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β1B Integrin Subunit Contains a Double Lysine Motif That Can Cause Accumulation Within the Endoplasmic Reticulum

Wai Jing Kee, Elizabeth R. Li, and Fiona M. Watt*

Keratinocyte Laboratory, Imperial Cancer Research Fund, London, WC2A 3PX, United Kingdom

Abstract Human epidermal keratinocytes are one of the few cell types that express the β1B splice variant of the β1 integrin subunit. Although in transfection experiments β1B acts as a dominant negative inhibitor of cell adhesion, we found that β1B was expressed at very low levels in keratinocytes, both in vivo and in culture, and had a predominantly cytoplasmic distribution, concentrated within the endoplasmic reticulum. To examine why β1B accumulated in the cytoplasm, we prepared chimeras between CD8α and the β1A and β1B integrin cytoplasmic domains. In transfected HeLa cells, both constructs reached the cell surface but the rate of maturation of the β1B chimera was considerably retarded relative to β1A. The β1B cytoplasmic domain contains two lysine residues that resemble the double lysine motif characteristic of many proteins that are resident within the endoplasmic reticulum. Mutation of each lysine individually to serine had no effect on CD8β1B maturation, but when both residues were mutated the rate of CD8β1B maturation increased to that of CD8β1A. Further analysis of β1B function in keratinocytes must, therefore, take into account the low abundance of the isoform relative to β1A and the potential for β1B to accumulate in the endoplasmic reticulum. J. Cell. Biochem. 78:97–111, 2000.

Key words: keratinocytes; integrins; endoplasmic reticulum; cell adhesion

Extracellular matrix receptors of the β1 integrin family not only mediate cell adhesion and migration, but also control proliferation, morphogenesis, and differentiation in a wide range of tissues [reviewed by Adams and Watt, 1993; Boudreau et al., 1995; Schwartz et al., 1995; Hynes, 1996; Brakebusch et al., 1997]. The cytoplasmic domain of the β1 subunit has a key role in transduction of signals between the cell and the extracellular matrix [Clark and Brugge, 1995; Schwartz et al., 1995; Yamada and Miyamoto, 1995]. In addition to the classical form of β1, β1A, three other forms of the β1 subunit, β1B, β1C, and β1D, which differ in the amino acid sequence of the cytoplasmic domain, are generated through alternative splicing [reviewed by Fornaro and Languino, 1997].

The β1B variant is formed through a failure of RNA splicing at the 3′ end of exon 6 and premature termination of transcription as a result of a polyadenylation signal 1,135 nucleotides downstream of exon 6 [Altruda et al., 1990; Van der Flier et al., 1995; Baudoin et al., 1996]. The first 26 amino acids of the cytoplasmic domain of β1B are identical to β1A, but the final 12 amino acids are unique [Altruda et al., 1990] (Fig. 1). When transfected into CHO cells, the β1B subunit forms heterodimers with the endogenous α subunits that interact with fibronectin in an RGD-dependent fashion [Balzac et al., 1993]. The β1B subunit, however, does not localise to focal contacts, since it lacks the NPIY motif found in the β1A subunit [Reszka et al., 1992] and transfection of β1B has a dominant-negative effect on cell adhesion and motility [Balzac et al., 1994; Retta et al., 1998].

By Western blotting, the β1B integrin subunit is detected primarily in the skin and in liver [Balzac et al., 1993]. The observation that

W.J. Kee's present address is SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Harlow, Essex, CM19 5AW, United Kingdom.

*Correspondence to: Fiona M. Watt, Keratinocyte Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom. E-mail: watt@icrf.icnet.uk

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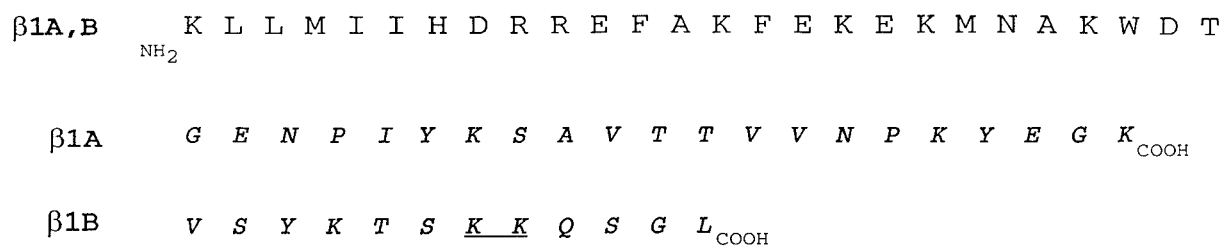

Fig. 1. Comparison of the amino acid sequences of the cytoplasmic domain of the β1A and β1B integrin subunits. Data are from Balzac et al. [1993]. The amino acids in plain type are common to both subunits. The lysine residues at the −5 and the −6 positions from the carboxy terminus of the β1B integrin subunit are underlined.

a β1 integrin variant that decreases adhesiveness is expressed in epidermal keratinocytes is of particular interest because loss of adhesiveness is a potent terminal differentiation stimulus for keratinocytes and essential for epidermal morphogenesis [reviewed by Watt and Hertle, 1994]. Our interest in β1B has been further stimulated by the fact that the β1B cytoplasmic domain contains two lysine residues, at the −5 and −6 positions from the carboxy terminus, resembling the double lysine motif for endoplasmic reticulum (ER) retention/retrieval, which is reported to be lysine at −3 and at either −4 or −5 [Nilsson et al., 1989; Jackson et al., 1993]. The hypothesis that the β1B integrin subunit contains such a motif raises the possibility that β1B could be involved in the inhibition of transport of integrin subunits that occurs when keratinocytes are placed in suspension to induce terminal differentiation [Hotchin and Watt, 1992; Hotchin et al., 1995]. The block in intracellular transport is keratinocyte- and integrin-specific and is believed to ensure that newly synthesised integrins do not reach the cell surface at a time when receptors already on the surface have been converted to an inactive, non-ligand binding, state [Hotchin et al., 1995; see also Dalton et al., 1995].

Set against these possible functions for β1B is the observation that the β1B variant is not encoded in the mouse genome [Baudoin et al., 1996]. If β1B were of general significance in the regulation of cell adhesion, one might expect that it would be conserved during mammalian evolution. Furthermore, little is known about the level of expression of β1B relative to β1A in keratinocytes or other cell types and this information is necessary in order to evaluate the physiological significance of the β1B integrin subunit.

METHODS

Cell Culture

Keratinocytes (strains z, kb, km) isolated from neonatal human foreskin were cultured on a feeder layer of J2-3T3 cells in 1 part Ham's F12 medium, 3 parts DMEM, 1.8×10^{-4} M adenine, 10% foetal calf serum (FCS), 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin, and 10 ng/ml EGF (FAD + FCS + HICE) [Watt, 1998]. HeLa cells were cultured in DMEM with 10% FCS. J2-3T3 cells were cultured in DMEM with 10% donor calf serum.

Construction of the CD8β1 Expression Vectors

The expression vector used was pCMU IV, a generous gift from T. Nilsson [Nilsson et al., 1989]. cDNAs corresponding to the entire cytoplasmic domain of the β1A and β1B integrin subunits were generated by RT-PCR and included a 5′ Xbal site and a 3′ BamHI site for cloning. pCMU IV was digested with Xbal and BamHI and ligated to the PCR fragments. Sequences of the β1A and β1B cytoplasmic domains were confirmed by sequencing directly from the pCMU IV vector. The final construct encoded the extracellular domain and transmembrane region of CD8α and the first 5 amino acids of the cytoplasmic domain of the E3/19K adenoviral protein, KYKSR, followed by the complete cytoplasmic domain of the β1A or β1B integrin subunit.

Mutants of the β1B cytoplasmic domain, SSQSGL, KSQSGL, and SKQSGL, were generated by PCR using CD8β1B as template and cloned into pCMU IV as described above. The mutations were confirmed by sequencing directly from the pCMU IV vector.

Transfection

HeLa cells were transfected at 50% confluence with 20 μg DNA per 100 mm dish via calcium phosphate precipitation. In transient transfection experiments, cells were analysed 48–72 h after transfection. Stable transfectants were isolated by co-transfecting the CD8 constructs and a retroviral vector expressing the puromycin resistance gene (pBabe puro) [Morgenstern and Land, 1990], in the ratio 17.5 μg CD8 DNA and 2.5 μg pBabe puro. Twenty-four hours after transfection, cells were harvested and replated at dilutions ranging from 1:3–1:10, and 48 h later 0.25 μg/ml puromycin was added. Three weeks after transfection, clones of surviving cells were picked, expanded, and screened for CD8 expression. Since CD8 staining revealed heterogeneous expression, the cells were subjected to single cell cloning in 96-well plates. Thirteen clones of CD8β1B, 2 clones of CD8β1A, 2 clones of CD8, and 3 clones of CD8E19 transfectants were established.

Antibodies

The following mouse monoclonal antibodies were used. P5D2 recognises an epitope on the extracellular domain of human β1 integrins (Developmental Studies Hybridoma Bank) [Dittel et al., 1993]. CD8 was detected with UCHT4 [Beverley, 1982], unconjugated (gift of P.C.L. Beverley) or FITC-conjugated (Sigma), and with OKT8 (European Catalogue of Animal Cell Cultures) [Thomas et al., 1980]. SY5 recognises human involucrin [Hudson et al., 1992]; AF8 recognises calnexin (gift of M.B. Brenner) [Hochstenbach et al., 1992]; 1D3 was generated against peptide KDDDKAVKDEL of protein disulphide isomerase (gift of S. Fuller) [Huovila et al., 1992]; M3F7 recognises type IV collagen (Developmental Studies Hybridoma Bank) [Foellmer et al., 1983]. M3F7 was conjugated to FITC essentially as described by Harlow and Lane [1988]. Secondary antibodies conjugated to FITC, Texas Red or HRP were purchased from Jackson ImmunoResearch and Amersham.

Three new antibodies to the β1B subunit were generated. WJK3 is a rabbit antiserum to the peptide DTVSYKTSKKQSGL and DH3 is a rabbit antiserum to the peptide KMNAKWDTVSYKTSKKQSGL. WJK16 is a mouse monoclonal antibody raised to the last 30 amino acids of the β1B integrin cytoplasmic domain, RREFAKFEKEKMNAKWDTVSYKTSKKQSGL. To generate WJK16, a female BALB/C mouse was immunised with the peptide and the spleen cells were fused with the SP2 myeloma cell line using the method of Harlow and Lane [1988]. The primary screen for monoclonals was an ELISA assay on immobilised immunogen; subsequent screening was carried out as described in Results. An antiserum to the β1B-specific peptide SYKTSKKQSGL was generously provided by G. Tarone [Balzac et al., 1993].

Flow Cytometry

To detect cell surface epitopes, cells were stained live with antibodies diluted in Hank's buffered saline solution containing 0.2% fish skin gelatin and 0.02% sodium azide and subsequently fixed in 3% paraformaldehyde in PBS for 5 min at 4°C or else analysed immediately following staining with 5 mg/ml propidium iodide added as a test for viability. Intracellular epitopes were detected by permeabilising cells in 0.3% saponin, 10% FCS in PBS (FSP) for 20 min at room temperature [Jacob et al., 1991] and incubating with antibodies diluted in FSP [Gandarillas and Watt, 1997]. Samples were analysed on a Becton-Dickinson FACScan machine, collecting data for 10,000 events.

Immunofluorescence Staining of Cultured Cells

To visualise intracellular membrane compartments, cells grown on glass coverslips were fixed in 3% paraformaldehyde for 20 min at room temperature, rinsed in PBS, incubated in 50 mM NH_4Cl for 10 min at room temperature, washed in PBS, and permeabilised in 0.1% Triton X-100 in PBS for 5 min at room temperature. To visualise focal adhesions, cells were fixed and permeabilised simultaneously in 3.7% formaldehyde and 0.4% Triton X-100 in PBS for 5 min at room temperature. Fixed cells were blocked in 0.2% fish skin gelatin (Sigma) in PBS for 20 min, then stained with primary and secondary antibodies diluted in block solution. Antibody incubations were for 1 h at room temperature and samples were rinsed in PBS after each antibody incubation. Samples were mounted in Gelvatol and examined with a Zeiss Axiophot microscope, a Nikon Diaphot 200 inverted microscope, or a Bio-Rad MRC-1000 scanning confocal microscope.

Immunofluorescence Staining of Tissue

Normal human neonatal foreskin embedded in OCT compound (BDH) was frozen for 1 min in isopentane cooled in liquid nitrogen. Frozen material was stored at −70°C. Sections (6 μm) were cut using a cryomicrotome. For staining, sections were air-dried at room temperature, blocked in 0.2% fish skin gelatin (Sigma) in PBS, and then incubated in primary and secondary antibodies diluted in block solution. Antibody incubations were for 1 h at room temperature. Sections were rinsed in PBS after each antibody incubation. Finally, sections were fixed in 3% paraformaldehyde, mounted in Gelvatol, and examined with a Zeiss Axiophot microscope, a Nikon Diaphot 200 inverted microscope, or a Bio-Rad MRC-1000 laser scanning confocal microscope.

Immunoprecipitation and Immunoblotting

For pulse labelling experiments, HeLa cells stably or transiently transfected with CD8β1A or CD8β1B were incubated in methionine-free medium for 20 min and then incubated in methionine-free medium containing 100 μCi/ml [^{35}S]-cysteine and -methionine for 20 min. The labelling medium was removed and the cells were washed three times in PBS. For the 0-h time point, cells were lysed immediately in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 25 mM iodoacetamide, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 mM benzamidine, and 40 μg/ml PMSF [Ponnambalam et al., 1994]. For subsequent time points, cells were incubated in normal growth medium supplemented with 5 mM cysteine and methionine prior to lysis.

For some experiments, cold lysates were immunoprecipitated as described above, then transferred to Immunobilon polyvinyldifluoride (PVDF) membrane (Millipore) as described by Hotchin et al. [1995]. After transfer, the membrane was blocked in 5% non-fat milk powder in PBS containing 0.05% Tween-20 (PBST) and then incubated in primary antibody followed by HRP-conjugated secondary antibody. HRP-conjugated antibody was detected using a chemiluminescence kit (ECL, Amersham). In some experiments, single cell suspensions of keratinocytes were surface labelled with biotin using NHS-LC-Biotin (Pierce), as described previously [Hotchin and Watt, 1992]. The cells were lysed and immunoprecipitated, transferred to PVDF membrane, incubated with HRP-conjugated streptavidin, and visualised using chemiluminescence (ECL, Amersham), as described previously [Hotchin and Watt, 1992].

Semi-Quantitative RT-PCR

Total RNA was extracted in guanidinium isothiocyanate buffer from keratinocytes, untransfected HeLa cells, and HeLa cells stably expressing CD8β1A or CD8β1B, essentially as described previously [Nicholson and Watt, 1991]. Specific 3′ primers for the β1A and β1B integrin subunits and, as a control, actin, were used in the first strand cDNA synthesis reaction. The first strand cDNA synthesis reaction was performed in a single tube and then individual aliquots were amplified by PCR using Taq DNA polymerase for different numbers of cycles [Braga et al., 1992]. The 5′ primers used for amplifying the β1A and β1B subunits were identical and spanned the region of the cytoplasmic domain proximal to the plasma membrane that is common to both subunits. Samples were analysed by electrophoresis on a 1.8% agarose gel following staining with ethidium bromide. The intensities of the β1A and β1B PCR products were quantitated in arbitrary units using the Image store 5000 programme. The following reaction conditions were used: "hot" start of 5 min at 95°C and 5 min at 72°C (during which Taq DNA polymerase was added) followed by 35 cycles of 94°C for 1 min, 65°C for 30 s, and 72°C for 1 min. The following primers were used:- Primer common to β1A and β1B: 5′-GCGGCTCTAGAAAGCTTTTAATGATAATTCATGACAG-3′; β1A-specific: 5′-GGCGCGGATCCTCATTTTCCCTCATACTTCGGATTG-3′; β1B-specific: 5′-GGCGCGGATCCTTATAAGCCACTTTGCTTTTTGGATG-3′; actin 5′: 5′-GTGGGCCGCTCTAGGCACCA-3′; actin 3′: 5′-TGGCCTTAGGGTGCAGGGGG-3′.

RESULTS

Preparation of Antibodies Specific for the β1B Subunit

In order to study the expression and potential functions of the β1B integrin subunit in keratinocytes, it was necessary to prepare β1B-specific antibodies. Preliminary observations with an antibody generously provided by G. Tarone [Balzac et al., 1993] suggested that β1B was not abundant in keratinocytes (data not

shown) and, thus, to facilitate antibody screening we prepared chimeric proteins consisting of the extracellular and transmembrane domains of CD8α and the cytoplasmic domains of the β1A and β1B integrin subunits. Mice and rabbits were injected with synthetic peptides comprising different regions of the β1B cytoplasmic domain and the resulting antibodies were screened against HeLa cells that had been stably transfected with CD8β1A or CD8β1B (Fig. 2). WJK3 is a rabbit antiserum to the C-terminal 14 amino acids of β1B; DH3 is a rabbit antiserum to the C-terminal 20 amino acids; and WJK16 is a mouse monoclonal antibody to the C-terminal 30 amino acids. By Western blotting, all three antibodies recognised a single band of approximately 30 kD in lysates of CD8β1B but not CD8β1A transfectants (Fig. 2A and data not shown). All three antibodies labelled CD8β1B, but not CD8β1A, transfectants by immunofluorescence, the staining pattern being predominantly perinuclear (Fig. 2B and data not shown).

The specificity of the antibodies was further confirmed by flow cytometry of permeabilised HeLa cells (Fig. 2C). CD8β1A and CD8β1B transfectants were positively labelled with UCHT4, a CD8-specific antibody, whereas the parental HeLa cells were not. Parental HeLa cells and CD8β1A transfectants did not label with any of the β1B-specific antibodies, whereas CD8β1B transfectants were positively labelled (Fig. 2C).

Expression and Subcellular Distribution of the β1B Subunit in Human Keratinocytes

The antibodies raised against the β1B integrin subunit were used to examine the distribution and abundance of β1B in human epidermis and in cultured human keratinocytes (Fig. 3). In the epidermis, labelling was confined to the basal layer: the labelling achieved with all three antibodies (Fig. 3A,B and data not shown) was much weaker than that observed with antibodies to the extracellular domain of β1 (Fig. 3C) and appeared diffusely cytoplasmic rather than membrane-associated (Fig. 3A,B; compare with C). The anti-β1B antibodies did not label focal adhesions in cultured keratinocytes (Fig. 3D), but labelled the cytoplasm in the same pattern as observed with antibodies to ER resident proteins such as protein disulphide isomerase (Fig. 3E).

To compare the level of β1B expressed on the surface of keratinocytes relative to total β1 integrins, single cell suspensions were surface labelled with biotin prior to extraction and immunoprecipitation (Fig. 3F). An antibody to the extracellular domain of β1 immunoprecipitated mature α and β1 subunits, together with the 80 kD fragment of the α3 subunit generated during trypsinisation of keratinocytes [Bishop et al., 1995]. An antibody (SY5) to an abundant cytoplasmic protein, involucrin, did not immunoprecipitate any labelled bands, establishing that the cells had not become permeable during biotinylation. WJK3, specific for β1B, immunprecipitated a band that comigrated with mature β1, but its abundance was considerably lower than total β1 integrins and any associated α subunits were below the level of detection of the method.

The immunofluorescence and immunoprecipitation results suggested that β1B was expressed at a low level in keratinocytes. In order to confirm this using a method that was independent of antibody quality, we used semiquantitative RT-PCR (Fig. 4). Total RNA was prepared from cultured human keratinocytes, parental HeLa cells and HeLa cells stably transfected with CD8β1A or CD8β1B. Specific 3′ primers for the β1A and β1B integrin subunits were used in the first strand synthesis. Aliquots of the first strand synthesis reactions were used in PCR with a 5′ primer that spanned a region of the cytoplasmic domain that is common to both subunits. Actin primers were used as a control. At 10 cycles of amplification and at every fifth cycle thereafter, up to a maximum of 30 cycles, an aliquot of each reaction was removed (Fig. 4A). The intensities of the amplified bands were quantitated and plotted against the number of amplification cycles (Fig. 4B). The slope of each line corresponded to the rate of amplification, which in turn is directly related to the number of template molecules prior to amplification [Braga et al., 1992].

Since the primers used to amplify β1A, β1B, and actin were different, comparison of the intensities of the different PCR products after the same number of amplification cycles does not give a sound indication of the relative abundance of the corresponding mRNAs. However, for a given primer pair, comparison of the intensities of the same PCR product from different RNAs does provide information about

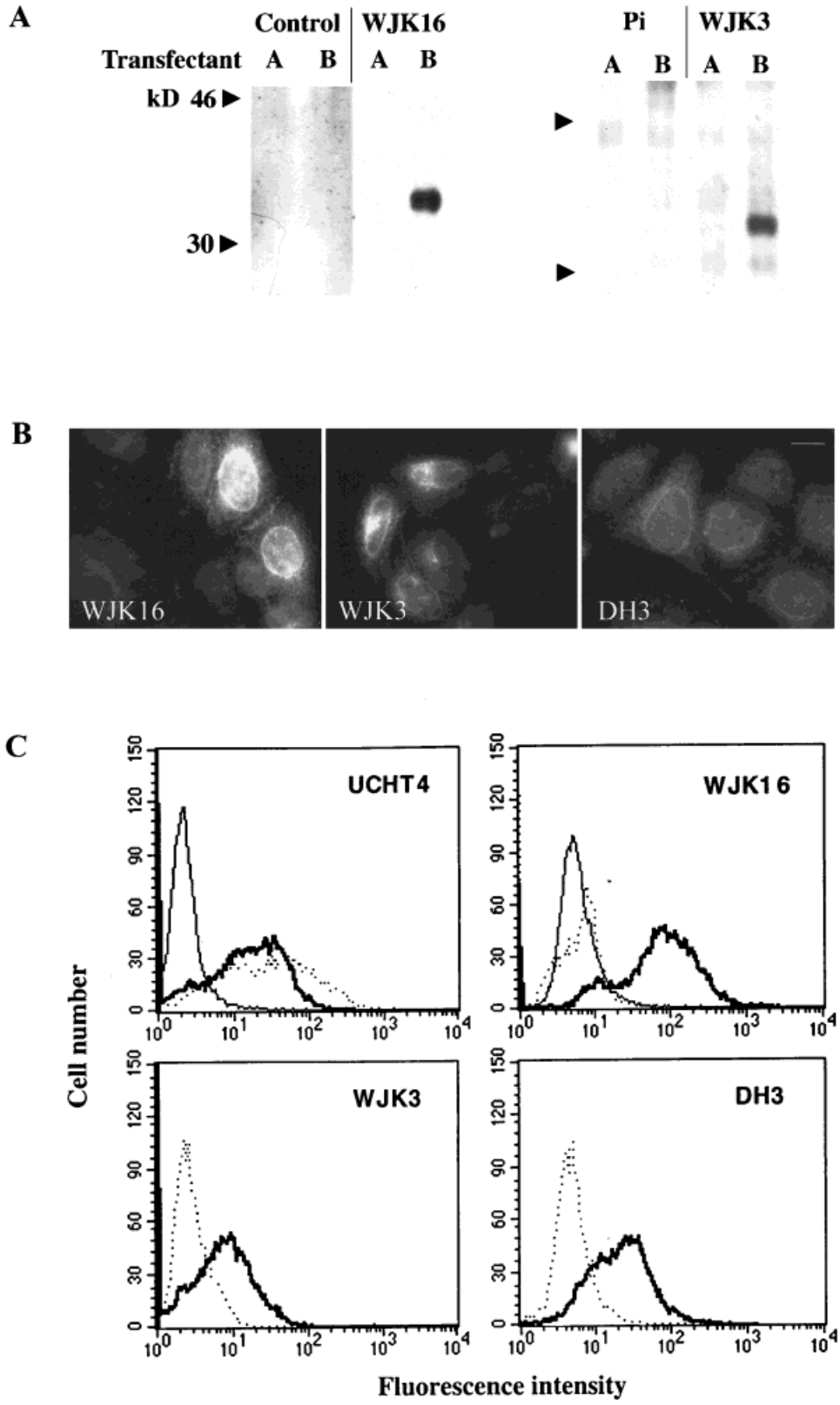
A
Control
WJK16
Transfectant
A
B
A
B
kD 46
30
Pi
WJK3
A
B
A
B
B
WJK16
WJK3
DH3
C
UCHT4
WJK16
WJK3
DH3
Cell number
Fluorescence intensity

Figure 2.

the relative abundance of the mRNAs. The actin primers acted as a control for a highly expressed mRNA from all cell populations and the actin PCR product was observed after only 15 cycles of amplification (Fig. 4A).

The β1A PCR product accumulated with approximately the same kinetics in keratinocytes, parental HeLa cells, CD8β1A and CD8β1B transfectants, suggesting that levels of β1A were similar (Fig. 4B). The level of the β1B PCR product accumulated at a similar rate to CD8β1A in CD8β1B transfectants. However, in keratinocytes, parental HeLa cells, and CD8β1A transfectants, the rate of accumulation of the CD8β1B PCR product was considerably lower than that of the CD8β1A product. We conclude that β1B mRNA is less abundant in keratinocytes than β1A mRNA, consistent with the conclusions regarding relative protein levels reached on the basis of immunofluorescence staining and immunoprecipitation experiments (Fig. 3).

β1B Is Partially Retained Within the ER

The cytoplasmic staining of the β1B integrin subunit observed by immunofluorescence suggested that intracellular transport and maturation could differ between the β1A and β1B integrin subunits, but the low abundance of β1B in keratinocytes precluded detailed biochemical analysis. We, therefore, examined β1B maturation using the CD8β1A and CD8β1B chimeric proteins. The integrin chimeras were compared with wild type CD8 and with CD8E19, a chimera containing the cytoplasmic domain of the E3/19K adenoviral protein, which contains an authentic double lysine motif for ER retention/retrieval [Nilsson et al., 1989; Jackson et al., 1993]. HeLa cells were examined following transient transfection or selection of stably transfected clones. In each case, the data obtained were the same for transient and stable transfectants and for different clones transfected with the same construct.

Consistent with earlier reports that full-length transfected β1B is expressed at the cell surface, but does not enter focal adhesions [Balzac et al., 1993, 1994] (see also Fig. 3D,F), both CD8β1A and CD8β1B were detected by live labeling of HeLa cells with anti CD8 (Fig. 5A,B), but only CD8β1A colocalised with vinculin in focal adhesions (Fig. 5C,D) [Bishop et al., 1998]. A further difference between the distribution of CD8β1A and β1B in permeabilised cells was the strong perinuclear labelling in CD8β1B transfectants (Fig. 5D). None of the HeLa transfectants showed any marked reduction in adhesiveness or spreading (data not shown) [see La Flamme et al., 1994; Lukashev et al., 1994].

The subcellular distribution of CD8β1A and CD8β1B was compared with CD8 and CD8E19 in cells that had been fixed and permeabilised under conditions that preserve intracellular organelles, as opposed to focal adhesions (Fig. 6). CD8 and CD8β1A were localised to vesicles throughout the cytoplasm (Fig. 6A,C) whereas CD8E19 and CD8β1B were concentrated in a perinuclear ring (Fig. 6B,D). Double label immunofluorescence of CD8β1B transfectants with anti CD8 and antibodies to calnexin or protein disulphide isomerase (PDI), ER resident proteins [Hochstenbach et al., 1992; Huovila et al., 1992], showed that the perinuclear distribution of CD8β1B corresponded to a subcompartment of the ER (Fig. 6E).

Taken together, the flow cytometry and immunofluorescence microscopy results suggested that although CD8β1B could reach the cell surface its maturation was less efficient than that of CD8β1A. We examined this by pulse labelling stably transfected HeLa cells with [^{35}S]-cysteine and -methionine and then immunoprecipitating with anti-CD8 (Fig. 7). As controls, HeLa cells stably transfected with CD8 or CD8E19 were examined first (Fig. 7A). At time 0, a single band (doublet in case of CD8) of about 30 kD was observed that corresponded to the immature, nonglycosylated protein. At 2 h, the immature protein was no longer detected in the CD8 transfectants and a band of about 35 kD, corresponding to the ma-

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Fig. 2. Characterisation of antibodies specific for CD8β1B. **A:** Protein lysates from HeLa cells stably transfected with CD8β1A or CD8β1B were immunoprecipitated with an anti-CD8 antibody, OKT8. Immunoprecipitated proteins were transferred onto a membrane that was probed with the mouse monoclonal antibody, WJK16, and irrelevant monoclonal antibody as a negative control (Control), or with the rabbit antiserum WJK3 and pre-immune serum (Pi). **B:** HeLa cells stably transfected with CD8β1B were fixed with paraformaldehyde, permeabilised and stained with WJK16, WJK3, or DH3. Scale bar = 10 μm. **C:** HeLa cells stably transfected with CD8β1A or CD8β1B were permeabilised using 3% saponin. Parental non-transfected HeLa cells (thin solid line), CD8β1A (dotted line), and CD8β1B (thick solid line) transfectants were stained with an anti-CD8 antibody, UCHT4, or with WJK16, WJK3, or DH3.

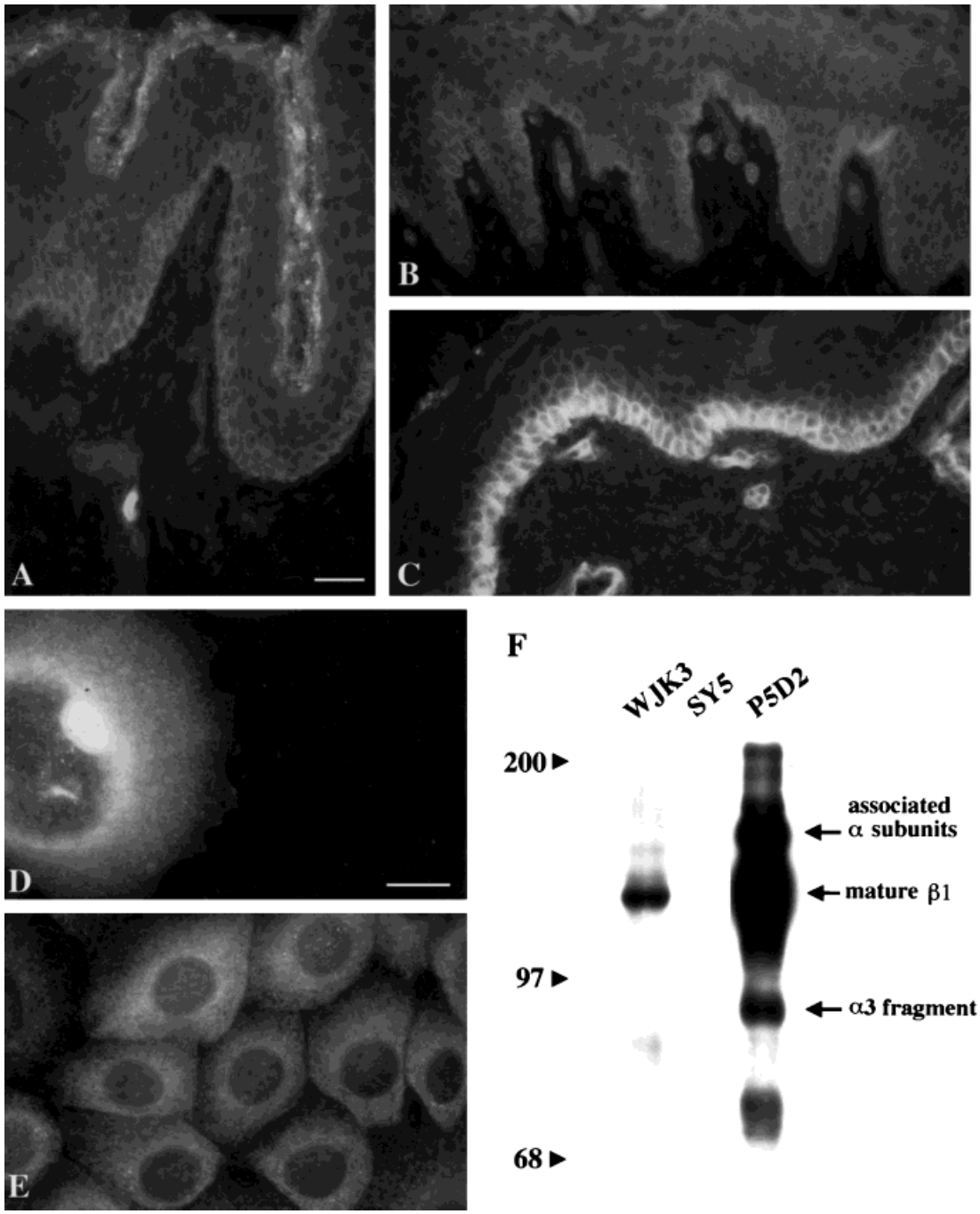

Fig. 3. β1B integrin subunit expression in human keratinocytes. **A–C:** Frozen sections of human foreskin epidermis were stained with the anti β1B antibodies DH3 (A) and WJK16 (B). P5D2, to the β1 integrin extracellular domain, was included as a positive control (C). **D,E:** Human keratinocytes cultured on glass coverslips were fixed, permeabilised, and stained with WJK3 (D) or ID3 antibody to protein disulphide isomerase (E). Scale bar = 25 μm (A–C), 10 μm (D,E). **F:** A single cell suspension of keratinocytes was surface biotinylated and immunoprecipitated with the antibodies shown. Samples were run under non-reducing conditions. Positions of molecular weight markers (kDa) are shown.

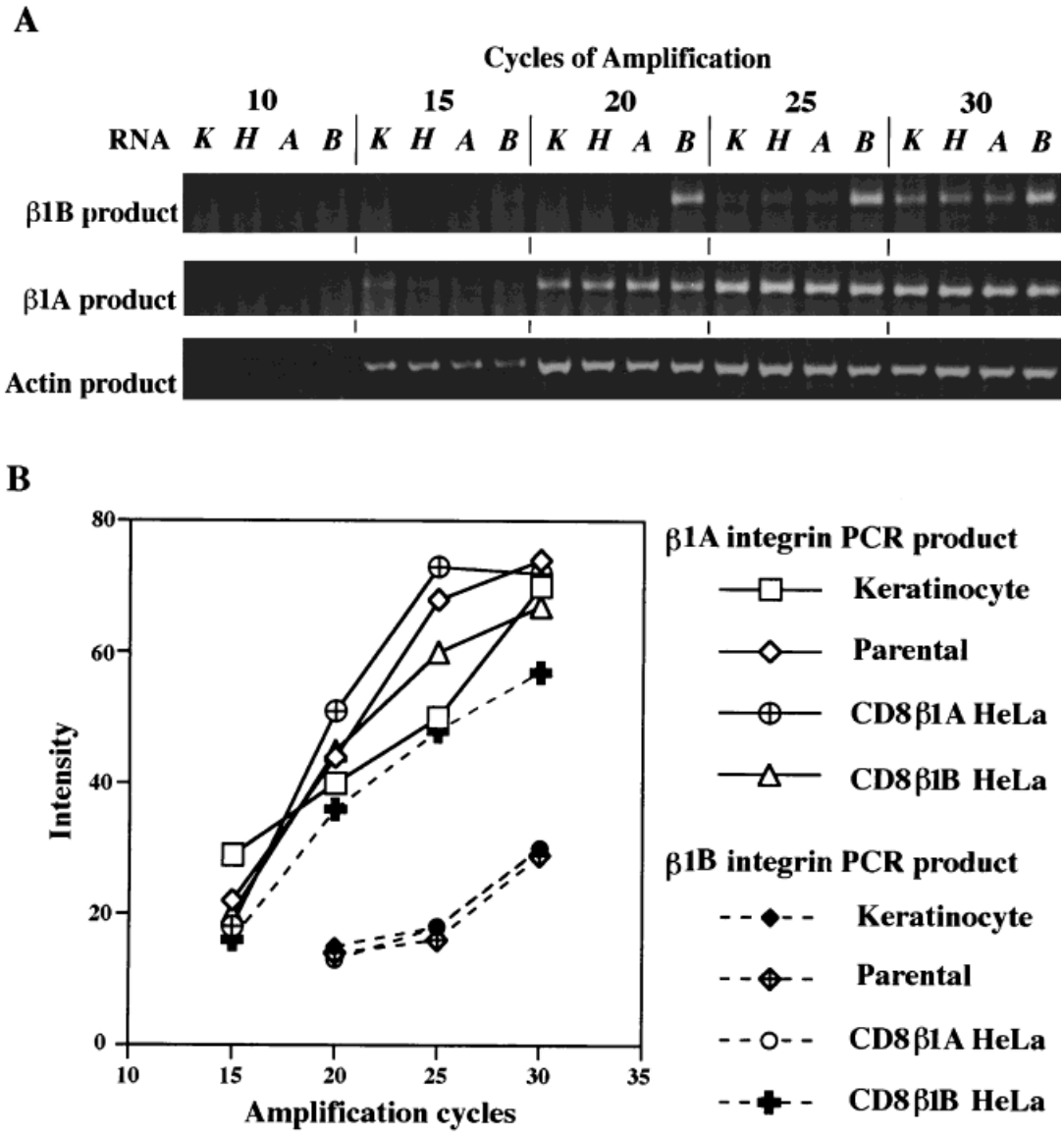

Fig. 4. Abundance of the β1B integrin subunit mRNA in keratinocytes. Total RNA prepared from newly confluent keratinocytes (K), HeLa cells stably expressing CD8β1A (A) or CD8β1B (B) and non-transfected parental HeLa cells (H) were used in RT-PCR reactions. Aliquots were removed after the number of amplification cycles shown (**A**). The intensities of the β1A and the β1B PCR products were quantitated in arbitrary units and plotted against the number of amplification cycles (**B**).

ture protein, had appeared [Nilsson et al., 1989]. In contrast, maturation of CD8E19 did not occur, even after a chase period of 4 h [Nilsson et al., 1989]. When CD8β1A and CD8β1B transfectants were examined at time 0, a single band of approximately 30 kD, corresponding to the immature, underglycosylated form of each chimera, was observed. By 2 h, a second band, of about 35 kD, corresponding to the fully glycosylated form, was observed. From 4 h onwards, the mature form of CD8β1A was more abundant than that of the immature form. In contrast, the immature form was as abundant as the mature form in CD8β1B immunoprecipitates as late as 8 h into the chase period. After overnight incubation, very little labelled CD8β1A or CD8β1B remained.

Double Lysine Motif in β1B Is Responsible for the Delay in β1B Maturation Relative to β1A

In order to test whether the lysine residues at the C terminus of β1B (Fig. 1) were respon-

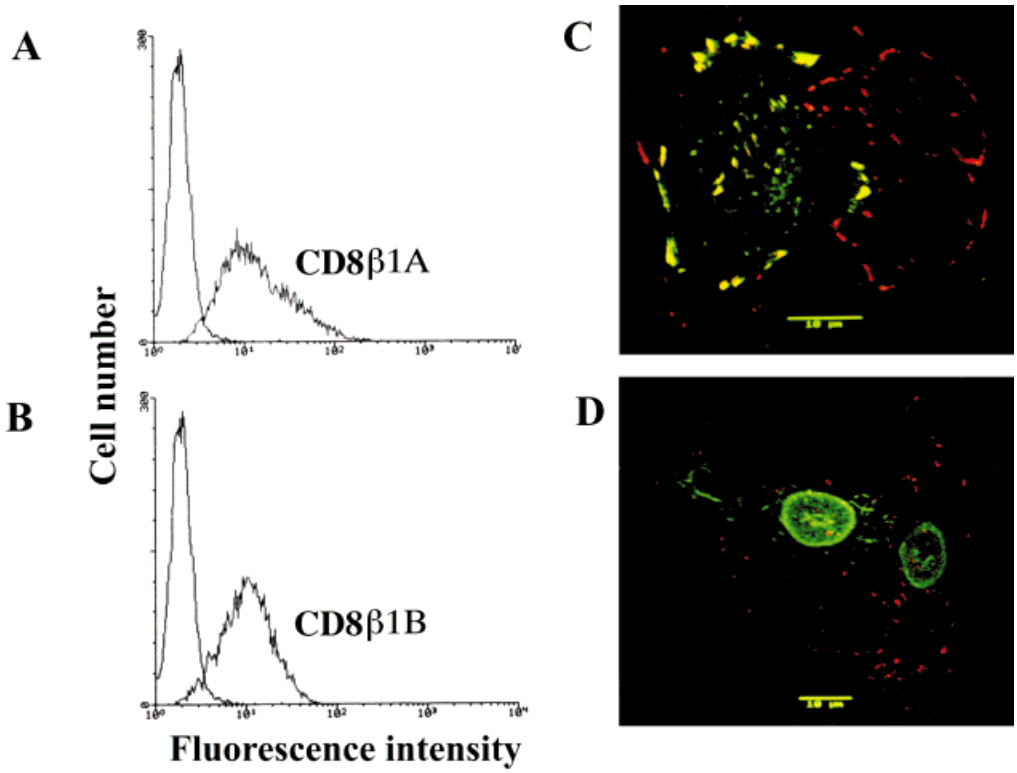

Fig. 5. Cell surface distribution of CD8β1A and CD8β1B chimeras. **A,B:** HeLa cells stably transfected with CD8β1A or CD8β1B were stained live with the anti-CD8 antibody, UCHT4-FITC, or with a negative control antibody, M3F7-FITC. Dead cells were gated out using propidium iodide and data were collected for 10,000 events. In each graph, the peak on the left-hand side corresponds to M3F7 labelling while the peak on the right-hand side corresponds to UCHT4. **C,D:** HeLa cells transiently transfected with CD8β1A (C) or CD8β1B (D) were permeabilised and stained with anti-vinculin antibody (red) and with anti-CD8 antibody (green). The staining pattern was visualised using confocal microscopy.

sible for the partial retention of β1B in the ER, each residue was mutated to a serine residue. Serine introduces a change in charge from positive to neutral, but still maintains the solubility of the cytoplasmic domain. Each lysine was mutated individually and, in addition, both lysines were mutated. The plasmids were used to transiently transfect HeLa cells and the distribution of CD8 was examined by immunofluorescence labelling of permeabilised cells. Mutation of one lysine residue, at the −5 or −6 position, did not change the intracellular location of CD8, which remained perinuclear and diffuse throughout the cytoplasm (Fig. 8A). However, when both lysine residues were mutated, the perinuclear staining was less intense and staining of vesicles was observed, as seen in the case of the CD8β1A chimera (Fig. 8A; compare with Fig. 6C).

The effect of mutating the lysine residues on maturation of CD8β1B was also examined by pulse labelling transiently transfected HeLa cells with [^{35}S]-cysteine and -methionine (Fig. 8B). Cells were extracted at time 0 and after a 2-h chase. When neither lysine was mutated, the majority of the labelled chimeric protein remained in the immature form at 2 h, confirming the result shown in Figure 7B. This was also the case when one lysine was mutated. However, when both were mutated, the maturation rate was increased, to the extent that the mature and immature bands were equally abundant at 2 h, as observed for CD8β1A (Fig. 8B; compare with Fig. 7B).

DISCUSSION

Important functions have been reported for each of the β1 integrin cytoplasmic domain splice variants. In transfection experiments, β1B acts as a dominant negative inhibitor of adhesion [Balzac et al., 1994; Retta et al., 1998] while β1C inhibits cell cycle progression [Meredith et al., 1995, 1999; Fornaro et al., 1998]. β1D, the only splice variant to be found

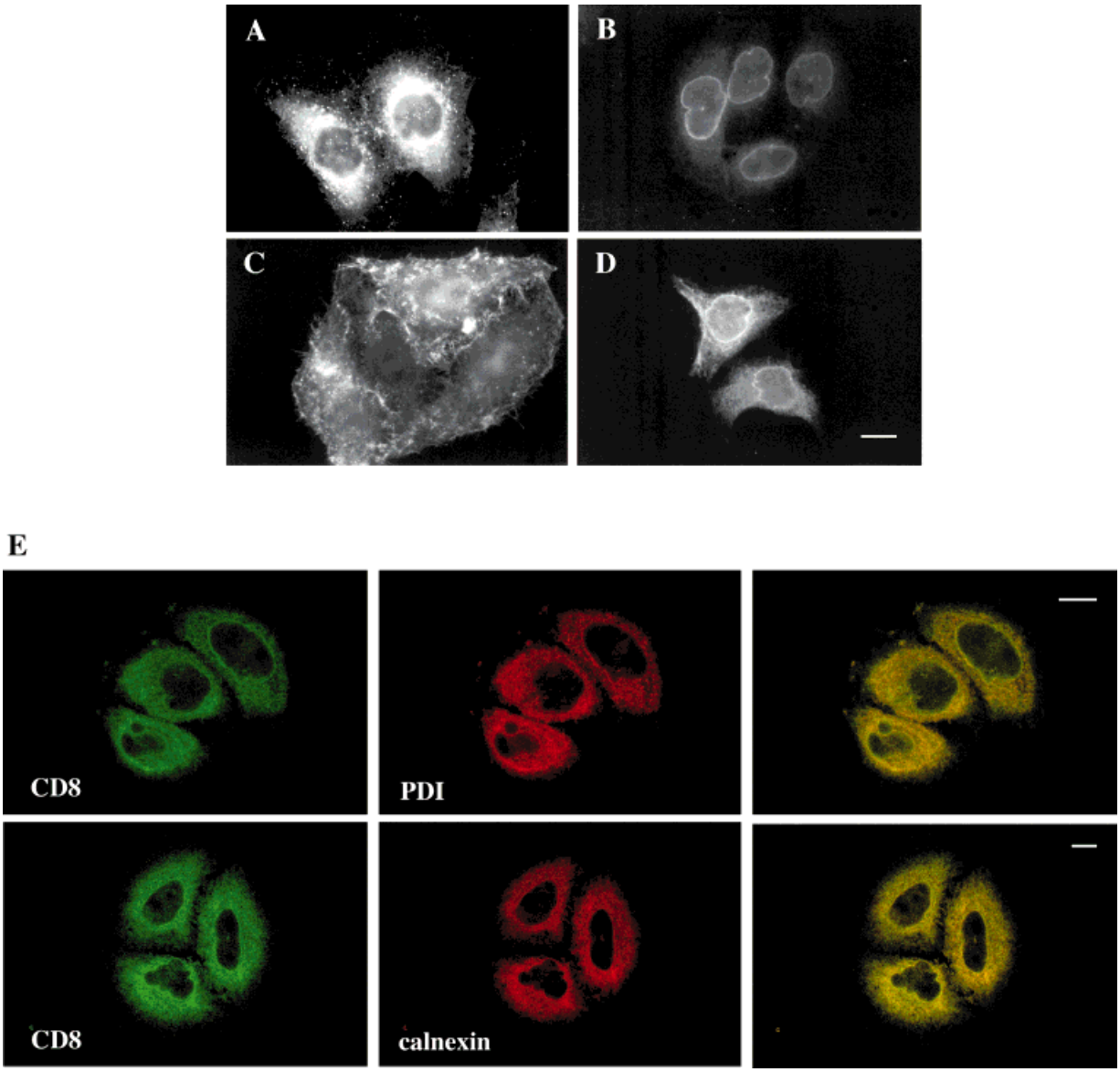

Fig. 6. Immunofluorescence localisation of CD8 and chimeric CD8 proteins in transfected HeLa cells. HeLa cells transiently transfected with CD8 (**A**), CD8E19 (**B**), CD8β1A (**C**), or CD8β1B (**D**) were fixed in paraformaldehyde, permeabilised and stained with an anti-CD8 antibody, UCHT4-FITC. **E:** Double label immunofluorescence of HeLa cells stably transfected with CD8β1B: UCHT4-FITC (green), antibodies to the ER-resident proteins, calnexin and protein disulphide isomerase (PDI) (red). Scale bars = 10 μm.

in the absence of β1A, plays an important role in muscle cells [Van der Flier et al., 1995; Belkin et al., 1996, 1997; Baudoin et al., 1998; Belkin and Retta, 1998]. However, the biological significance of β1B and β1C has been questioned because, unlike β1A and β1D, β1B and β1C are not conserved in the mouse genome [Baudoin et al., 1996]. The aim of our experiments was to analyse β1B expression in keratinocytes, which are one of the few cell types known to express β1B [Balzac et al., 1993] and are a cell type in which decreased adhesiveness is linked to initiation of terminal differentiation [Adams and Watt, 1990; Hotchin et al., 1993].

Using RT-PCR, immunoprecipitation and immunofluorescence, we found that β1B was expressed at a low level relative to total β1 integrins, both in the epidermis and in cultured keratinocytes. Immunofluorescence staining of epidermis, cultured keratinocytes, and CD8β1B transfectants showed cytoplasmic

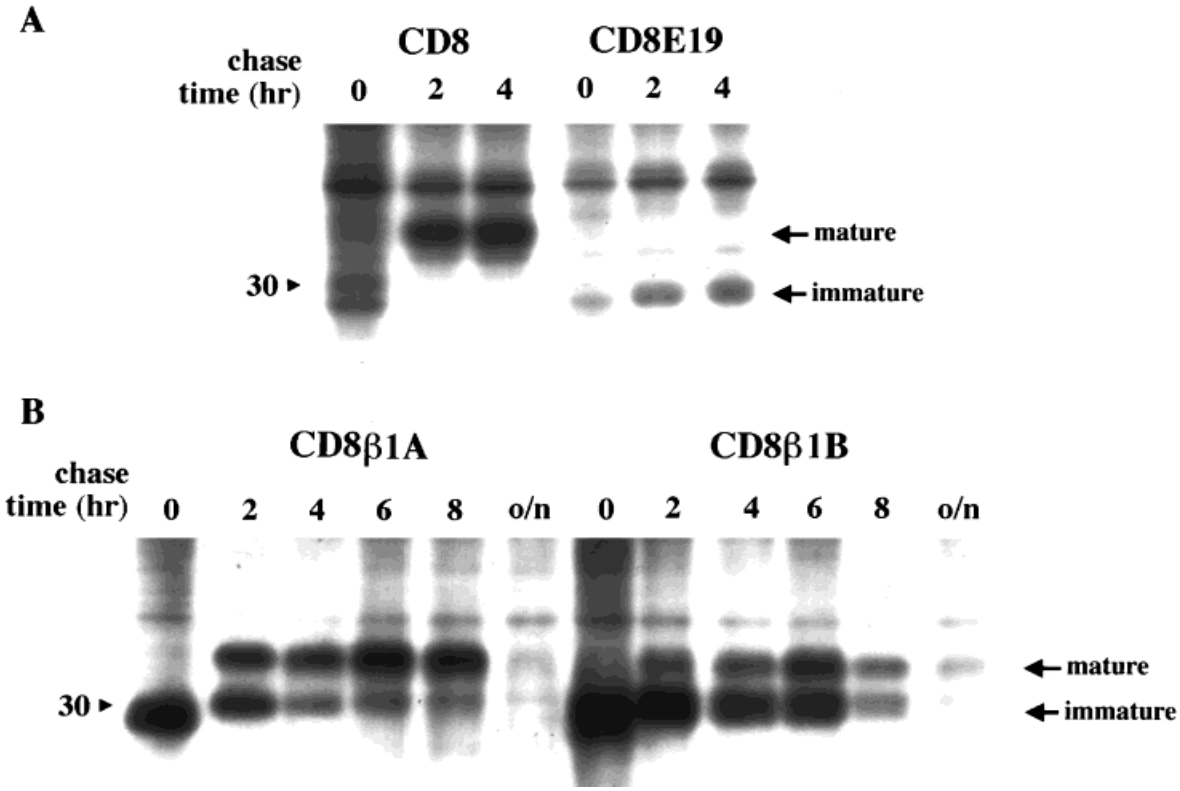

Fig. 7. Maturation of CD8β1A, CD8β1B, CD8, and CD8E19 in HeLa cells. **A:** HeLa cells stably transfected with CD8 or CD8E19 were pulse labelled for 20 min in medium containing [^{35}S]-cysteine and -methionine and then chased in medium containing excess unlabelled cysteine and methionine for 0, 2, or 4 h. Equal volumes of lysate were immunoprecipitated with OKT8, an anti-CD8 antibody. **B:** HeLa cells stably transfected with CD8β1A or CD8β1B were pulse labelled as described in A, but were examined at additional time points. o/n: overnight. Positions of the mature and immature forms of CD8, CD8E19, CD8β1A, and CD8β1B are indicated. Position of molecular weight marker (kDa) is shown.

accumulation of β1B in the ER. Although the endogenous β1B subunit in keratinocytes and CD8β1B in HeLa transfectants did reach the cell surface, CD8β1B underwent slower maturation than CD8β1A. Mutagenesis experiments showed that the lysines at the −5 and −6 positions of β1B were responsible for the reduced maturation rate.

KKXX is a signal for retrograde movement of ER proteins [Nilsson et al., 1989; Jackson et al., 1993] and retrieval is via COP I-coated vesicles [Cosson and Letourneur, 1994; Letourneur et al., 1994; Schutze et al., 1994; Teasdale and Jackson, 1996]. KKXX-containing proteins have been reported never to reach the cell surface [Jackson et al., 1990; Martire et al., 1996] although it now appears that, depending on the length and structure of the cytoplasmic domain, KKXX is not completely incompatible with cell surface expression [Vincent et al., 1998].

It remains to be investigated whether or not the KK motif in β1B associates with COP I-coated vesicles, or whether β1B is recognised as a mis-folded protein and retained by a different mechanism [Helenius et al., 1992]. In favour of the idea that it acts as a specific signal, both lysine residues were required for the reduced maturation of CD8β1B and both lysine residues are required for ER retrieval of KKXX proteins [Nilsson et al., 1989]. However, whether the KK motif is responsible for the cytoplasmic accumulation of β1B in keratinocytes has still to be established. Pulse chase experiments in transfected CHO cells did not reveal any differences in maturation rate of full-length β1A or β1B (data not shown) and transport of β1B to the cell surface may occur as a result of steric masking of the KK motif when β1B forms heterodimers with integrin α subunits [Teasdale and Jackson, 1996]. Furthermore β1C

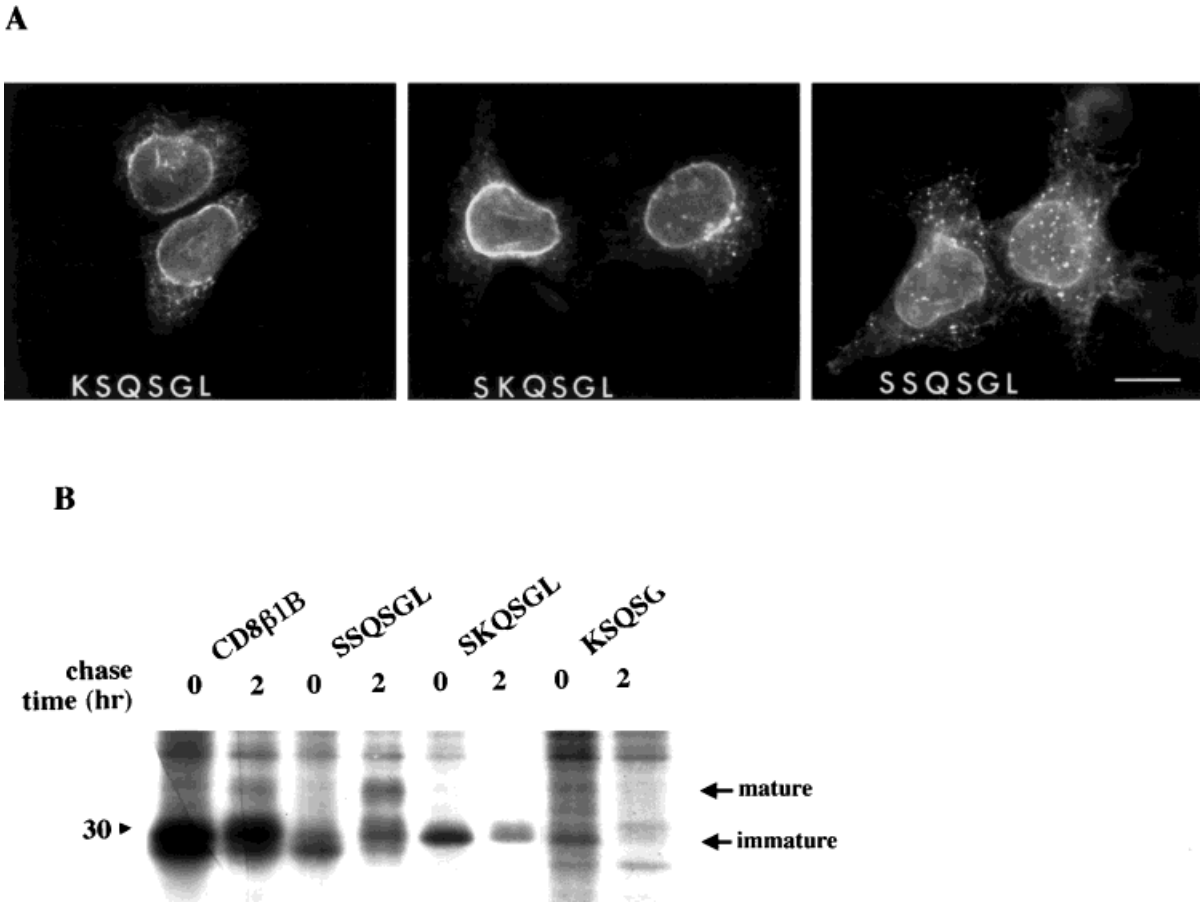

Fig. 8. Mutation of the two lysine residues closest to the carboxy terminus of CD8β1B affects maturation and subcellular distribution. **A:** Transiently transfected HeLa cells were permeabilised and stained with anti-CD8 antibody. Scale bar = 10 μm. **B:** Transient transfectants were pulse labelled and analysed at time 0 and after a 2-h chase period as described in the legend to Figure 7. Positions of mature and immature forms of CD8βB1B are indicated. Molecular weight standard: 30 kDa.

does not have a KK motif, yet in some cells it is also retained in the endoplasmic reticulum and degraded [Svineng and Johansson, 1999]. The fate of specific integrin isoforms may depend on the cell type in which they are expressed and on which integrin subunits are coexpressed.

Although β1B could potentially contribute both to the reduced adhesiveness of human keratinocytes [Adams and Watt, 1990; Hotchin et al., 1993] and to the block in intracellular transport of integrins to the cell surface [Hotchin and Watt, 1992; Hotchin et al., 1995] that occurs during terminal differentiation it is important to bear in mind that mouse keratinocytes also lose adhesiveness to the extracellular matrix on commitment to differentiation [Romero et al., 1998] even though they lack the β1B subunit [Baudoin et al., 1996]. Given that growth inhibition by the β1C integrin isoform can occur in the absence of membrane targeting [Meredith et al., 1999], it is possible that β1B could affect keratinocyte behaviour without reaching the cell surface. However, since the abundance of the β1B integrin subunit compared to total β1 integrins is so low, both in culture and in the epidermis, it is also possible that β1B lacks major functional significance in keratinocytes. At present, interest in the β1B integrin subunit must lie as much in what it tells us about the function of the β1A cytoplasmic domain [Retta et al., 1998] as in its physiological significance in normal cells and tissues.

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