Biochemical Characterization and Tissue Distribution of the A and B Variants of the Integrin α6 Subunit

F. Hogervorst,* L. G. Admiraal,* C. Niessen,* I. Kuikman,* H. Janssen,§ H. Daams,‡ and A. Sonnenberg*

*Department of Immunohematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; and Divisions of §Cell Biology and ‡Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Abstract. Two cytoplasmic variants of the α6 integrin, α6A and α6B, have been identified previously (Hogervorst, F., I. Kuikman, A. G. van Kessel, and A. Sonnenberg. 1991. *Eur. J. Biochem.* 199:425–433; Cooper, H. M., R. N. Tamura, and V. Quaranta. 1991. *J. Cell Biol.* 115:843–850). Using synthetic peptides, containing sequences of their cytoplasmic domains, we have produced mAbs specific for either of the variants. These antibodies reacted with a variety of different epithelial tissues. In some tissues (e.g., salivary gland) both variants could be detected while in others only one of the variants was found (e.g., α6A in epidermis and α6B in kidney). Among nonepithelial cells and tissues, perineural fibroblasts and Schwann cells in peripheral nerves and platelets reacted with anti-α6A, while microvascular endothelia reacted with both anti-α6A and anti-α6B. From our immunohistochemical results there is no evidence that combination with β1 or β4 is restricted to one of the two variants of α6. This was confirmed by immunoprecipitation studies which showed that both β1 and β4 were coprecipitated by both anti-α6A or anti-α6B antibodies from cells. Also, the distribution of α6A and α6B subunits associated with β1 on cells attached to laminin was similar: both were found in focal contacts colocalizing with vinculin. In contrast, the α6A subunit, associated with β4 in cultures of a squamous cell carcinoma cell line, was found to codistribute with bullous pemphigoid antigen 230 in hemidesmosomal-like structures.

The α6A and α6B variants, immunoprecipitated from various cell lines, exhibited slightly different electrophoretic mobilities. Analysis of the antigens under reducing conditions showed that the mobility of the light chains, but not of the heavy chains, is different. In addition, in some cells the light chains of α6A and α6B, each are of two different sizes. Treatment with *N*-glycanase showed that these two light chain variants of α6A and α6B are not due to differences in N-linked glycosylation, and may therefore represent alternative proteolytic products of the α6 precursor.

We further demonstrate that α6A, but not α6B, is a major target for PMA-induced phosphorylation. Phosphorylated α6A contained phosphoserine and a small amount of phosphotyrosine. There are also two variants of the integrin α3 subunit with different cytoplasmic domains, but in the cell lines examined only α3A could be demonstrated by RT-PCR. The α3A subunit exhibits increased phosphorylation in cells treated with PMA but to a lesser degree than does α6A. Like α6A, the α3A subunit is phosphorylated both on serine and weakly on tyrosine. Phosphorylation of the A but not of the B variants of the α6 subunit may indicate different regulation mechanisms for the two subunits. Furthermore, phosphorylation of α subunits as they occur in α3 and α6 might influence their function.

Over the past few years, the number of identified integrins has considerably increased. In addition to novel α and β subunits, new combinations of α and β subunits have been recognized (1, 16, 29). The complexity of the integrin family was further increased by the discovery that, due to alternative splicing, variants of α and β subunits occur (16). The integrin α6 subunit has been shown to combine with either β1 (11, 34) or β4 (12, 19, 36). The α6β1 integrin is expressed on platelets, lymphocytes, epithelial cells, and a variety of other cell types (37), and functions as a laminin receptor (3, 35, 38, 41). The tissue distribution of the α6β4 integrin is more restricted. It is found in various epithelial tissues, endothelia, and peripheral nerves (22, 37). In skin and cornea, α6β4 is concentrated in hemidesmosomes, suggesting a role in epithelial cell–basement membrane interaction (18, 40, 43). It has been shown that α6β4 on colon carcinoma cells binds to laminin (25, 26). The ligand for α6β4 on other cell types is not known. Keratinocytes which express

F. Hogervorst, C. Niessen, I. Kuikman, and A. Sonnenberg's present address is Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

high levels of $\alpha6\beta4$ poorly bind to laminin (6, 9). Moreover, $\alpha6\beta4$ on normal mammary cells and mammary tumor cell lines has no detectable affinity for laminin, in contrast to $\alpha6\beta1$ expressed on these cells (38, 41).

The $\beta4$ subunit is a transmembrane molecule with an extracellular domain that is homologous to that of other β subunits, but with a unique, long cytoplasmic domain of ~1,000 amino acids (14, 44). Furthermore, there is evidence that the $\beta4$ mRNA can be alternatively spliced, leading to variants of $\beta4$ with different cytoplasmic domains (14, 44, 49).

Recently, we and others have shown that the $\alpha6$ subunit is encoded by two different mRNA's (7, 15, 50). The two $\alpha6$ variants, called $\alpha6A$ and $\alpha6B$, have unique cytoplasmic domains of 36 and 54 amino acids, respectively. The GFFKR sequence, which is located immediately carboxyterminal to the transmembrane domain of every α subunit cloned so far, is the only homologous sequence of the $\alpha6A$ and $\alpha6B$ cytoplasmic domains. PCR analysis has demonstrated that most carcinoma cell lines contain both $\alpha6A$ and $\alpha6B$ mRNAs, although the relative amounts of the two variants seem to vary (15). By contrast, most normal tissues appear to have mRNA for only one of the two $\alpha6$ variants (50).

In this report we describe the production of $\alpha6A$ and $\alpha6B$ specific mAbs. By immunohistochemistry using these mAbs we show that the $\alpha6A$ and $\alpha6B$ subunits have distinct distribution patterns. Furthermore, we provide evidence for the alternative usage of proteolytic cleavage sites in the precursor $\alpha6$ subunit. We also show that in several carcinoma cell lines $\alpha6A$ but not $\alpha6B$ is a major target of phorbol 12-myristate 13-acetate (PMA)-induced phosphorylation.

Materials and Methods

Cell Lines and mAbs

The following human cell lines were used: HBL-100, normal mammary epithelial cells; OVCAR-4, ovarian carcinoma cells; A549, breast carcinoma cells; T24, bladder carcinoma cells; Tera-2 clone 13, an embryonal carcinoma cell line (obtained as a gift from Dr. C.L. Mummery, Hubrecht Lab, Utrecht, The Netherlands); and UMSSC-22B, squamous cell carcinoma cell line (kindly provided by Dr. T.E. Carey, University of Michigan, Ann Arbor, Michigan). Cell lines were cultured in DME, supplemented with 10% FCS. The rat mAb GoH3, which is specific for the integrin $\alpha6$ subunit and the mouse mAb C17 against the integrin $\beta3$ subunit have been described previously (34, 53). The mouse mAb J143, which recognizes the integrin $\alpha3$ subunit (J143) was a gift of Dr. A. Albino (20). Dr. M. Hemler kindly provided the mouse mAb A-1A5 against the $\beta1$ (10) and Dr. S. Kennel provided the rat mAb 439-9B against the integrin $\beta4$ subunit (21). Rabbit anti-bullous pemphigoid antigen 230 (BPA 230) was produced by immunization with a fusion protein containing the carboxy-terminal part of BPA 230 (51). It was kindly provided by Dr. J. R. Stanley (National Institutes of Health, Bethesda, Maryland).

Generation of Hybridomas to $\alpha6A$ and $\alpha6B$

To immunize mice against the $\alpha6A$ or $\alpha6B$ antigens, we synthesized a 28-amino acid-long peptide corresponding to the COOH-terminal portion of the cytoplasmic domain of $\alpha6A$, KKDHYDATYHKAEIHAQPSDKERLTSDA, and a 34-amino acid peptide corresponding to an internal sequence of the cytoplasmic domain of $\alpha6B$, SRYDDSVPRYHAVRIRKEEREIKDEKYIDNLEKK. An additional cysteine residue was synthesized at the NH_2 terminus to couple the peptides to keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, MO.). Both free and coupled peptides were used for immunization of Balb/c mice. After four immunizations the mice were sacrificed for fusion. The fusion was carried out with polyethylene glycol (PEG) 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 in a 96-well tissue culture plate and after 1 d, selection medium (hypoxanthine/azaserine) was added. 10–14 d after fusion the supernatants were collected and screened for antibodies in an ELISA, as described below. Selected hybridomas were cloned by limiting dilution and were injected into pristane-primed Balb/c mice for production of ascitic fluid.

ELISA for Screening of Hybridoma Supernatants

Maxisorb-plates (Nunc, Roskilde, Denmark) were coated overnight with 100 ng peptide/well in PBS at 4°C. Plates were subsequently washed with PBS/Tween-20 (0.05%) and incubated for 1 h at room temperature with PBS/FCS (2%) to reduce nonspecific binding. After an additional wash the plates were incubated with hybridoma supernatant for 1 h at room temperature. The plates were washed three times with PBS/Tween-20 after which goat anti-mouse Ig conjugated to alkaline phosphatase (Promega Corp., Madison, WI) was added. After incubation at room temperature for 1 h, the plates were washed. Antibody binding was detected by incubation with *p*-nitrophenyl phosphate substrate (Sigma Chemical Co.).

Reverse Transcription and PCR

cDNA synthesis of total RNA or messenger RNA and amplification with the PCR were performed as described (15). The primers used for the $\alpha6$ PCR were: (*a*) sense primer position 2656–2675, 5'-CTAACGGAGTCTCACAACTC-3', and (*b*) antisense primer position 3514–3532, 5'-CGAAGGTACAGTTTTAACT-3' (15), and for the $\alpha3$ PCR: (*a*) sense primer position 2963–2977, 5'-ACCATCAACATGGAG-3'; and (*b*) antisense primer position 3613–3630, 5'-CTTACAACGCCGAGTGCA-3' (48). Amplified DNA was analyzed on 1.25% agarose gels.

Immunoprecipitation and Electrophoresis

Adherent cells and human platelets were surface labeled with ^{125}I by the lactoperoxidase/hydrogen peroxide method as previously described (34), washed and solubilized in lysis buffer containing 1% (vol/vol) NP-40, 20 mM Tris, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml leupeptin. The lysates were clarified at 15,000 rpm and precleared by incubation with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). After incubation with mAbs and rabbit anti-mouse or anti-rat antibodies, immune complexes were collected with protein A-Sepharose. Treatment of the immunoprecipitates with *N*-glycanase (Genzyme Corp., Cambridge, MA) was performed as described (37). Immunoprecipitated material was analyzed by SDS-PAGE.

Immunoperoxidase

Immunoperoxidase staining of cryostat sections of various human tissues was carried out by an indirect procedure using HRP-conjugated second antibody (33). Reactivity was visualized using hydrogen peroxide and 3'3'-diaminobenzidine as substrate. The sections were counterstained with hematoxylin and mounted in Depex (British Drug House Chemicals, Poole, UK). Control reactions were performed with Sp2/O culture medium.

Immunofluorescent Staining of Focal Contacts

Glass coverslips were coated overnight at 4°C with 20 μg/ml laminin in PBS. After washing with PBS, the coverslips were incubated with PBS/BSA (0.35% wt/vol) to reduce nonspecific binding of cells to the glass. Cultured adherent OVCAR-4 cells were detached with trypsin/EDTA and washed twice with serum-free culture medium containing 0.35% BSA. The cells were allowed to attach to the coated coverslips for 2 h at 37°C. The nonadherent cells were then removed by washing the coverslips with PBS. The attached cells were fixed with 1% (wt/vol) paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.5% (vol/vol) Triton X-100 in PBS for 5 min at room temperature, and then blocked by incubation in PBS/BSA. They were incubated for 30 min at 37°C with primary antibody, washed with PBS and then incubated for 30 min at 37°C with fluorescein isothiocyanate coupled goat antibodies against mouse IgG (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) or Texas red-coupled donkey anti-rabbit IgG (Amersham Corp., Buckinghamshire, UK). The coverslips were washed, mounted in PBS containing 50% glycerol and 10% (wt/vol) 1.4 diazobicyclo-2,2,2,-octane (Janssen Biochemica, Beerse, Belgium) and viewed under a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence or a confocal scanning laser microscope (BioRad, Richmond, CA).

Table I. Binding of mAbs 1A10 and 6B4 to Immobilized Peptides in ELISA

No.	Peptide	Reactivity in ELISA 1A10	6B4
α6A(28)	(C) KKDHYDATYHKAE I HTQPSDKERLTSDA*	+++	–
47	NKKDHYDATYHKAE I	–	–
14	TYHKAE I HTQPSDK	–	–
15	HTQPSDKERLTSDA	++	–
α3A(29)	(C) RTRALYEAKRQKAEMKSQPSETERLTDDY	–	–
α6B(47)	(C) SRYDDSVPRYHAVR I RKEERE I KDEKY I DNLEKKQW I TKWNRNESYS	–	+++
α6B(34)	(C) SRYDDSVPRYHAVR I RKEERE I KDEKY I DNLEKK*	–	+++
76	PRYHAVR I RKEER	–	–
48	AVR I RKEERE I KDE	–	±
74	ERE I KDEKY I DNLE	–	++
30	EKY I DNLEKKQW I TK	–	–
α3B(32)	(C) TRYYQ I MPKYHAVR I REEERYPPPGSTLPTKK	–	–

* Peptides used for immunization.

Immunofluorescent Staining of Stable Anchoring Contacts

For stable anchoring contact (SAC)[1] staining, coverslips were used that were not coated, but only washed with alcohol. After plating UMSSC-22B cells on these coverslips, they were cultured for 2 d in DME, supplemented with 10% FCS. Cells were fixed as above and incubated with primary antibody, followed by appropriate secondary antibodies coupled with fluorescein dyes. BODIPY-conjugated goat anti-rabbit IgG and Texas red-conjugated goat anti-rat IgG were purchased from Molecular Probes, Inc. (Eugene, OR) and Texas red-conjugated sheep anti-mouse IgG was from Amersham Corp.

^{32}P-labeling and PMA Treatment

Cell cultures (25 cm^2, 80% confluency) were washed twice with phosphate-free MEM (Flow Laboratories, Irvine, UK), incubated in the same medium for 10–20 min at 37°C, and labeled for 2 h at 37°C with 0.5 mCi/ml [^{32}P]orthophosphate (carrier free; Amersham). PMA (Sigma Chemical Co.) was added for 15 min at the end of the labeling period at a final concentration of 20 ng/ml. Labeled cells were washed twice with cold PBS and lysed in lysis buffer containing 2 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium phosphate, pH 7.5. The lysate was then used for immunoprecipitation analysis.

Phosphoamino Acid Analysis

Bands were cut out of the fixed and autoradiographed SDS-gels and proteins were boiled for 10 min in 50 mM NH_4HCO_3, 0.1% SDS and 5% β-mercaptoethanol and extracted for 3 h at 37°C. The extracted proteins were precipitated by TCA (15%) and ribonuclease (20 μg/ml) was used as carrier protein. The precipitates were washed twice with ice-cold acetone and, after drying, dissolved in 75 μl 6 N HCl. The proteins were hydrolyzed for 1 h at 110°C. The amino acid mixture was resolved by electrophoresis on thin layer cellulose plates in two dimensions at pH 1.9 and pH 3.5 in the presence of unlabeled phosphoamino acids (5). The plates were dried and the positions of the unlabeled phosphoamino acids were visualized by staining with 0.15% (wt/vol) ninhydrin in acetone. Labeled phosphoamino acids were identified after autoradiography.

Results

Production of mAbs to α6A and α6B

Hybridomas were produced from spleen cells of mice immunized with peptides comprising the main portion of the cytoplasmic domains of the α6A and α6B variants, which were coupled to keyhole limpet hemocyanin. Two mAbs, 1A10 and 6B4, were selected because they reacted in ELISA with either α6A or α6B, respectively. These two antibodies were classified as IgG1.

Fine Mapping of the Epitopes Recognized by 1A10 and 6B4

To identify more precisely the epitopes recognized by the mAbs 1A10 and 6B4, overlapping peptides corresponding to regions of the cytoplasmic domains of α6A and α6B were synthesized. Furthermore α3A and α3B peptides of regions similar to those from α6A and α6B used for immunization were synthesized. Table I shows the reactivity of the mAbs with the various peptides in ELISA. The 1A10 antibody reacted strongly with the peptide used for immunization and with one of the three smaller peptides, namely the one representing the 14 carboxyl-terminal amino acids of the cytoplasmic domain of α6A. No reactivity was observed with the α3A peptide. The 6B4 mAb reacted with the 35-amino acid peptide used for immunization, as well as with the 48 mer, representing the almost complete cytoplasmic domain of α6B. The antibody also showed reactivity with no. 74 of the smaller peptides and reacted very weakly with no. 48. The antibody did not react with the α3B peptide.

Expression of mRNAs for the A and B Variants of α3 and α6 in Cell Lines

For further characterization of the antibodies, cell lines expressing α6A and/or α6B were needed. To identify such cell lines, reverse transcription (RT)-PCR analysis of the two α6 mRNAs as well as those for the two α3 variants was performed. PCRs were carried out on cDNAs prepared from the various cell lines, using α6 and α3 primer sets covering the alternatively spliced regions. The results are shown in Fig. 1. The α6 mRNA in UMSSC-1, T24, and a keratinocyte cDNA library was almost exclusively for the A variant, whereas it was mainly for the B variant in Colo 320. For the present study we selected the following cell lines: HBL-100, OVCAR-4, T24, and Tera-2 (which contains solely α6B mRNA) (13*b*) and A549.

In contrast to the α6 PCR, the α3 PCR showed only one

1. *Abbreviations used in this paper*: RT-PCR, reverse transcription-PCR; SAC, stable anchoring contact.

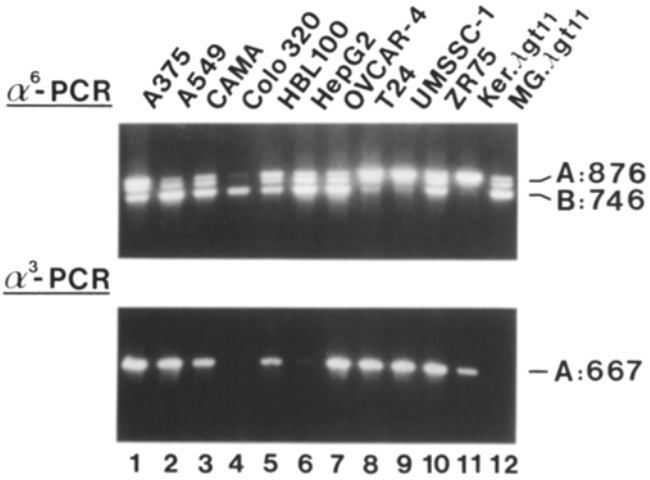

Figure 1. Detection of α3 and α6 mRNAs in different cell lines by PCR. PCR was performed as described in Materials and Methods. cDNA was synthesized from mRNA of A375 (melanoma, lane *1*), A549 (mamma carcinoma, lane *2*), CAMA (mamma carcinoma, lane *3*), Colo 320 (colon carcinoma, lane *4*), HBL-100 (normal mammary cells, lane *5*), HepG2 (hepatoma, lane *6*), OVCAR-4 (ovarian carcinoma, lane *7*), T24 (bladder carcinoma, lane *8*), UMSSC-1 (squamous carcinoma, lane *9*), ZR-75 (mamma carcinoma, lane *10*), and total phage DNA from human keratinocyte (lane *11*) and human mammary gland λgt11 cDNA libraries (lane *12*). The results of the α6 PCR are shown in the top panel and those of the α3 PCR at the bottom. The PCR products were separated on 1.25% agarose gels and stained with ethidium bromide.

product, corresponding to the α3A mRNA, in all cell lines tested. This suggests that the regulation of splicing of α3 and α6 is not coordinated.

Specificity of the α6A and α6B mAbs

When a lysate of ^{125}I-surface-labeled HBL-100 cells was used for immunoprecipitation with the 1A10 antibody, both α6β1 and α6β4 integrins were recovered (Fig. 2). The 1A10 antibody also immunoprecipitated these two integrins in the presence of 100 μg/ml α6B peptide, while the same concentration of α6A peptide completely blocked this precipitation. Similarly, the immunoprecipitation of α6β1 and α6β4 by 6B4 was blocked by the α6B peptide. The α6A and α6B peptides had no effect on the recovery of α6β1 and α6β4 when the mAb GoH3 was used for immunoprecipitation. These results confirm the specific reactivity of the 1A10 and 6B4 antibodies with the α6A and α6B variants, respectively, and demonstrate that both α6A and α6B subunits can associate with β1 or β4 (50). Fig. 2 also shows that under nonreducing conditions the α6B band migrated slightly faster than the α6A band (see also below).

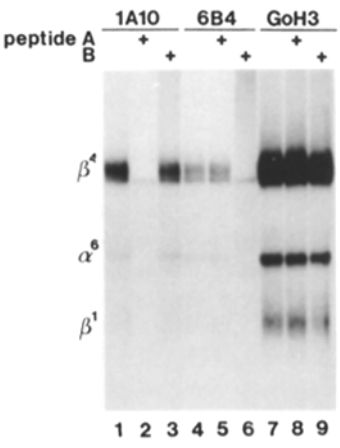

Figure 2. Specificity of mAbs GoH3, 1A10, and 6B4 in immunoprecipitation analysis. Lysates of surface-iodinated HBL-100 cells were immunoprecipitated with 1A10 (lanes *1–3*), 6B4 (lanes *4–6*) or GoH3 (lanes *7–9*) in the absence or presence (+) of the indicated peptides (100 μg/ml). The immunoprecipitates were analyzed by SDS-PAGE on 5% polyacrylamide gels under nonreducing conditions.

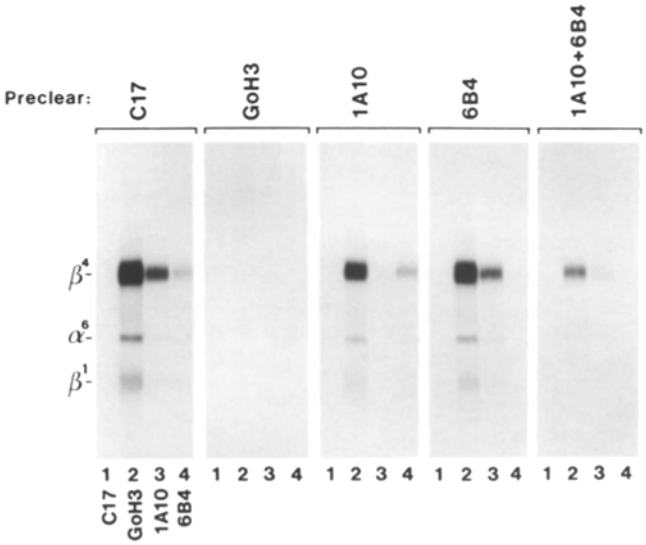

Figure 3. Preclearing analysis of proteins recognized by GoH3, 1A10, and 6B4. A ^{125}I-labeled lysate of HBL-100 cells was precleared using control mAb (C17), GoH3, 1A10, 6B4, or a combination of 1A10 and 6B4, as indicated. After two successive preclearing steps, the lysate was used for immunoprecipitations with C17 (lanes *1*), GoH3 (lanes *2*), 1A10 (lanes *3*) and 6B4 (lanes *4*). Samples were analyzed by SDS-PAGE on a 5% polyacrylamide gels under nonreducing conditions.

To confirm that the integrins precipitated by the 1A10 and 6B4 antibodies are subsets of the molecules recognized by the α6-specific antibody GoH3, preclearing experiments were carried out. Lysates of HBL-100 cells were first precleared with control mAb (C17, an anti-β3 mAb), GoH3, 1A10, 6B4, or a combination of 1A10 and 6B4. Then, aliquots were immunoprecipitated with the same panel of antibodies. After preclearing with a control mAb, the α6β1 and α6β4 complexes could be readily immunoprecipitated with all three anti-α6-mAbs (Fig. 3). However, after preclearing with GoH3, no precipitation with this antibody or with the α6A and α6B specific antibodies was seen. Similar results were obtained when preclearing was performed using a combination of 1A10 and 6B4, although some precipitation was still seen with GoH3. Preclearing with 1A10 decreased the amount precipitated by GoH3, but had no effect on the precipitation by 6B4. Conversely, preclearing with 6B4 partially decreased precipitation by GoH3, but did not affect precipitation by 1A10. Thus, the α6 subunits precipitated with GoH3 from HBL-100 cells consist of a mixture of α6A and α6B variants.

Different Forms of α6A and α6B

Previous studies have shown that the α6 subunit is synthesized as a single subunit that is proteolytically cleaved into

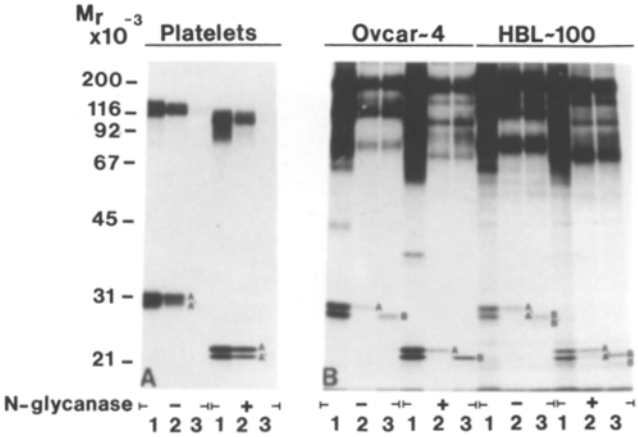

Figure 4. Analysis of the α6A and α6B light chains from platelets, OVCAR-4 and HBL-100 cells. Lysates of surface-labeled platelets (*A*), OVCAR-4 and HBL-100 cells (*B*) were immunoprecipitated with GoH3 (lane *1*), 1A10 (lane 2), and 6B4 (lane *3*). Immunoprecipitates were untreated (−) or treated (+) with *N*-glycanase (see Materials and Methods). Samples were analyzed by SDS-PAGE on a 10% polyacrylamide gel under reducing conditions.

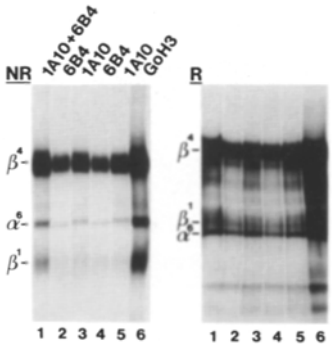

Figure 5. Analysis of α6A and α6B variants from HBL-100 cells by SDS-PAGE under nonreducing and reducing conditions. Lysates of surface-labeled HBL-100 cells were immunoprecipitated with 1A10+6B4 (lane *1*), 6B4 (lanes *2* and *4*), 1A10 (lane *3* and *5*) and GoH3 (lane *6*). Samples were analyzed by SDS-PAGE on a 5% polyacrylamide gels under nonreducing (*NR*) and reducing (*R*) conditions.

a heavy and a light chain, which are disulfide-bonded (36). The heavy chain contains the bulk of the extracellular part of α6, while the light chain contains the residual part of the extracellular domain, the transmembrane region, and the entire cytoplasmic domain (15, 49). Furthermore, analysis of the α6 subunit from various cell lines by SDS-PAGE revealed that the light chain consists of a doublet of polypeptides of 31 and 30 kD (37). We investigated whether these two light chains correspond to the α6A and α6B light chains or to alternatively cleaved products of a single precursor. Precipitation of α6 by GoH3 from platelet lysates yielded a light chain doublet of 31 and 30 kD (Fig. 4 *A*, lanes *1*). After treatment with *N*-glycanase, which removes both high-mannose and complex-type N-linked sugars (27), the doublet remained detectable but the size of the two polypeptides that constituted the doublet was reduced to 25 and 24 kD, consistent with previous results (37). Results of immunoprecipitation of platelet lysates with the mAb 1A10 were the same (Fig. 4 *A*, lanes 2). Thus, there are two α6A light chains, which were designated α6A and α6A′. Only very small amounts of α6B were precipitated by the mAb 6B4 from platelets (Fig. 4 *A*, lanes *3*).

Fig. 4 *B* shows the analysis of immunoprecipitates of α6A and α6B from the cell lines HBL-100 and OVCAR-4. From both cell lines, immunoprecipitation of α6 by GoH3 yielded the light chain doublet of 31 and 30 kD, whose mobility again shifted after *N*-glycanase treatment to 25 and 24 kD (Fig. 4 *B*, lanes *1*). Immunoprecipitation with the mAbs 1A10 (Fig. 4 *B*, lanes 2) and 6B4 (Fig. 4 *B*, lanes *3*) showed that the 31-kD band was specifically recognized by 1A10, and the 30-kD band by 6B4. This is essentially different from the results with platelets from which the mAb 1A10 immunoprecipitated two light chains.

From HBL-100 cells, 1A10 also immunoprecipitated two light chains, but one was much more strongly labeled than the other. The weakest band migrated slightly faster than the prominent α6A band and it almost comigrated with the α6B band. It seems likely that this band corresponds to the α6A′ light chain precipitated from platelets. A similar smaller polypeptide was observed in the precipitation by 6B4 and this we named α6B′.

The observation that the α6B light chains migrated faster than the α6A light chains was unexpected because it did not correlate with the predicted differences in size of the two α6 light chain variants, the α6B light chain being 18 amino acids longer than the α6A light chain (7, 15).

As seen in Fig. 5, SDS-PAGE analysis of α6A and α6B under nonreducing conditions also indicated that the intact α6B subunit migrated faster than the intact α6A subunit. However, upon reduction the heavy chains of α6A and α6B comigrated at the 120-kD position. This suggests that the light chains are responsible for the difference in mobility of the intact subunits.

PMA Induces Phosphorylation of the α6A But Not the α6B Subunit

Shaw et al. have recently reported that in mouse macrophages the α6 subunit becomes phosphorylated when these cells are treated with PMA (30). To investigate whether both α6 variants can be phosphorylated, cell lines which express primarily α6A or α6B, or both were labeled with [^{32}P]orthophosphate and treated with PMA or not. Lysates from these cells were then immunoprecipitated with the 1A10 and

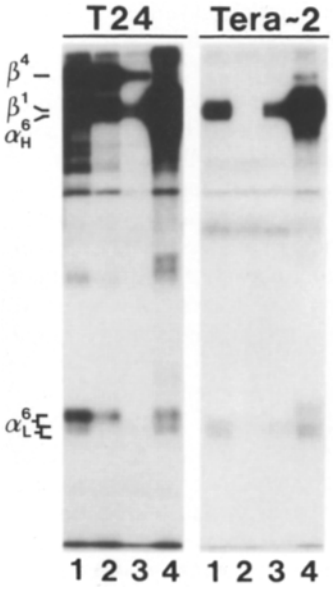

Figure 6. Analysis of α6 integrins from T24 and Tera-2 cells. ^{125}I-labeled cell lysates were immunoprecipitated with GoH3 (lanes *1*), 1A10 (lanes 2), 6B4 (lanes *3*), and A-1A5 (lanes *4*). The precipitates were analyzed by SDS-PAGE on a 10% polyacrylamide gel under reducing conditions.

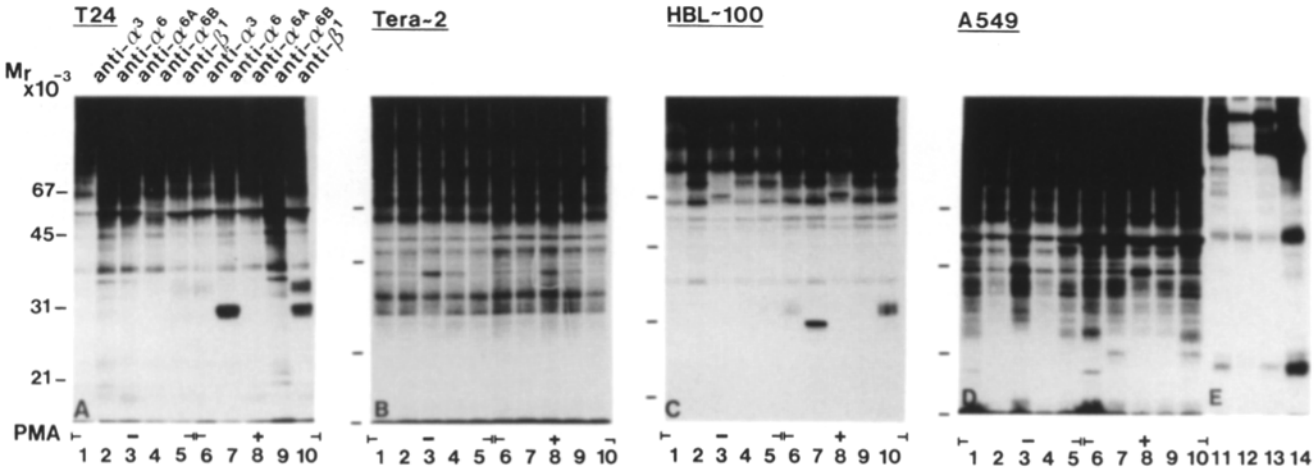

Figure 7. Analysis of the effect of PMA on the phosphorylation of α6. Cell cultures of T24 (*A*), Tera-2 (*B*), HBL-100 (*C*), and A549 (*D*) were metabolically labeled with [^{32}P]orthophosphate for 2 h and incubated with (+) or without (−) PMA (20 ng/ml) for 15 min. Cell lysates were immunoprecipitated with J143 (lanes *1* and *6*), GoH3 (lanes *2* and *7*), 1A10 (lanes *3* and *8*), 6B4 (lanes *4* and *9*) and A-1A5 (lanes *5* and *10*). Samples were analyzed by SDS-PAGE on a 10% polyacrylamide gel under reducing conditions. *E* shows the immunoprecipitation analysis of ^{125}I-labeled A549 cells with GoH3 (lane *11*), 1A10 (lane *12*), 6B4 (lane *13*), and A-1A5 (lane *14*).

6B4, as well as with the GoH3 and the A-1A5 (anti-β1) antibodies. A mAb against the α3 subunit, which is constitutively phosphorylated (20), was included as a positive control.

Immunoprecipitation analysis of ^{125}I-labeled T24 cells revealed that they predominantly express α6A (Fig. 6), which is only weakly phosphorylated (Fig. 7 *A*, lanes *2* and *5*). However, after the cells were treated with PMA for 15 min, the intensity of the α6A band increased significantly (Fig. 7 *A*, lanes *7* and *10*). Some phosphorylation is also seen in a band migrating slightly faster than the α6A band which is probably due to phosphorylation of α6A′ and not of α6B; the α6A′ band migrates to practically the same position as α6B. Interestingly, the mAb 1A10 (Fig. 7 *A*, lanes *3* and *8*) did not precipitate the phoshorylated form of α6A. This suggests that phosphorylation of the cytoplasmic domain of α6A affects recognition of its epitope by the mAb. Phosphorylated light chains of α3 were detected in both α3 and β1 immunoprecipitates (Fig. 7 *A*, lanes *1* and *5*, respectively). Like the α6 light chain, the α3 light chain runs as a closely spaced doublet (35 and 34 kD) and its phosphorylation is increased after treatment with PMA (Fig. 7 *A*, lanes *6* and *10*).

Tera-2 cells only express α6B as is shown in immunoprecipitation of ^{125}I-labeled cells by the GoH3 and 6B4 antibodies (Fig. 6). The α6B light chain in these cells is not phosphorylated, and in contrast to what happens with α6A/A′ in T24 cells, phosphorylation was not induced by treatment with PMA. No precipitation with either GoH3, 6B4 or A-1A5 was seen at the position of phosphorylated α6B light chains (Fig. 7 *B*, lanes *7*, *9*, and *10*). Tera-2 cells do not express α3 (13*b*) and consequently, no phosphorylated α3 light chains were detected.

Because phosphorylation of cytoplasmic domains of integrin subunits might be inefficient in Tera-2 cells, we have also tested two other cell lines HBL-100 and A549, which express both α6A and α6B. The presence of α6A in these cells allows us to judge the efficiency of phosphorylation by PMA. Because on SDS-PAGE, as already discussed, the α6B light chain migrates slightly faster than α6A, we can distinguish between phosphorylated α6A and α6B in immunoprecipitations by GoH3.

Analysis of HBL-100 cells which have almost equal amounts of α6A and α6B (Fig. 1) again showed that α6A but not α6B was phosphorylated by PMA treatment (Fig. 7 *C*, lane *7*). A minor phosphorylated band seen just below the α6A band probably represents α6A′. mAb 1A10 did not precipitate phosphorylated α6A, which confirms the results obtained with the T24 cell line (Fig. 7 *C*, lane *8*). Immunoprecipitation with the 6B4 antibody was negative (Fig. 7 *C*, lanes *4* and *9*), confirming that phosphorylation of α6B cannot be detected. In addition to the α6 variants, the HBL-100 cell line expresses the α3β1 integrin (38) and as seen in Fig. 7 *C*, the light chain doublet of this subunit was weakly phosphorylated in untreated cells (Fig. 7 *C*, lanes *1* and *5*). PMA treatment (Fig. 7 *C*, lanes *6* and *10*) increased the amount of phosphorylated α3 light chains.

Fig. 7 *D* shows the analysis of the A549 cell line. This cell line contains more α6B than α6A (Fig. 7 *E*). As observed in T24 and HBL-100 cells the α6A light chains became phosphorylated by PMA treatment (Fig. 7 *E*, lanes *7* and *10*), but again there was no evidence for phosphorylation of α6B. Similar results were obtained with the OVCAR-4 and Colo-320 cell lines (not shown). Thus, in several cell lines, α6A but not α6B could be shown to become phosphorylated by treatment with PMA.

Phosphoamino acid analysis was performed to determine which amino acids were phosphorylated in the cytoplasmic domains of the α6A and α3A subunits. Phosphorylated α6A and α3A proteins, precipitated from PMA-treated T24 cells, were isolated from SDS–gel, partially hydrolyzed with acid and analyzed by thin-layer electrophoresis. Fig. 8 shows that both α6A and α3A were predominantly phosphorylated on serine residues. A low level of tyrosine phosphorylation was also observed for both proteins, but no threonine phosphorylation was apparant. Phosphoamino acid analysis of the β4 subunit revealed that serine was the major phosphorylated amino acid, but phosphorylated tyrosine and threonine were also detectable.

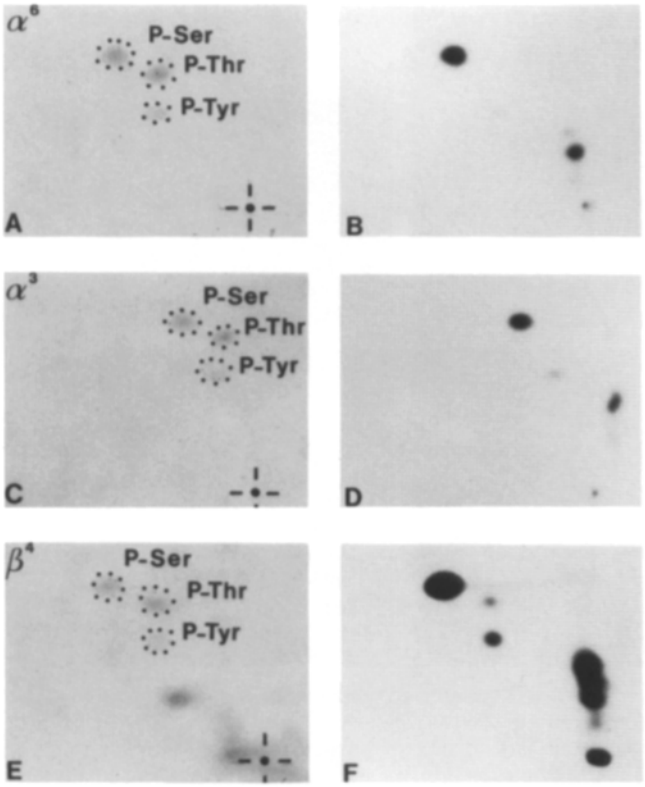

Figure 8. Phosphoamino acid analysis of α6A, α3A and β4 subunits. T24 cells were labeled with [^{32}P]orthophosphate, treated with PMA for 15 min and lysed. The lysate was used for precipitation of α6A and α3A light chains and the β4 subunit. After extraction, these polypeptides were subjected to acid hydrolysis and further analyzed using thin layer electrophoresis. (*A* and *B*) analysis of the α6A light chains; (*C* and *D*) analysis of the α3A light chains; (*E* and *F*) analysis of the β4 subunits. *A*, *C*, and *E* show the ninhydrin staining of the phosphoamino acid standards and *B*, *D*, and *F* show the autoradiograms.

Distribution of α6A and α6B Variants in Focal Contacts and Stable Anchoring Contacts

The α6β1 integrin localizes in focal contacts when OVCAR-4 cells attach to and spread on laminin (41). To see whether they contain both α6A and α6B subunits, OVCAR-4 cells were grown on laminin-coated coverslips and stained in double immunofluoresence with anti-vinculin to visualize focal contacts and with the mAbs 1A10 or 6B4. Staining of OVCAR-4 by GoH3 (Fig. 9 *a*) or A-1A5 (Fig. 9 *d*) showed distinct localization patterns, at the periphery of the cells. Staining with anti-vinculin confirms that these elements are focal contacts (Fig. 9, *e* and *h*). The staining patterns with the mAbs 1A10 and 6B4 were similar (Fig. 9, *b* and *c*) and there was complete codistribution with anti-vinculin (Fig. 9, *f* and *g*). The staining reactions by these mAbs, however, were weaker. These results indicate that both α6 variants can localize in focal contacts.

In cultured keratinocytes adhering to secreted extracellular matrix, the α6β4 integrin localized in SACs These SACs resemble hemidesmosomes in skin and are associated with BPA 230 (6, 42). Because keratinocytes only express the A variant of α6, we assume that these adhesion structures will be stained by 1A10. To find support for this assumption, we examined the distribution of α6Aβ4 in SACs of UMSSC-22B cells, human squamous carcinoma cells, that form SACs upon adherence to secreted extracellular matrix. As shown in Fig. 10 staining with 1A10 produced a brush-like pattern that codistributed with SACs as revealed by staining with anti-BPA 230 antiserum. The staining patterns produced by mAb GoH3, directed to α6 that reacts with both its variants, and mAb 439-9B, against β4 were similar to that by 1A10. This shows that the integrin in SACs is α6Aβ4. No staining was seen with 6B4 which is consistent with the finding that these cells express only low levels of α6B as was detected by immunoprecipitation and RT-PCR (not shown). As expected, when UMSSC-22B cells were stained with anti-vinculin, a focal distribution is observed which shows little colocalization with SACs as stained by anti-α6 (not shown).

Tissue Distribution of α6A and α6B

Immunoperoxidase staining of frozen tissue sections with the 1A10 and 6B4 antibodies revealed that the α6A and α6B subunits have distinct patterns of distribution (Table II). In skin, 1A10 stained the basement membrane zone (BMZ) of the epidermis (Fig. 11 *b*). Staining was also seen in sweat glands (Fig. 12 *b*), peripheral nerves (Fig. 13 *b*), sebaceous glands and some blood vessels present in the dermis. In sweat glands, the reactivity of 1A10 was confined to myoepithelial cells; lumenal cells were not stained. In peripheral nerves, reactivity was prominent in perineurium and in (myelinated) Schwann cells, whereas other cells in the tissue showed only weak reactivity. The 6B4 antibody reacted weakly with sweat glands (Fig. 12 *c*) and sebaceous glands and blood vessels, but not with epidermis (Fig. 11 *c*) or peripheral nerves (Fig. 13 *c*). In the mammary gland, myoepithelial cells of ducts and alveoli in the mammary gland stained with 1A10 (Fig. 11 *e*), but not with 6B4 (Fig. 11 *f*). In contrast, kidney tubules reacted with 6B4 (Fig. 12 *f*) but not with 1A10 (Fig. 12 *e*). Neither 1A10 or 6B4 antibodies reacted with glomeruli. Furthermore, 6B4 stained epithelium of colonic crypts (Fig. 12 *i*). These cells reacted weakly with 1A10 (Fig. 12 *h*). Finally, in salivary gland, both 1A10 and 6B4 stained the BMZ of ducts and acini (Fig. 11, *h* and *i*).

With two exceptions, all structures which were stained by either 1A10 or 6B4 were also reactive with the GoH3 antibodies (Figs. 11–13, *a*, *d*, and *g*). The exceptions are smooth muscle cells (muscularis of the intestine and smooth muscle cells in large vessel walls) and striated muscle cells, which displayed strong and weak reactivity with 1A10, respectively, but did not react with GoH3 (not shown).

Discussion

In this paper, we describe the characterization of mAbs directed against two variants of α6, α6A, and α6B. Using peptides that represent the major portion of the cytoplasmic domains of α6A and α6B for immunization, we prepared several mAbs reacting with either α6A or α6B peptides. Immunohistochemistry and immunoprecipitation studies performed with these mAbs, revealed that the tissue expression of the α6A and α6B variants is different and that their structures are heterogenous.

Most tissues appeared to contain either the α6A and α6B

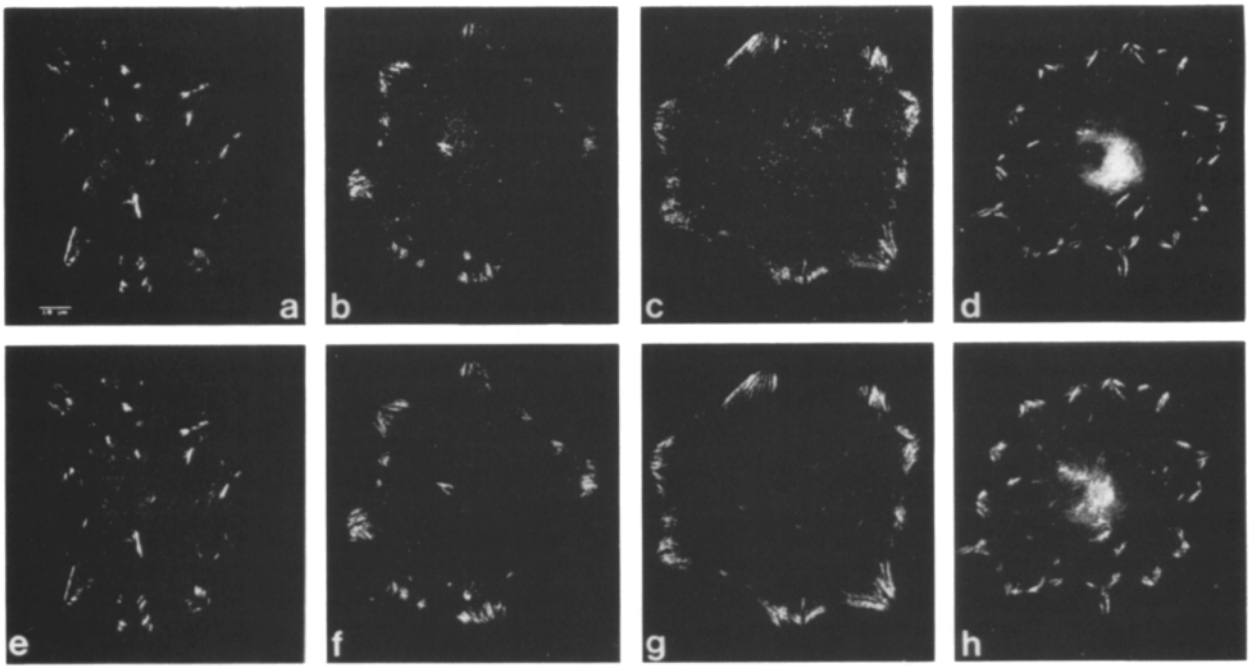

Figure 9. Localization of α6 variants in focal contacts. OVCAR-4 cells were allowed to adhere and spread on mouse laminin-coated glass coverslips for 2 h, fixed with paraformaldehyde, permeabilized with Triton X-100 and stained with mAbs GoH3 to α6 (*A*), 1A10 to α6A (*B*), 6B4 to α6B (*C*), and A-1A5 to β1 (*D*), and then labeled with FITC-conjugated goat anti-mouse antibody. *E–H* shows the staining of vinculin performed on the same cells using a rabbit polyclonal antibody to vinculin as primary antibody and a Texas red-conjugated donkey anti-rabbit secondary antibody.

subunit. In some tissues, e.g., salivary gland and intestine, both variants are expressed, the reactivity with anti-α6B being stronger than with anti-α6A. There was no correlation between the presence in tissues of α6A or α6B and the presence of β1 or β4. Platelets and renal tubuli, which contain β1 but not β4, express only α6A and α6B, respectively, which suggests that the two variants of α6 can combine with β1. However, because the affinity of α6 for β4 is much greater than that for β1 (12, 21), it can be assumed that in tissues which express both β1 and β4 the two variants of α6 will mainly combine with β4.

There is good agreement between our results and those of the RT-PCR assays described by Tamura et al. (50). These authors found that kidney only contains transcripts for α6B, whereas transcripts for both α6A and α6B were found in salivary gland and intestine. These results correspond with the reactions we found with the variant specific mAbs. Apart from the fact that our studies confirm and extend the distri-

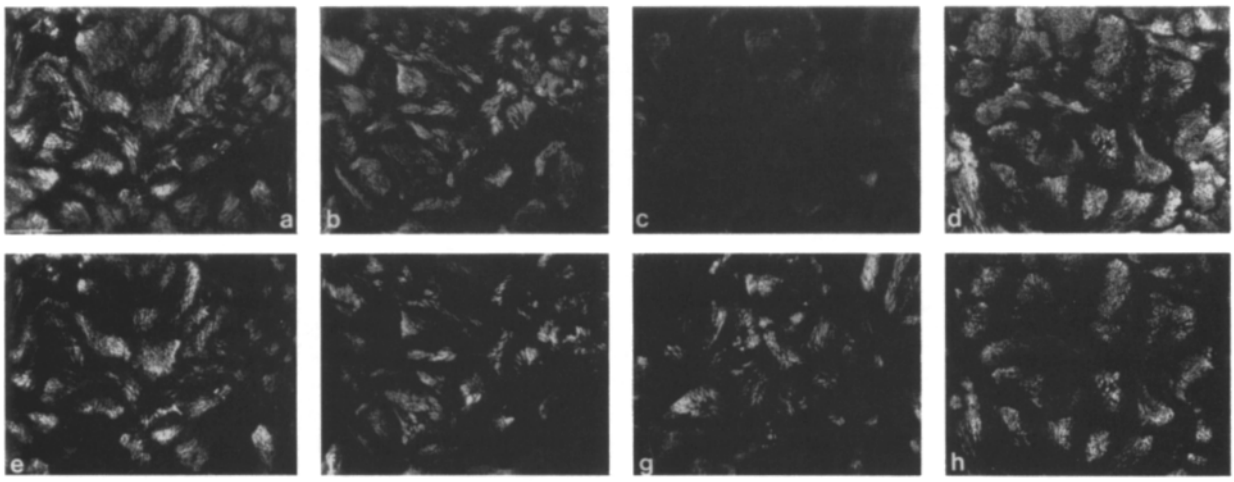

Figure 10. Localization of the A variant of α6 in SACs. UMSCC-22B cells were plated on alcohol-washed coverslips and cultured for 2 d. They were then fixed with paraformaldehyde, permeabilized with Triton X-100 and stained with mAbs GoH3 to α6 (*A*), 1A10 to α6A (*B*), 6B4 to α6B (*C*), and 439-9B to β4 (*D*), and then labeled with Texas red-conjugated secondary antibodies. *E–H* shows the staining of BPA 230 performed on the same cells using rabbit anti-BPA 230 and BODIPY-conjugated goat anti-rabbit as a secondary antibody.

Table II. Immunoperoxidase Staining of Human Tissues

Tissue	1A10	6B4	GoH3
Skin			
Epidermis	++	−	++
Sweat gland	++	±	++
Sebaceous gland	+	±	+
Mammary gland			
Aveoli	++	−	++
Ducts	++	−	++
Salivary gland			
Acini	+	++	++
Ducts	+	++	++
Large intestine			
Crypts	±	+	++
Muscularis	++	−	−
Kidney			
Glomeruli	−	−	±
Proximal tubuli	−	+	++
Distal tubuli	−	+	++
Peripheral nerve			
Perineurium	++	−	++
Schwann cells	++	−	++
Vascular system			
Capillaries			
Endothelial cells	+	±	+
Arterioles and veins			
Smooth muscle cells	++	−	−
Endothelial cells	−	−	−
Striated muscle	±	−	−

(++) very strong; (+) strong; (±) weak; and (−) no staining.

bution pattern as obtained by RT-PCR, it also provides information about the expression of the α6A and α6B variants at the single-cell level. For example, it is clear from the immunohistochemical but not from the RT-PCR analysis, that both variants are expressed in the same ductal and acinar cells of the salivary gland.

In spite of the good correlation between the results of our antibodies and those obtained by RT-PCR, it should be kept in mind that the anti-α6A antibody, 1A10, does not react with phosphorylated α6A. Therefore, a negative result obtained with this antibody does not conclusively mean that α6A is absent.

In epidermis α6β4 has been localized in hemidesmosomes, suggesting that it might be essential in the formation of these structures (18, 40, 43). α6β4 is also present in perineural fibroblasts and Schwann cells of peripheral nerves and the question arises whether in these cells it is also localized in hemidesmosomes. We could not define hemidesmosomes in these cells by electron microscopy nor could we detect the hemidesmosomal associated protein BPA 230 (42) in them. This is consistent with the absence of these structures in perineural fibroblasts and Schwann cells. Perhaps it is interesting to mention that another component of hemidesmosomes, the 500-kD HD1 protein (13) could be demonstrated both in Schwann cells and perineural fibroblasts (Daams, H, and A. Sonnenberg, unpublished data). Thus, the question remains whether the absence of hemidesmosomes could be due to a different role of the two variants of α6 in their assembly. This is unlikely because our results show that in both epidermis and peripheral nerves α6 is of the A variant. Alternatively, epidermis and peripheral nerves might express different forms of the cytoplasmic domain of β4. In any case, the results show that the presence of α6Aβ4 by itself is insufficient to ensure hemidesmosome formation. All tissues which reacted with either 1A10 or 6B4 also reacted with mAb GoH3. The only two exceptions were striated and smooth muscle cells, which were stained by 1A10, but not by GoH3 and other α6 specific mAbs (Daams, H., and A. Sonnenberg, unpublished data). This indicates that the α6A mAb shows crossreactivity, possibly with α subunits, like α3 and α7 which closely resemble α6. However, 1A10 does not react with α3A peptide and does not immunoprecipitate α3β1 from cell lysates. Recently, the cDNA for the α7 subunit, which is expressed in muscle cells, has been cloned (32). There is no homology between the protein sequence of the cytoplasmic domains of α7 and α6A. Instead, there is partial homology between α7 and the α3B and α6B cytoplasmic domains. Therefore, the reactivity of 1A10 with smooth and striated muscle cells cannot be explained by crossreactivity with α6-like α molecules. Immunoprecipitation analysis of surface-labeled, cultured smooth muscle cells with 1A10 did not result in the identification of the antigen recognized by this mAb. The exact nature of the epitope recognized by 1A10 in muscle cells remains therefore unknown.

We have previously reported on the structural complexity of the α6 subunit (37, 39). Variations in the glycosylation of the heavy and light chains of α6 were found in platelets and various cell lines. Furthermore, we have shown that the α6 light chains, which contain the entire cytoplasmic domain of the α6 subunit, had different polypeptide backbones, which run as a doublet in SDS-gels under reducing conditions. Two explanations were suggested: (*a*) alternative splicing of the α6 mRNA and/or (*b*) differences in proteolytic processing of the α6 precursor. Our present studies produced evidence for both options. First, immunoprecipitation with the variant specific mAbs showed that, in most cell lines, the doublet contains both α6A and α6B light chains; the upper band of the doublet represents the α6A and the lower band the α6B light chain. The relative intensity of the two bands in most instances reflects the relative amounts of α6A and α6B mRNA as detected by RT-PCR analysis (Fig. 1) (15).

Second, both anti-α6 (GoH3) and anti-α6A (1A10) mAbs immunoprecipitated a light chain doublet (α6A and α6A′) from platelets. Two distinct light chains remained after treatment with *N*-glycanase, indicating that differences in length of the polypeptide backbones may be responsible for the different electrophoretic mobilities of α6A and α6A′. Since we have not observed alternative splicing of α6 mRNA in addition to that responsible for the generation of α6A and α6B, we hypothesize that the two variants of the α6A light chain result from alternative proteolytic processing. We also detected a doublet of α6B light chains, α6B and α6B′, before and after *N*-glycanase treatment, which may similarly represent alternative proteolytic products of α6B.

Besides α6, several other α subunits of the integrin family are endoproteolytically cleaved into a heavy and a light chain (2, 4, 24, 28, 46–48). Analysis of the NH_2 terminus of the αv and α4 light chains showed that the cleavage site is located after two consecutive basic amino acids (45, 52). Re-

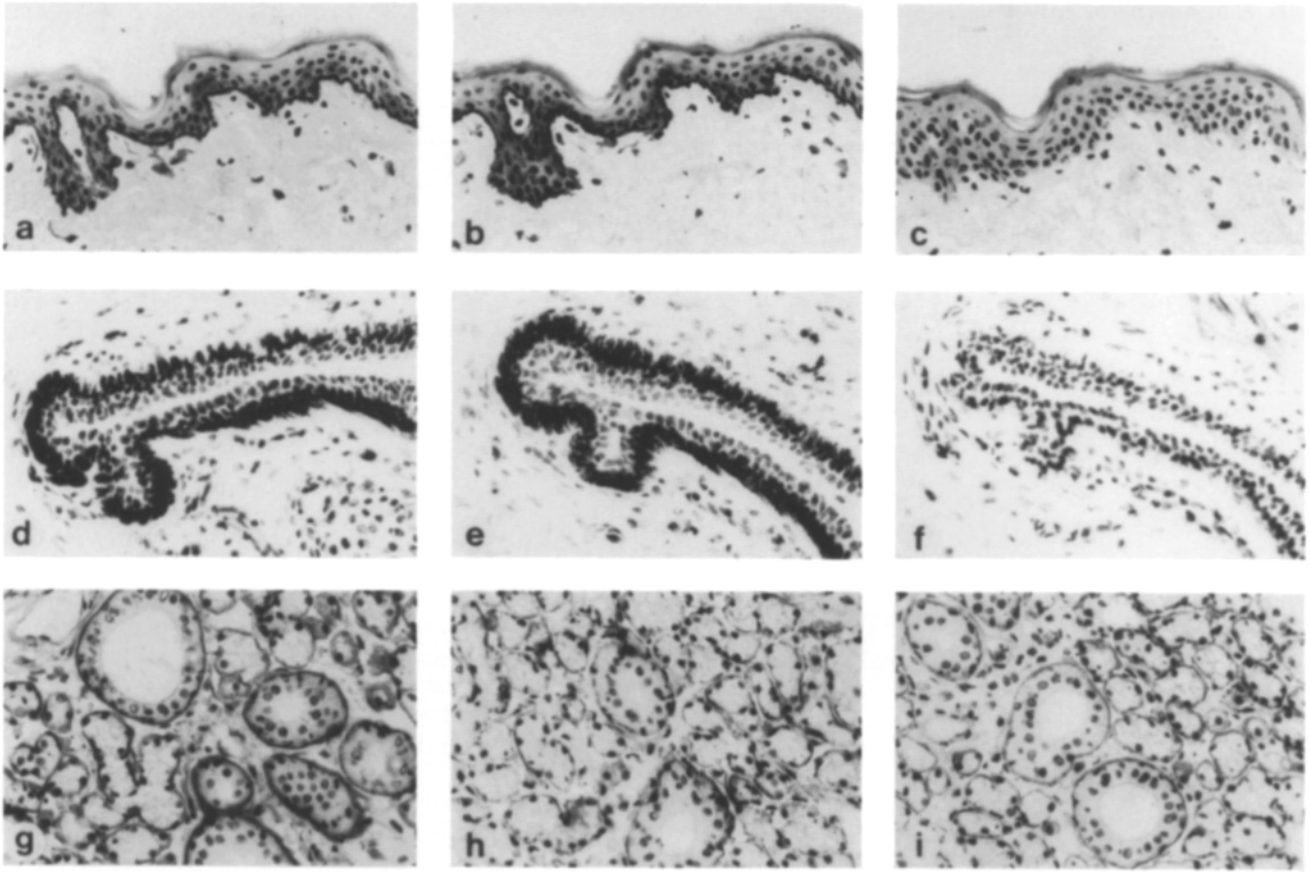

Figure 11. Immunoperoxidase staining of human tissue sections with mAbs GoH3 (*a*, *d*, and *g*), 1A10 (*b*, *e*, and *h*) and 6B4 (*c*, *f*, and *i*). Skin sections (*a*, *b*, and *c*) showing linear GoH3 and 1A10 reactivity of the basement membrane zone. mAb 6B4 did not stain. Sections of mammary gland (*d*, *e*, and *f*) showing reactivity of GoH3 and 1A10 in myoepithelial cells. Salivary gland sections (*g*, *h*, and *i*) showing acini and ducts stained with all three antibodies.

cently, Kolodziej et al. (23) determined the cleavage site in GPIIb, using site-directed mutagenesis. They found that the basic amino acids, lysine and arginine, at positions 858–859 were essential for cleavage.

The α6 sequence contains four dibasic sequences in the region corresponding to that in which GPIIb and αv are cleaved (15, 49). Two of these sequences are consecutive (876–877 and 878–879) and the others are located at positions 890–891 and 898–899. The sequence, located at positions 890–891, is not conserved in chicken α6 cDNA (8) or in other cleaved α subunits and is therefore unique for human α6. Because of the homology between the α subunits, we assume that the α6 precursor might be cleaved after arginine 879 or lysine 899. Analysis of α6A and α6B precipitates on SDS-PAGE under reducing conditions showed that the heavy chains of α6A and α6B comigrated as a single band. Therefore, we suspect that there is a primary cleavage site, located after position 879, and a second, alternatively used, site after position 899. We also observed that α3A light chains consist of a doublet. Taking into account the strong homology between α3 and α6, we assume that α3 is also alternatively cleaved at similar positions as α6.

Although the light chain of α6B is 18 amino acids longer than that of α6A, the former migrated faster both before and after *N*-glycanase treatment. We suspect that the posttranslational cleavage products of the two variants do not differ, but that their migration is influenced by conformational differences of their cytoplasmic domains.

The question is whether the cytoplasmic variants of α6, α6A, and α6B, and their different cleavage products influence the function of the α6β1 integrin. From what is known about other integrins, cleavage of the α subunits apparently does not influence the affinity for their ligands. Kolodziej et al. (23) showed that mutations induced in GPIIb cDNA which resulted in the production of uncleaved mature GPIIb, did not alter the ability of the complex to bind to fibrinogen (23). The capacity of α4 containing integrins to bind to their ligands was also independent of whether or not they were cleaved (52).

The following observations also do not indicate a functional difference between α6A and α6B. We have previously found that platelets and Tera-2 cells adhere to laminin and that this adhesion could be blocked by the mAb GoH3 (3, 35). In this study, we have shown that platelets have almost exclusively α6Aβ1, while Tera-2 cells only express α6Bβ1. Furthermore, both variants of α6 colocalized with vinculin in focal adhesions when OVCAR-4 cells adhered to laminin, suggesting that they both can interact with this ligand. It also suggests that at least with respect to interaction with the cytoskeleton, α6Aβ1 and α6Bβ1 integrins do not differ. However, the conditions for the interaction of the two variants with their ligand and the cytoskeleton remain to be es-

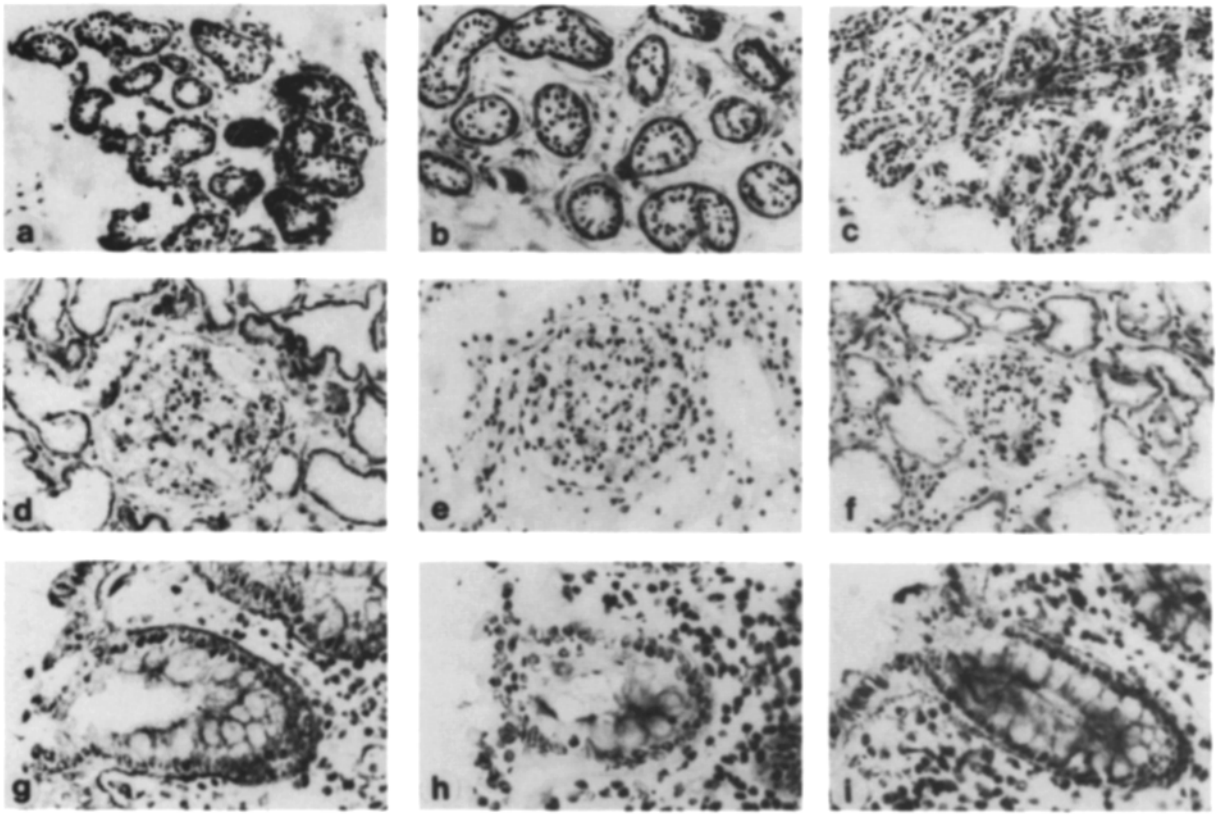

Figure 12. Immunoperoxidase staining of human tissue sections with mAbs GoH3 (*a*, *d*, and *g*), 1A10 (*b*, *e*, and *h*) and 6B4 (*c*, *f*, and *i*). Sections of sweat glands (*a*, *b*, and *c*) showing staining of myoepithelial cells with GoH3 and 1A10. A weak staining was observed with 6B4. Sections of kidney tissue (*d*, *e*, and *f*) showing reactivity of GoH3 and 6B4 with the tubules. mAb 1A10 did not stain. Sections of colonic crypts (*g*, *h*, and *i*) showing staining of the epithelial layer with GoH3 and 6B4. The same epithelial cells are weakly stained by 1A10.

tablished. Because α6B is not detectably phosphorylated, phosphorylation cannot be a major condition for the interaction of the α6Bβ1 integrin with its ligand and/or the cytoskeleton. On the other hand, phosphorylation of α6Aβ1 might be required for its interaction either with both the ligand and cytoskeleton or only the latter. In fact, PMA induced-phosphorylation of α6β1 in macrophages has been associated with the binding of this integrin to the actin-containing cytoskeleton and with an increased affinity for laminin (receptor activation) (30). Also the α6β1 integrin on T-cells is activated by PMA (31). Platelets, however, can adhere directly to laminin without being activated with PMA or other stimuli (17, 35). Because the 1A10 mAb, not recognizing phosphorylated α6A, reacted almost as strongly as GoH3 with α6Aβ1 on platelets, both in immunoprecipitation analysis (Fig. 4) and in immunohistochemistry (not shown), the majority of the α6A subunits apparently are not phosphorylated. This was further confirmed by metabolic labeling of platelets with [^{32}P]orthophosphate (not shown). Thus, while in some cells (e.g., macrophages) phosphorylation of the α6A subunit might be essential for their interaction with laminin, this appears not to be the case in platelets. Furthermore, in epidermis, α6Aβ4 is localized in hemidesmosomes of basal keratinocytes and interaction of this integrin with both ligand and cytoskeleton is presumed to occur (18, 40, 43). Basal keratinocytes react with the 1A10 mAb, as strongly as GoH3. Therefore, also in keratinocytes, phosphorylation of α6A may not be essential for either ligand recognition or cytoskeletal interactions by α6Aβ4. This conclusion is further supported by the observation that with the mAb 1A10 the α6A subunit is localized in the SAC structures of cultured UMSSC-22B cells. However, in these cells α6Aβ4 is likely to interact with the keratin filament system rather than with the actin-containing cytoskeleton as in macrophages. Finally, there is increasing evidence for the involvement of integrins in signal transduction (16) and it is therefore possible that the two variants transduce signals in a different way in response to interaction with the same ligand. All these points as well as the question whether different cleavages also influence the intracellular function for α6β1 are subjects of further investigation.

In conclusion, we have described the production and characterization of anti-α6A and anti-α6B mAbs. We have shown that the expression of the α6 variants is tissue-specific and that both forms may be subject to alternative endoproteolytic cleavage. Finally, we provide evidence that α6A but not α6B is a major target of PMA-induced phosphorylation.

We thank Dr. A. P. Albino, M. E. Hemler, B. Geiger, I. R. Stanley, and S. J. Kennel for providing antibodies; C. Mummery and T. E. Carey for the Tera-2 and UMSSC-22B cell lines, respectively; E. Schuuring and A. Genissen for RNA samples; L. Oomen for confocal scanning laser microscopy analysis; and P. W. Modderman and C. P. Engelfriet for reviewing the manuscript. This work was supported by a grant from the Netherlands Or-

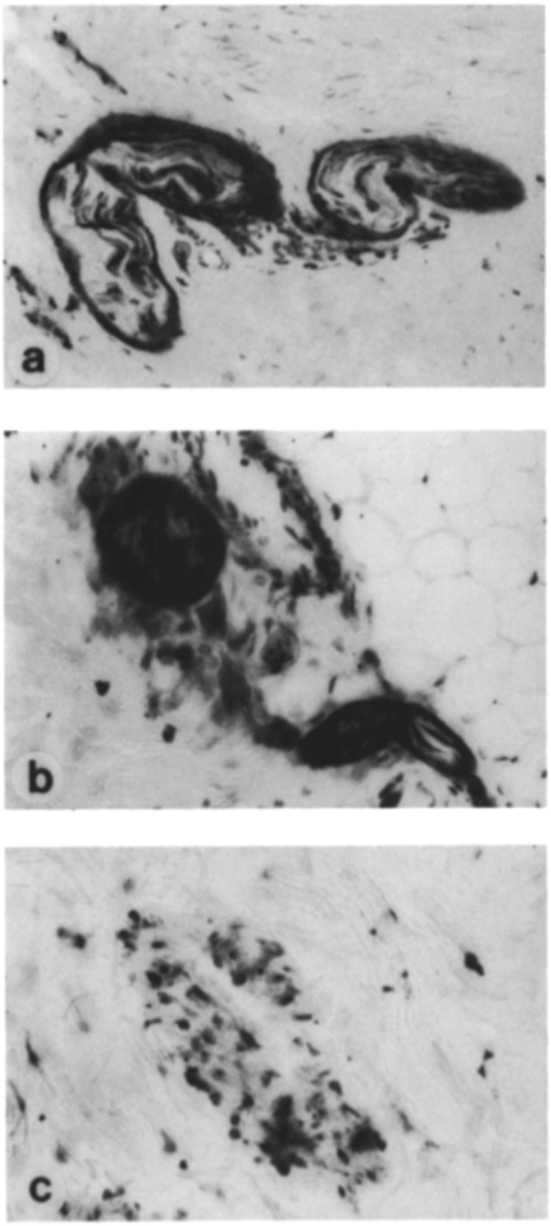

Figure 13. Immunoperoxidase staining of sections of human peripheral nerves. The perineurium and the Schwann cells were stained with GoH3 (*a*) and 1A10 (*b*). The mAb 6B4 (*c*) did not stain.

ganization for Scientific Research (NWO) (900-526-106) and by a grant from the Dutch Cancer Society (NKI 91-260).

Received for publication 2 June 1992, and in revised form 22 December 1992.

References

1. Albelda, S. M., and C. A. Buck. 1990. Integrins and other cell adhesion molecules. *FASEB. (Fed. Am. Soc. Exp. Biol.) J.* 4:2868–2880.
2. Argraves, W. S., R. Pytela, S. Suzuki, J. L. Millan, M. D. Pierschbacher, and E. Ruoslahti. 1986. cDNA sequences from the alpha subunit of the fibronectin receptor predict a transmembrane domain and a short cytoplasmic peptide. *J. Biol. Chem.* 261:12922–12924.
3. Aumailley, M., R. Timpl, and A. Sonnenberg. 1990. Antibody to integrin alpha 6 subunit specifically inhibits cell-binding to laminin fragment 8. *Exp. Cell Res.* 188:55–60.
4. Bogaert, T., N. Brown, and M. Wilcox. 1987. The Drosophila PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell.* 51:929–940.
5. Boyle, W. J., P. van der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *In* Methods in Enzymology, Vol. 201. T. Hunter, and B. M. Sefton, editors. Academic Press, Inc., San Diego, CA. 110–149.
6. Carter, W. G., P. Kaur, S. G. Gil, P. J. Gahr, and E. A. Wayner. 1990. Distinct functions for integrins $\alpha 3\beta 1$ in focal adhesions and $\alpha 6\beta 4$/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.* 111:3141–3154.
7. Cooper, H. M., R. N. Tamura, and V. Quaranta. 1991. The major laminin receptor of mouse embryonic stem cells is a novel isoform of the $\alpha_6\beta_1$ integrin. *J. Cell Biol.* 115:843–850.
8. De Curtis, I., V. Quaranta, R. N. Tamura, and L. F. Reichardt. 1991. Laminin receptors in the retina: sequence analysis of the chick integrin α_6 subunit. Evidence for transcriptional and posttranslational regulation. *J. Cell Biol.* 113:405–416.
9. DeLuca, M., R. N. Tamura, S. Kajiji, S. Bondanza, R. Candedda, P. Rosino, P. C. Marchisio, and V. Quaranta. 1990. Polarized integrin mediates keratinocyte adhesion to the basal lamina. *Proc. Natl. Acad. Sci. USA.* 87:6888–6892.
10. Hemler, M. E., C. Huang, and L. Schwarz. 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.
11. Hemler, M. E., C. Crouse, Y. Takada, and A. Sonnenberg. 1988. Multiple very late antigen (VLA) heterodimers on platelets. Evidence for distinct VLA-2, VLA-5 (fibronectin receptor), and VLA-6 structures. *J. Biol. Chem.* 263:7660–7665.
12. Hemler, M. E., C. Crouse, and A. Sonnenberg. 1989. Association of the VLA alpha 6 subunit with a novel protein. A possible alternative to the common VLA beta 1 subunit on certain cell lines. *J. Biol. Chem.* 264:6529–6535.
13. Hieda, Y., Y. Nishizawa, J. Uematsu, and K. Owaribe. 1992. Identification of a new hemidesmosomal protein, HD1: a major, high molecular mass component of isolated hemidesmosomes. *J. Cell Biol.* 116:1497–1506.

13*b*. Hierck, B. P., S. Thorsteinsdóttir, C. M. Niessen, E. Freund, L. van Iperen, A. Feyen, F. Hogervorst, R. E. Poelmann, C. L. Mummery, and A. Sonnenberg. 1993. Variants of the $\alpha 6\beta 1$ laminin receptor in early marine development: distribution, molecular cloning and chromosomal localization of the mouse integrin $\alpha 6$ subunit. *Cell Adhesion and Communication.* In press.

14. Hogervorst, F., I. Kuikman, A. E. von dem Borne, and A. Sonnenberg. 1990. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:765–770.
15. Hogervorst, F., I. Kuikman, A. G. Van Kessel, and A. Sonnenberg. 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.
16. Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.
17. Ill, C. R., E. Engvall, and E. Ruoslahti. 1984. Adhesion of platelets to laminin in the absence of activation. *J. Cell Biol.* 99:2140–2145.
18. Jones, J. C. R, M. A. Karpakus, H. M. Cooper, and V. Quaranta. 1991. A function for the integrin $\alpha 6\beta 4$ in the hemidesmosomes. *Cell Regul.* 2:427–438.
19. Kajiji, S., R. N. Tamura, and V. Quaranta. 1989. A novel integrin (alpha E beta 4) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:673–680.
20. Kantor, R. R., M. J. Mattes, K. O. Lloyd, L. J. Old, and A. P. Albino. 1987. Biochemical analysis of two cell surface glycoprotein complexes, very common antigen 1 and very common antigen 2. Relationship to very late activation T cell antigens. *J. Biol. Chem.* 262:15158–15165.
21. Kennel, S. J., L. J. Foote, R. Falcioni, A. Sonnenberg, C. D. Stringer, C. Crouse, and M. E. Hemler. 1989. Analysis of the tumor-associated antigen TSP-180. Identity with alpha 6-beta 4 in the integrin superfamily. *J. Biol. Chem.* 264:15515–15521.
22. Kennel, S. J., V. Godfrey, L. Y. Ch'ang, T. K. Lankford, L. J. Foote, and A. Makkinje. 1992. The β_4 subunit of the integrin family is displayed on a restricted subset of endothelium in mice. *J. Cell Sci.* 101:145–150.
23. Kolodziej, M. A., G. Vilaire, D. Gonder, M. Poncz, and J. S. Bennett. 1991. Study of the endoproteolytic cleavage of platelet glycoprotein IIb using oligonucleotide-mediated mutagenesis. *J. Biol. Chem.* 266:23499–23504.
24. Kramer, R. H., K. A. McDonald, E. Crowley, D. M. Ramos, and C. H. Damsky. 1989. Melanoma cell adhesion to basement membrane mediated by integrin-related complexes. *Cancer Res.* 49:393–402.
25. Lee, E. C., M. M. Lotz, G. D. Steele, Jr., and A. M. Mercurio. 1992. The integrin $\alpha 6\beta 4$ is a laminin receptor. *J. Cell Biol.* 117:671–678.
26. Lotz, M. M., C. A. Korzelius, and A. M. Mercurio. 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.
27. Plummer, T. H., Jr., J. H. Elder, S. Alexander, A. W. Phelan, and A. L. Tarentino. 1984. Demonstration of peptide: N-glycosidase F activity in endo-β-N-acetylglucosaminidase F preparations. *J. Biol. Chem.* 259:

10700–10704.
28. Poncz, M., R. Eisman, R. Heidenreich, S. M. Silver, G. Vilaire, S. Surrey, E. Schwartz, and J. S. Bennett. 1987. Structure of the platelet membrane glycoprotein IIb. Homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. *J. Biol. Chem.* 262:8476–8482.
29. Ruoslahti, E. 1991. Integrins. *J. Clin. Invest.* 87:1–5.
30. Shaw, L. M., J. M. Messier, and A. M. Mercurio. 1990. The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the alpha 6 beta 1 integrin. *J. Cell Biol.* 110:2167–2174.
31. Shimizu, Y., G. A. Van Seventer, K. J. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA (beta 1) integrin receptors on T cells. *Nature. (Lond.).* 345:250–253.
32. Song, W. K., W. Wang, R. F. Foster, D. A. Bielser, and S. J. Kaufman. 1992. H36-alpha 7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. *J. Cell Biol.* 117:643–657.
33. Sonnenberg, A., H. Daams, M. A. Van der Valk, J. Hilkens, and J. Hilgers. 1986. Development of mouse mammary gland: identification of stages in differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. *J. Histochem. Cytochem.* 34:1037–1046.
34. Sonnenberg, A., H. Janssen, F. Hogervorst, J. Calafat, and J. Hilgers. 1987. A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. *J. Biol. Chem.* 262:10376–10383.
35. Sonnenberg, A., P. W. Modderman, and F. Hogervorst. 1988. Laminin receptor on platelets is the integrin VLA-6. *Nature. (Lond.).* 336:487–489.
36. Sonnenberg, A., F. Hogervorst, A. Osterop, and F. E. Veltman. 1988. Identification and characterization of a novel antigen complex on mouse mammary tumor cells using a monoclonal antibody against platelet glycoprotein Ic. *J. Biol. Chem.* 263:14030–14038.
37. Sonnenberg, A., C. J. Linders, J. H. Daams, and S. J. Kennel. 1990. The alpha 6 beta 1 (VLA-6) and alpha 6 beta 4 protein complexes: tissue distribution and biochemical properties. *J. Cell Sci.* 96:207–217.
38. Sonnenberg, A., C. J. Linders, P. W. Modderman, C. H. Damsky, M. Aumailley, and R. Timpl. 1990. Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that alpha 6 beta 1 but not alpha 6 beta 4 functions as a major receptor for fragment E8. *J. Cell Biol.* 110:2145–2155.
39. Sonnenberg, A., P. W. Modderman, P. van der Geer, M. Aumailley, and R. Timpl. 1990. Structure and function of platelet glycoprotein IC-IIA (VLA-6). *In* Platelet Immunology: Fundamental and Clinical Aspects. C. Kaplan-Gouet, N. Schlegel, Ch. Salmon, and J. McGregor, editors. John Libbey Eurotext, Paris, London. 75–95.
40. Sonnenberg, A., J. Calafat, H. Janssen, H. Daams, L. M. H. Van der Raaij-Helmer, R. Falcioni, S. J. Kennel, J. D. Aplin, J. Baker, M. Loizidou, and D. Garrod. 1991. Integrin $\alpha 6/\beta 4$ complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. *J. Cell Biol.* 113:907–917.
41. Sonnenberg, A., K. R. Gehlsen, M. Aumailley, and R. Timpl. 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.
42. Stanley, J. R., P. Hawley Nelson, S. H. Yuspa, E. M. Shevach, and S. I. Katz. 1981. Characterization of bullous pemphigoid antigen: a unique basement membrane protein of stratified squamous epithelia. *Cell.* 24: 897–903.
43. Stepp, M. A., S. Spurr Michaud, A. Tisdale, J. Elwell, and I. K. Gipson. 1990. Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc. Natl. Acad. Sci. USA.* 87:8970–8974.
44. Suzuki, S., and Y. Naitoh. 1990. Amino acid sequence of a novel integrin beta 4 subunit and primary expression of the mRNA in epithelial cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:757–763.
45. Suzuki, S., W. S. Argraves, R. Pytela, H. Arai, T. Krusius, M. D. Pierschbacher, and E. Ruoslahti. 1986. cDNA and amino acid sequences of the cell adhesion protein receptor recognizing vitronectin reveal a transmembrane domain and homologies with other adhesion protein receptors. *Proc. Natl. Acad. Sci. USA.* 83:8614–8618.
46. Suzuki, S., W. S. Argraves, H. Arai, L. R. Languino, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the vitronectin receptor alpha subunit and comparative expression of adhesion receptor mRNAs. *J. Biol. Chem.* 262:14080–14085.
47. Takada, Y., M. J. Elices, C. Crouse, and M. E. Hemler. 1989. The primary structure of the alpha 4 subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1361–1368.
48. Takada, Y., E. Murphy, P. Pil, C. Chen, M. H. Ginsberg, and M. E. Hemler. 1991. Molecular cloning and expression of the cDNA for α_3 subunit of human $\alpha_3\beta_1$ (VLA-3), an integrin receptor for fibronectin, laminin, and collagen. *J. Cell Biol.* 115:257–266.
49. Tamura, R. N., C. Rozzo, L. Starr, J. Chambers, L. F. Reichardt, H. M. Cooper, and V. Quaranta. 1990. Epithelial integrin alpha 6 beta 4: complete primary structure of alpha 6 and variant forms of beta 4. *J. Cell Biol.* 111:1593–1604.
50. Tamura, R. N., H. M. Cooper, G. Collo, and V. Quaranta. 1991. Cell type-specific integrin variants with alternative α chain cytoplasmic domains. *Proc. Natl. Acad. Sci. USA.* 88:10183–10187.
51. Tanaka, T., N. J. Korman, H. Shimizu, R. A. Eady, V. Klaus Kovtun, K. Cehrs, and J. R. Stanley. 1990. Production of rabbit antibodies against carboxy-terminal epitopes encoded by bullous pemphigoid cDNA. *J. Invest. Dermatol.* 94:617–623.
52. Teixidó, J., C. M. Parker, P. D. Kassner, and M. E. Hemler. 1992. Functional and structural analysis of VLA-4 integrin α^4 subunit cleavage. *J. Biol. Chem.* 267:1786–1791.
53. Tetteroo, P. A., P. M. Lansdorp, O. C. Leeksma, and A. E. von dem Borne. 1983. Monoclonal antibodies against human platelet glycoprotein IIIa. *Br. J. Haematol.* 55:509–522.