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Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain

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The α6β4 complex is a member of the integrin superfamily of adhesion receptors. A human keratinocyte lambda gt11 cDNA library was screened using a monoclonal antibody directed against the β4 subunit. Two cDNAs were selected and subsequently used to isolate a complete set of overlapping cDNA clones. The β4 subunit consists of 1778 amino acids with a 683 amino acid extracellular domain, a 23 amino acid transmembrane domain and an exceptionally long cytoplasmic domain of 1072 residues. The deduced amino-terminal sequence is in good agreement with the published amino-terminal sequence of purified β4. The extracellular domain contains five potential N-linked glycosylation sites and four cysteine-rich homologous repeat sequences. The extracellular part of the β4 subunit sequence shows 35% identity with other integrin β subunits, but is the most different among this class of molecules. The transmembrane region is poorly conserved, whereas the cytoplasmic domain shows no substantial identity in any region to the cytoplasmic tails of the known β sequences or to other protein sequences. The exceptionally long cytoplasmic domain suggests distinct interactions of the β4 subunit with cytoplasmic proteins.

Key words: cDNA sequence/integrin/β4 subunit

Introduction

Integrins are αβ subunit cell surface heterodimers which mediate cell–cell and cell–matrix interactions (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). The integrin family is divided into three subfamilies; the VLA protein family (Hemler *et al.*, 1987), the Leu–Cam proteins (Springer *et al.*, 1987) and the cytoadhesins (Ginsberg *et al.*, 1988). These subfamilies are characterized by a common β subunit (β1, β2 and β3) that can associate with one of a number of α subunits.

Recently, three further β subunits, β4, βx (or βs) and βp, have been identified that form complexes with previously described α subunits (Sonnenberg *et al.*, 1988a; Cheresh *et al.*, 1989; Freed *et al.*, 1989; Hemler *et al.*, 1989; Holzmann *et al.*, 1989; Kajiji *et al.*, 1989).

The α6 subunit can associate with either the β1 or the β4 subunit. Complexes of α6β1 (VLA-6) are found on platelets and on a variety of different epithelial cell types (Sonnenberg *et al.*, 1987, 1988a; Hemler *et al.*, 1988, 1989). The α6β1 complex functions as a receptor for laminin (Sonnenberg *et al.*, 1988b). Recent studies have indicated that the site which is recognized by the α6β1 complex is located on the E8 fragment of laminin (Sonnenberg *et al.*, manuscript submitted). The same laminin fragment has previously been reported to promote neurite outgrowth (Edgar *et al.*, 1984) and cell adhesion (Goodman *et al.*, 1987; Aumailley *et al.*, 1987). The ligand of α6β4 is not known, but because cells expressing high levels of α6β4 do not adhere to the E8 fragment of laminin, it is probably different from that of the α6β1 complex.

Immunohistochemical analysis showed that β4 expression is limited to epithelial cells and Schwann cells (Sonnenberg *et al.*, manuscript submitted). The β4 subunit is particularly strongly expressed on squamous epithelial cells and is localized exclusively at the basal side of cells. This suggests a cell–matrix function for heterodimeric complexes of this subunit. A major difference between β4 and all other β subunits described so far, is its much larger size (M_r 205 000 versus 90–130 000).

To study the relationship of β4 with other β subunits and to determine the basis for the unusual size of β4, detailed structural information was needed. In this report, we describe the isolation and sequence analysis of β4 cDNA. We show that the β4 subunit has an extracellular part that is homologous to other β subunits and a unique large cytoplasmic part. A search of the GenBank data base revealed no significant similarities of the cytoplasmic domain to other proteins.

Results

Isolation of β4 cDNA

A lambda gt11 cDNA library prepared from poly(A)$^+$ RNA isolated from human keratinocytes was screened with the 439-9B monoclonal antibody against the integrin β4 subunit (Kennel *et al.*, 1989). Screening of ~500 000 recombinants identified two positive clones; clone 134 contained an insert of 4.3 kb and clone 140 of 2.5 kb. Restriction fragment analysis showed that the insert of clone 140 was almost completely contained within clone 134 (Figure 1). The cDNAs hybridized to a single mRNA species of ~6 kb (Figure 2). This mRNA was present in the normal mammary cell line HBL-100 and the mammary tumor cell line T47D, which express the β4 protein. Very low levels of β4 mRNA, seen only on long exposed blots, were detected in OVCAR-4 and A375 cells and this is in accord with the low expression of the β4 subunit in these lines. No mRNA was detected in the erythroid cell line K562. Both the distribution and the level of the 6 kb mRNA thus correspond to those of the β4 protein. The two cDNAs were subsequently sequenced. The deduced amino acid sequence contained a cysteine-rich domain which was previously found to be conserved in the β1, β2 and β3 subunits.

Overlapping cDNA clones that extended more to the 5′ end of the β4 cDNA were isolated by screening the same

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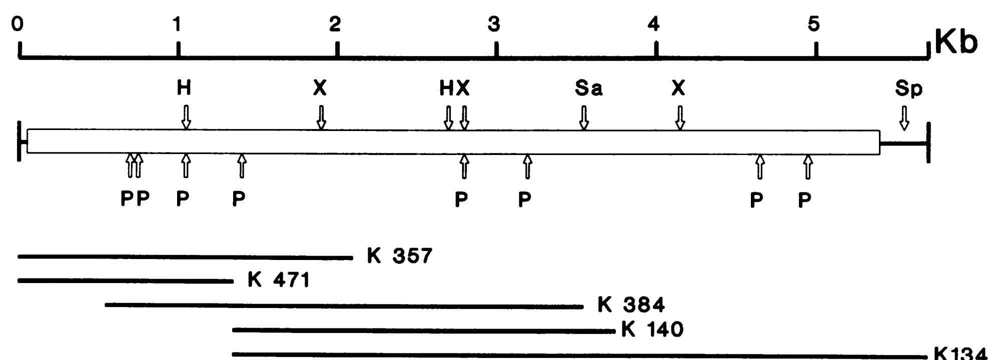

Fig. 1. Restriction map of the $\beta 4$ cDNA clones. The open reading frame is shown as an open box. The lines indicate the size and order of the various cDNA clones. Relevant restriction sites are *Pst*I (P), *Xma*I (X), *Hin*dIII (H), *Sac*I (Sa) and *Sph*I (Sp).

cDNA library with a radiolabeled probe from the most 5′ portion of clone 140. An additional round of screening, using a 5′ fragment of the newly isolated clone 357 (see Figure 1) as a probe, did not result in cDNA clones extending further in the 5′ direction. The cDNA clones together spanned a stretch of ~5.7 kb. This size corresponds closely to the size of the mRNA detected in the Northern blot analysis (Figure 2).

$\beta 4$ cDNA and deduced amino acid sequence

The cDNA sequence and the deduced amino acid sequence of $\beta 4$ are shown in Figure 3. The nucleotide sequence of 5696 bp contains a small 9 bp 5′ untranslated leader followed by a single open reading frame of 5415 bp encoding polypeptide of 1805 amino acids and by a 3′ untranslated region of 272 bp. The 3′ untranslated region contains the polyadenylation signal AATAAA, followed 16 bases further by a poly(A) stretch.

The open reading frame starts with an ATG methionine codon that is flanked by sequences that meet the requirement for the initiation of protein translation (Kozak, 1984). Following the ATG methionine codon, there is a stretch of 27 mostly hydrophobic amino acids (−27 to −1) with the characteristics of a signal peptide (von Heijne, 1984). We assume, therefore, that the ATG codon represents the initiation site for translation, although no in-frame stop codon is observed in the 9-base sequence preceding this site. The deduced amino-terminal sequence of the mature protein matches, except at two positions, the amino-terminal amino acid sequence of purified $\beta 4$ (Hemler *et al.*, 1989; Kajiji *et al.*, 1989). However, the amino acids at these two positions in the peptide sequence have not yet been unambiguously identified. Therefore, we conclude that the isolated cDNA is authentic for the $\beta 4$ subunit.

The mature protein contains an extracellular region of 683 amino acids, a hydrophobic region of 23 amino acids and an exceptionally long cytoplasmic domain of 1072 amino acids. There are five potential N-linked glycosylation sites in the extracellular part of the $\beta 4$ subunit. N-glycanase treatment of $\beta 4$ resulted in a reduction of the size by ~10 000 dalton (Sonnenberg *et al.*, manuscript submitted). This may suggest that four of the five sites (average mol. wt per site is 2500 dalton) are glycosylated *in vivo*. The calculated mol. wt of the mature $\beta 4$ subunit is 198 011 daltons; this value is close to the 195 000 daltons determined by SDS gel electrophoresis for the deglycosylated $\beta 4$ subunit. The extracellular portion of $\beta 4$ has a relatively high cysteine content, 48 out of 683 amino acids (7%), in contrast to the cytoplasmic portion which only contains 15 cysteine residues (1.4%). Most of the cysteines of the extracellular portion of $\beta 4$ are clustered at the amino terminus and in four homologously repeated cysteine-rich domains. Six of 15 cysteine residues in the cytoplasmic domain are located in a 15 amino acid stretch immediately following the transmembrane domain. These cysteine residues may be palmitoylated which may provide a better anchorage of the $\beta 4$ subunit in the plasma membrane (Magee *et al.*, 1989).

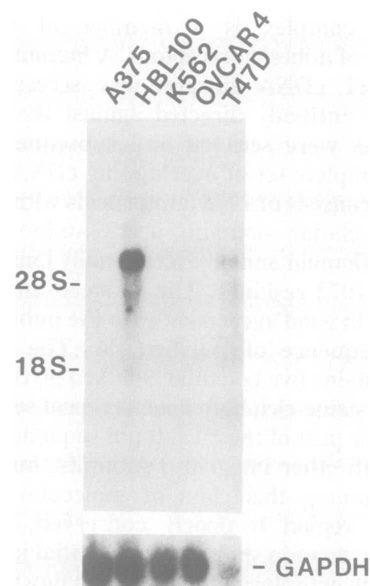

Fig. 2. Northern blot analysis. Total RNA (10 μg per lane) from A375, HBL100, K562, OVCAR-4 and T47D cell lines was electrophoretically separated on 1% agarose−formaldehyde gel, transferred to nitrocellulose filters and probed with $\beta 4$ subunit cDNA (clone 134, 4.3 kb). In a control experiment (below) the same RNA samples were probed with glyceraldehydephosphatedehydrogenase cDNA.

Comparison with β subunits and other proteins

Figure 4 shows the alignment of the deduced amino acid sequence of $\beta 4$ with the $\beta 1$, $\beta 2$ and $\beta 3$ sequences. The overall identity between the extracellular part of the mature

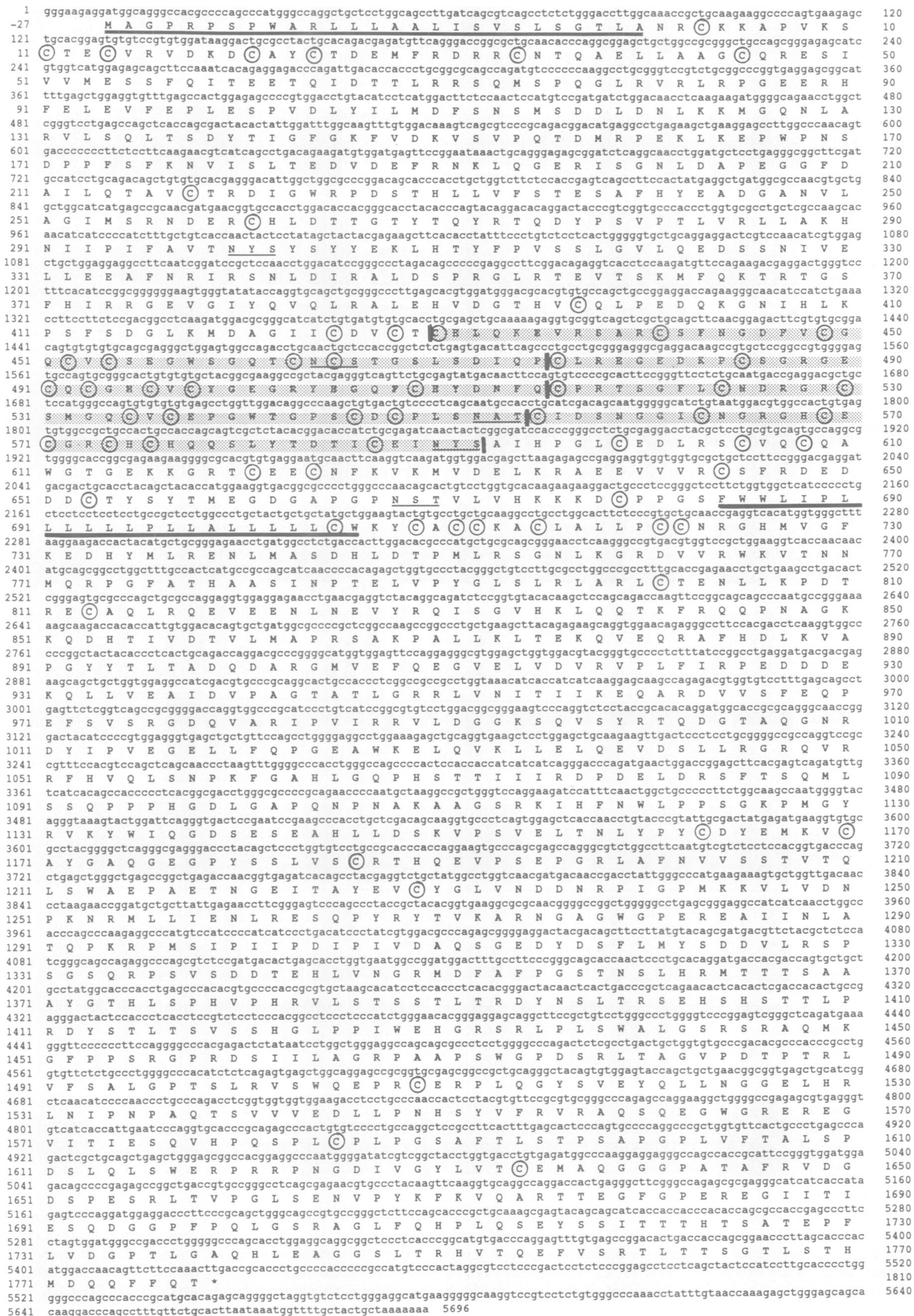

Fig. 3. Nucleotide sequence and the deduced amino acid sequence of β4 cDNA. The positions of the signal peptide (double underlined), the transmembrane region (heavily underlined) and the putative N-linked glycosylation sites (thinly underlined) are marked. The cysteine-rich domains are indicated with a shaded background and cysteine residues are circled.

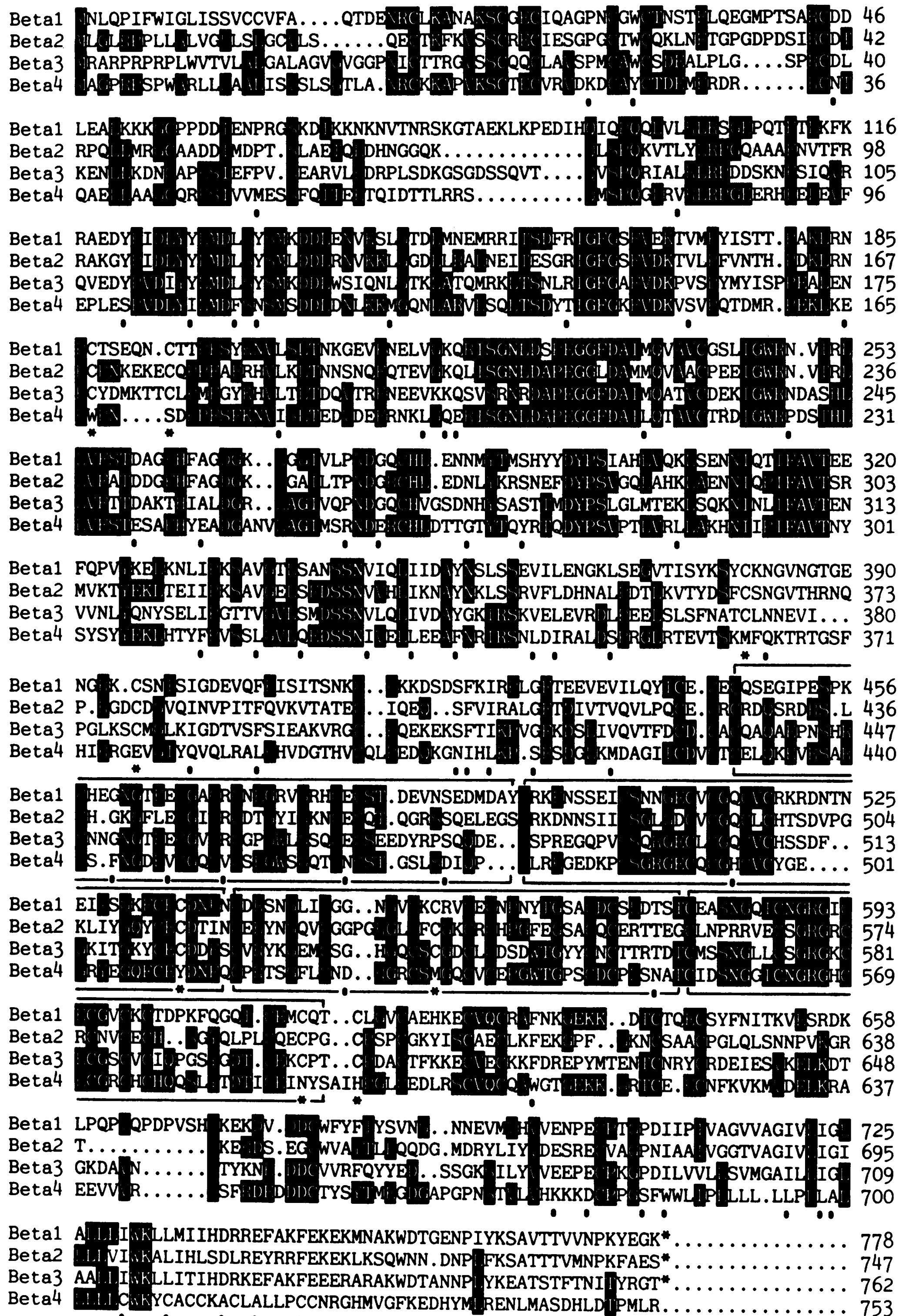

Fig. 4. Alignment of the $\beta4$ protein sequence with integrins β subunits. The $\beta4$ sequence is compared to three human β subunit sequences; $\beta1$ (Argraves *et al.*, 1987), $\beta2$ (Kishimoto *et al.*, 1987; Law *et al.*, 1987) and $\beta3$ (Fitzgerald *et al.*, 1987). Amino acids identical between the $\beta4$ subunit and any of the other three β subunits are indicated by inverse printing. Except for cysteine residues, all residues which are conserved in $\beta1$, $\beta2$ and $\beta3$ but not in $\beta4$ are indicated by solid circles. The cysteines lacking in $\beta4$ are marked with an asterisk and the cysteine-rich domains are boxed. The major part of the cytoplasmic domain of $\beta4$ has been omitted, because there is no corresponding sequence in $\beta1$, $\beta2$ or $\beta3$.

$\beta4$ subunit and each of the three other β subunits is ~35%. Identities in the order of ~45% were found in two long stretches of amino acids, one of 269 residues from positions 73 to 342 and another of 183 residues from positions 425 to 608. The latter region corresponds closely to the four homologous cysteine-rich repeat sequences. Between these two long homologous regions, there is a region of 83 amino acids with almost no identity. Of the 15 amino acids that are shared between all three $\beta1$, $\beta2$ and $\beta3$ subunits, only two are conserved in the $\beta4$ sequence. The first 72 amino

acids of the amino terminus and the 76 amino acids between the last cysteine-rich repeat sequence and the transmembrane domain are weakly conserved (20–30%) in β4. Also, the transmembrane region of β4 (residues 684–706) is weakly conserved, whereas the cytoplasmic portion of β4 is entirely unique.

Although the extracellular part of the β4 subunit is clearly similar to that of the three other β subunit sequences, this similarity is less than that between these three β subunit sequences (~35 versus ~45%). Furthermore, the β4 subunit lacks eight of the 56 cysteine residues that are conserved in all other three β subunit sequences. The positions of these residues are marked with an asterisk. Three of them are located in the cysteine-rich domains.

Computer searches of protein data bases revealed no significant similarities of the β4 subunit to proteins other than the β1, β2 and β3 subunits. Nevertheless, at the nucleotide level, a large region of β4 seems to be homologous with the non-muscle myosin heavy-chain gene of *Acanthamoeba* (Hammer *et al.*, 1987), i.e. 50% identity with multiple gaps in a stretch of 1400 nucleotides. This percentage is large, considering the evolutionary distance between the two species. However, because this homology is not found at the amino acid level, its significance is not clear. Probably, the fact that both regions are highly G/C-rich may account for the nucleotide homology of these two sequences.

In conclusion, it seems that the cytoplasmic domain of β4 is unique, not only in size but also in amino acid sequence.

Discussion

We here report the primary structure of β4, one of the newly characterized integrin β subunits. There are several observations that indicate that the sequence presented is authentic.

First, it is in accord with the amino-terminal sequence of the β4 subunit as published by Kajiji *et al.* (1989) and Hemler *et al.* (1989). Second, the RNA hybridization is consistent with the expression of β4 on cell lines. Third, the protein sequence has typical integrin β subunit-like structures. Finally, the predicted mol. wt of the polypeptide chain of 198 011 daltons agrees with the value determined after SDS–PAGE analysis of the deglycosylated β4 protein (Sonnenberg *et al.*, manuscript submitted).

Previously, Kajiji *et al.* (1989) have suggested that the larger size of β4 is due to the presence of a large amount of sialic acid, but from our present results it seems that the high mol. wt value of β4 is due to a very large cytoplasmic part comprising 1072 amino acids (~118 kd). In addition to the large cytoplasmic part, there are other distinctive features of the β4 subunit. The amino acid sequence of the extracellular part of β4 comprises the smallest number of conserved amino acids of all β subunits. In this regard, the β4 subunit lacks eight of the 56 cysteine residues, conserved in the three other human β subunits and in β1 sequences from the mouse (Holers *et al.*, 1989), chicken (Tamkun *et al.*, 1986) and *Xenopus laevis* (DeSimone and Hynes, 1988) and in the *Drosophila melanogaster* integrin β subunit, absent in *lethal myospheroid* mutants (Mackrell *et al.*, 1988). As the tertiary structure of β subunits is thought to be determined by intrachain disulfide bonds, the absence of these residues in the β4 subunit may have important structural and functional implications. Furthermore, a region of 63 amino acids, from position 109 to 171 in β3 implicated in RGD-mediated adhesion (D'Souza *et al.*, 1988), is strongly conserved in the β1, β2 and β3, but not in the β4 sequence. Only 28 of 48 amino acids match the conserved sequence for this region. Finally, in one of the least conserved regions (residues 324–425) β1, β2 and β3 share 15 amino acids, only two of which are present in the sequence of β4.

A further distinctive feature of β4 is its susceptibility to proteolytic degradation (Hemler *et al.*, 1989). The molecule can be degraded stepwise from a 205 via a 183 to a 150 kd product. Because the amino-terminal sequence of the 150 kd product matches the deduced sequence of the 205 kd mature protein, it can be concluded that both the 183 and 150 kd products have shorter versions of the cytoplasmic domain. A search in the cytoplasmic domain for endopeptidase cleavage sites, which usually consist of two or more basic residues, yielded eight of such sites. Cleavage at three of these sites may generate the β4 degradation products of 183 or 150 kd. They are located at positions 1621, 1294 and 1244. Recently, it has been shown that only the 205 kd band, but not the proteolytic degradation products of 183 and 150 kd, is phosphorylated (Kennel *et al.*, 1989). Therefore, the phosphorylation sites are assumed to be located within the last 20 kd region of the carboxy terminus. Indeed, there are several potential phosphorylation sites in this region for both serines and threonines. A tyrosine residue (residue 1668) might also be phosphorylated, because there is homology between the region around it and the phosphorylation site of β1 (residue 763) and other tyrosine kinase acceptor sites (Tamkun *et al.*, 1986).

Because the β1 and β4 but not the other β subunits associate with the α6 subunit, we searched for amino acids common to the sequences of only the first two. In the aligned sequences, 36 amino acids were found to be exclusively shared by the β1 and β4 subunits. A region immediately following the last cysteine-rich domain (residues 605–624) contained six of these exclusively shared amino acids, three of which were consecutive, i.e. EKK. This EKK sequence is also found on the β1 subunit of the mouse (Holers *et al.*, 1989) and chicken (Tamkun *et al.*, 1986). It is known that α6β1 complexes occur in the mouse (Sonnenberg *et al.*, 1988a) but not whether they also occur in chicken. The sequence is absent from the β1 subunit from *Xenopus laevis* (DeSimone *et al.*, 1988) and the β subunit from *Drosophila melanogaster* (Mackrell *et al.*, 1988), but whether these species possess α6β1 is unknown.

Integrins interact via their cytoplasmic domains with several other proteins to form transmembrane connections with the actin-cytoskeleton. These linker molecules include talin (Horwitz *et al.*, 1986), vinculin (Burridge and Mangeat, 1984), α-actinin (Chen and Singer, 1982) and the newly detected cytoplasmic protein, fibulin (Argraves *et al.*, 1989). It is tempting to speculate that one of the functions of the cytoplasmic domain of β4 is to interact directly with the cytoskeleton without the involvement of these linker proteins. We found no similarity of the β4 subunit to chicken α-actinin and vinculin. Unfortunately, the sequence of talin is not known and therefore, a direct comparison was not possible. Of the sequence of fibulin, only 15 amino acids of the amino terminus are known. Four of these amino acids, three of which are consecutive (LLE), are identical to amino acids in the sequence of the cytoplasmic part of β4 (residues

1032–1046). Therefore, there is at the moment no argument that part of the cytoplasmic tail of β4 is similar to linker proteins.

Materials and methods

cDNA cloning

The human keratinocyte lambda gt11 cDNA library (Clontech, Palo Alto, CA) was screened with the 439-9B rat monoclonal antibody against β4 (Kennel *et al.*, 1989) using the protoblot immunoscreening system of Promega. Clones that expressed insert-encoded protein reactive with the 439-9B antibody were isolated and plaque purified. The lambda DNAs were prepared by Lambda sorb immunoprecipitation (Promega Biotec) and the *Eco*RI inserts were subcloned into pUC18 or pUC19 for restriction mapping (Maniatis *et al.*, 1982). Additional screenings were done with radiolabeled DNA fragments from the 5′ portions of the previously isolated cDNA inserts as probes. DNA fragments were ^{32}P-labeled by random oligonucleotide priming (Feinberg and Vogelstein, 1984).

Sequence analysis

DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using a modified T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH) and 5′α [^{35}S]dATP as radioactive nucleotide. Sequences were obtained from one end of subcloned restriction fragments by using universal M13/pUC-sequencing primers. The internal sequence of large fragments was obtained from oligonucleotide primers that were synthesized on the basis of preceding sequence. Sequencing artefacts were avoided by determining all sequences using both dGTP and the nucleotide analogue deoxyinosine triphosphate (dITP) and by sequencing both strands of the cDNA insert.

Northern blot analysis

Total cellular RNA was isolated by the ureum/lithium chloride method (Barlow *et al.*, 1963) from cell lines which express high levels (HBL-100 and T47D), low levels (OVCAR-4 and A375) and no (K562) β4 protein. RNA was electrophoresed in a 1% agarose gel containing formaldehyde and transferred to nitrocellulose by standard procedures (Maniatis *et al.*, 1982). Hybridizations were carried out for 16 h at 64°C with ^{32}P-labeled insert probes in 3 × SSC containing 10% dextran sulphate, 5 × Denhardt's solution (1 × = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) and 50 μg/ml salmon sperm DNA. The filters were washed once with 3 × SSC, 0.1% SDS for 30 min, once with 1 × SSC, 0.1% for 30 min and twice with 0.1 × SSC, 0.1% SDS for 30 min at 64°C, dried and exposed to Kodak XAR-5 film with Dupont Cronex Lightning-Plus intensifying screens.

Data handling and analysis

Sequence data were analysed using GCG software (Devereux *et al.*, 1984). Nucleotide sequence and deduced amino acid sequences were used to search for homology in the Genbank (release 60.0).

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