laminin. J. Biol. Chem. *268*, 25865–25875.

Domloge-Hultsch, N., Gammon, W.R., Briggaman, R.A., Gil, S.G., Carter, W.G., and Yancey, K.B. (1992). Epiligrin, the major human keratinocyte integrin ligand, is a major target in both an acquired autoimmune and an inherited subepidermal blistering skin disease. J. Clin. Invest. *90*, 1628–1633.

Ehrig, K., Leivo, I., Argraves, W.S., Ruoslahti, E., and Engvall, E. (1990). Merosin, a tissue-specific basement membrane protein, is a laminin-like protein. Proc. Natl. Acad. Sci. USA *87*, 3264–3268.

Elices, M.J., and Hemler, M.E. (1989). The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. Proc. Natl. Acad. Sci. USA *86*, 9906–9910.

Elices, M.J., Urry, L.S., and Hemler, M.E. (1991). Receptor functions for the integrin VLA-3: fibronectin, collagen, and laminin binding are differentially influenced by ARG-GLY-ASP peptide and by divalent cations. J. Cell Biol. *112*, 169–181.

Fox, J.W., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Mann, K., Timpl, R., Krieg, T., Engel, J., and Chu, M. (1991). Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. EMBO J. *10*, 3137–3146.

Gehlsen, K.R., Dickerson, K., Argraves, W.S., Engvall, E., and Ruoslahti, E. (1989). Subunit structure of a laminin-binding integrin and localization of its binding site on laminin. J. Biol. Chem. *264*, 19034–19038.

Gehlsen, K.R., Dillner, L., Engvall, E., and Ruoslahti, E. (1988). The human laminin receptor is a member of the integrin family of cell adhesion receptors. Science *241*, 1228–1229.

Green, T.L., Hunter, D.D., Chan, W., Merlie, J.P., and Sanes, J.R. (1992). Synthesis and assembly of the synaptic cleft protein S-laminin by cultured cells. J. Biol. Chem. *267*, 2014–2022.

Hemler, M.E., Sánchez-Madrid, F., Flotte, T.J., Krensky, A.M., Burakoff, S.J., Bhan, A.K., Springer, T.A., and Strominger, J.L. (1984). Glycoproteins of 210,000 and 130,000 M.W. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. J. Immunol. *132*, 3011–3018.

Higuchi, R., Krummel, B., and Saiki, R.K. (1988). A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. *16*, 7351–7367.

Hogervorst, F., Admiraal, L., Niessen, C., Kuikman, I., Janssen, H., Daams, H., and Sonnenberg, A. (1993). Biochemical characterization and tissue distribution of the A and B variants of the integrin $\alpha 6$ subunit. J. Cell Biol. *121*, 179–191.

Hogervorst, F., Kuikman, I., Van Kessel, A.G., and Sonnenberg, A. (1991). Molecular cloning of the human $\alpha 6$ integrin subunit: alternative splicing of $\alpha 6$ mRNA and chromosomal localization of the $\alpha 6$ and $\beta 4$ genes. Eur. J. Biochem. *199*, 425–433.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Isberg, R.R., and Leong, J.M. (1990). Multiple $\beta 1$ chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell *60*, 861–871.

Jaspars, L.H., van der Linden, H.J., Scheffer, G.L., Scheper, R.J., and Meyer, C.J.L.M. (1993). Monoclonal antibody 4C7 recognizes an endothelial basement membrane component that is selectively expressed in capillaries of lymphoid follicles. J. Pathol. *170*, 121–128.

Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B., and Tryggvason, K. (1992). A truncated laminin chain homologous to the B2 chain: structure, spatial expression, and chromosomal assignment. J. Cell Biol. *119*, 679–693.

Kantor, R.R.S., Mattes, M.J., Lloyd, K.O., Old, L.J., and Albino, A.P. (1987). Biochemical analysis of two cell surface glycoprotein complexes, very common antigen 1 and very common antigen 2. J. Biol. Chem. *262*, 15158–15165.

Keizer, G.D., te Velde, A.A., Schwarting, R., Figdor, C.G., and de Vries, J.E. (1987). Role of p150,95 in adhesion, migration, chemotaxis and phagocytosis of human monocytes. Eur. J. Immunol. *17*, 1317–1322.

Kennel, S.J., Epler, R.G., Lankford, T.K., Foote, L.J., Dickas, V., Canamucio, M., Cavalierie, R., Cosimelli, M., Venturo, I., Falcioni, R., and Sacchi, A. (1990). Second generation monoclonal antibodies to the human integrin $\alpha 6\beta 4$. Hybridoma *9*, 243–255.

Korhonen, M., Ylänne, J., Laitinen, L., and Virtanen, I. (1990). The $\alpha 1$-$\alpha 6$ subunits of integrins are characteristically expressed in distinct segments of developing and adult human nephron. J. Cell Biol. *111*, 1245–1254.

Kramer, R.H., Cheng, Y.F., and Clyman, R. (1990). Human microvascular endothelial cells use $\beta 1$ and $\beta 3$ integrin receptor complexes to attach to laminin. J. Cell Biol. *111*, 1233–1243.

Laemmli, Y.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Languino, L.R., Gehlsen, K.R., Wayner, E., Carter, W.G., Engvall, E., and Rouslahti, E. (1989). Endothelial cells use $\alpha 2\beta 1$ as a laminin receptor. J. Cell Biol. *109*, 2455–2462.

Leivo, I., and Engvall, E. (1988). Merosin, a protein specific for basement membranes of Schwann cells, striated muscle, and trophoblast, is expressed late in nerve and muscle development. Proc. Natl. Acad. Sci. USA *85*, 1544–1548.

Lindblom, A., Marsh, T., Fauser, C., Engel, J., and Paulsson, M. (1994). Characterization of native laminin from bovine kidney and comparison with other laminin variants. Eur. J. Biochem. *219*, 383–392.

Lotz, M.M., Korzelius, C.A., and Mercurio, A.M. (1990). Human colon carcinoma cells use multiple receptors to adhere to laminin: involvement of $\alpha 6\beta 4$ and $\alpha 2\beta 1$ integrins. Cell Regul. *1*, 249–257.

Marinkovich, M.P., Lunstrum, G.P., and Burgeson, R.E. (1992a). The anchoring filament protein kalinin is synthesized and secreted as a high molecular weight precursor. J. Biol. Chem. *267*, 17900–17906.

Marinkovich, M.P., Lunstrum, G.P., Keene, D.R., and Burgeson, R.E. (1992b). The dermalepidermal junction of human skin contains a novel laminin variant. J. Cell Biol. *119*, 695–703.

Masumoto, A., and Hemler, M.E. (1993). Multiple activation states of VLA-4: mechanistic differences between adhesion to CS1/fibronectin and to vascular cell adhesion molecule-1. J. Biol. Chem. *268*, 228–234.

Nurcombe, V., Aumailley, M., Timpl, R., and Edgar, D. (1989). The high-affinity binding of laminin to cells. Assignment of a major cell binding site to the long arm of laminin and of a latent cell binding site to its short arms. Eur. J. Biochem. *180*, 9–14.

Odermatt, E., Risteli, J., van Delden, V., and Timpl, R. (1983). Structural diversity and domain composition of a unique collagenous fragment (intima collagen) obtained from human placenta. Biochem. J. *211*, 295–302.

Ott, U., Odermatt, E., Engel, J., Furthmayer, H., and Timpl, R. (1982). Protease resistance and conformation of laminin. Eur. J. Biochem. *123*, 63–72.

Paulsson, M., Deutzmann, R., Timpl, R., Dalzoppo, D., Odermatt, E., and Engel, J. (1985). Evidence for coiled-coil α-helical regions in the long arm of laminin. EMBO J. *4*, 309–316.

Paulsson, M., and Saladin, K. (1989). Mouse heart laminin. J. Biol. Chem. *264*, 18725–18732.

Paulsson, M., Saladin, K., and Engvall, E. (1991). Structure of laminin variants. J. Biol. Chem. *266*, 17545–17551.

Pfaff, M., Aumailley, M., Specks, U., Knolle, J., Zerwes, H.G., and Timpl, R. (1993). Integrin and Arg-Gly-Asp dependence of cell adhesion to the native and unfolded triple helix of collagen type VI. Exp. Cell Res. *206*, 167–176.

Pytela, R., Pierschbacher, M.D., and Ruoslahti, E. (1985). Identification and isolation of a 140 kDa cell surface glycoprotein with properties expected of a fibronectin receptor. Cell *40*, 191–198.

Rousselle, P., Lunstrum, G.P., Keene, D.R., and Burgeson, R.E. (1991). Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. J. Cell Biol. *114*, 567–576.

Sanes, J.R., Engvall, E., Butkowski, R., and Hunter, D.D. (1990). Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J. Cell Biol. *111*, 1685–1699.

Sonnenberg, A., Gehlsen, K.R., Aumailley, M., and Timpl, R. (1991). Isolation of $\alpha 6\beta 1$ integrins from platelets and adherent cells by affinity chromatography on mouse laminin fragment E8 and human laminin pepsin fragment. Exp. Cell Res. *197*, 234–244.

Sonnenberg, A., Janssen, H., Hogervorst, F., Calafat, J., and Hilgers, J. (1987). A complex of platelet glycoprotein Ic and IIa identified by a rat monoclonal antibody. J. Biol. Chem. *262*, 10376–10383.

Sonnenberg, A., Linders, C.J.T., Modderman, P.W., Damsky, C.H., Aumailley, M., and Timpl, R. (1990). Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that α6β1 but not α6β4 functions as a major receptor for fragment E8. J. Cell Biol. *110*, 2145–2155.

Sonnenberg, A., Modderman, P.W., and Hogervorst, F. (1988). Laminin receptor on platelets is the integrin VLA-6. Nature *336*, 487–489.

Takada, Y., Murphy, E., Pil, P., Chen, C., Ginsberg, M.H., and Hemler, M.E. (1991). Molecular cloning and expression of the cDNA for α3 subunit of human α3β1 (VLA-3), an integrin receptor for fibronectin, laminin, and collagen. J. Cell Biol. *115*, 257–266.

Takada, Y., Wayner, E.A., Carter, W.G., and Hemler, M.E. (1988). Extracellular matrix receptors, ECMRII and ECMRI, for collagen and fibronectin correspond to VLA-2 and VLA-3 in the VLA family of heterodimers. J. Cell. Biochem. *37*, 385–393.

Tamura, R.N., Rozzo, C., Starr, L., Chambers, J., Reichardt, L.F., Cooper, H.M., and Quaranta, V. (1990). Epithelial integrin α6β4: complete primary structure of α6 and variant forms of β4. J. Cell Biol. *111*, 1593–1604.

Timpl, R. (1989). Structure and biological activity of basement membrane proteins. Eur. J. Biochem. *180*, 487–502.

Timpl, R., Paulsson, M., Dziadek, M., and Fujiwara, S. (1987). Basement membranes. Methods Enzymol. *145*, 363–391.

Tomaselli, K.J., Hall, D.E., Reichardt, L.T., Flier, L.A., Gehlsen, K.R., Turner, D.C., and Carbonetto, S. (1990). A neuronal cell line (PC12) expresses two β1-class integrins—α1β1 and α3β1—that recognize different neurite outgrowth promoting domains in laminin. Neuron *5*, 651–662.

Van de Wiel-van Kemenade, E., van Kooyk, Y., de Boer, A.J., Huybens, R.J.F., Weder, P., van de Kasteele, W., Melief, C.J.M., and Figdor, C.G. (1992). Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be triggered through the β subunit of VLA. J. Cell Biol. *117*, 461–470.

Wayner, E.A., and Carter, W.G. (1987). Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. J. Cell Biol. *105*, 1873–1884.

Wayner, E.A., Carter, W.G., Piotrowicz, R.S., and Kunicki, T.J. (1988). The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. J. Cell Biol. *107*, 1881–1891.

Wayner, E.A., Gil, S.G., Murphy, G.F., Wilke, M.S., and Carter, W.G. (1993). Epiligrin, a component of epithelial basement membranes is an adhesive ligand for α3β1 positive T lymphocytes. J. Cell Biol. *121*, 1141–1152.

Weitzman, J.B., Pasqualini, R., Takada, Y., and Hemler, M.E. (1993). The function and distinctive regulation of the integrin VLA-3 in cell adhesion, spreading, and homotypic cell aggregation. J. Biol. Chem. *268*, 8651–8657.

Werb, Z., Tremble, P.M., Behrendtsen, O., Crowley, E., and Damsky, C.H. (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. *109*, 877–889.