LAMININ-5
Function of the γ2 chain in epithelial cell adhesion and migration, and expression in epithelial cells and carcinomas

SIRPA
SALO
Department of Biochemistry

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Abstract

Laminins are basement membrane glycoproteins consisting of three polypeptide chains $\alpha$, $\beta$ and $\gamma$. Until now 12 members of the protein family have been characterized and all isoforms have an $\alpha\beta\gamma$ chain composition, but they assemble in varying combinations of chain variants. The functional properties of laminins include cell adhesion, proliferation, differentiation, growth and migration. Laminin-5 has a chain composition of $\alpha3\beta3g2$ with the distribution mainly restricted to epithelial basement membranes, where its biological functions involve anchorage and locomotion of cells. The importance of this protein for the attachment of basal keratinocytes is clearly demonstrated by the fact that all genes encoding its chains have been shown to be mutated in the severe skin blistering disease Epidermolysis bullosa junctionalis.

The present study focused on investigations of the role of the laminin-5 isoform and particularly its $g2$ chain in cell adhesion and migration. The role of the short arm of the laminin $g2$ chain in the process of epithelial cell attachment is to serve as a kind of a bridging molecule to the extracellular environment, because it does not have any cell binding activity by itself. It was also shown that the newly synthesized $g2$ chain participates in the complex process of cell migration, probably as one of the first attachment components for moving cells. Thus, as a migration and differentiation-associated molecule, laminin-5 was considered a potential marker for detection of malignant processes where cell movement plays a role. Subsequently it was shown that the $g2$ chain is expressed not only in a restricted manner in human epithelial tissues, but also in a number of human epithelium-derived cancers. In some carcinomas, expression of the $g2$ chain appeared to be a characteristic of cancer cells with invasive properties. Examination of over 50 dysplasias and cervical tumors revealed that $g2$ chain antibodies were able to distinguish between lesions with or without invasive capacity. This is the first systematic study of epithelial cancers where $g2$ chain antibodies have been shown to be a useful marker in the histopathological diagnostics. In addition, this study showed in a mouse tumor model that the $g2$ chain of laminin-5 has a potential for being of use for in vivo tumor imaging.

Keywords: Basement membrane, cell anchorage, immunohistology, tumor imaging
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Oulu, August 1999
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbrecht-Holm-Swarm</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HD1</td>
<td>hemidesmosomal protein-1</td>
</tr>
<tr>
<td>IF</td>
<td>intermediate filament</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscularly</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenously</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase plasminogen activator receptor</td>
</tr>
</tbody>
</table>
Original articles

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Laminins are large heterotrimeric basement membrane glycoproteins composed of \( \alpha, \beta \) and \( \gamma \) chains (Timpl 1996). The number of laminins has enlarged noteworthy in recent years, and currently at least 12 different isoforms are known (Verrando et al. 1988, Beck et al. 1990, Engvall et al. 1990, Carter et al. 1991, Rousselle et al. 1991, Marinkovich et al. 1992a, Champliaud et al. 1996, Miner et al. 1997, Koch et al. 1999). All isoforms have \( \alpha \beta \gamma \) composition, but assemble in varying combinations of these chains. As an essential basement membrane component, laminins show a large variety of biological functions in cells and organs, including cell attachment, proliferation, differentiation, growth and migration. However, investigations of the exact functional properties of different laminin isoforms remain largely to be carried out.

Laminin-5, earlier also known as epiligrin, nicein, kalinin and ladsin, is one of the most investigated laminin isoforms in recent years. It has a chain composition of \( \alpha_3\beta_3\gamma_2 \) and its tissue distribution is mainly restricted to epithelial basement membranes (Verrando et al. 1988, Carter et al. 1991, Marinkovich et al. 1992b, Mizushima et al. 1996). The biological functions proposed for laminin-5 are as part of epithelial anchoring systems and cell locomotion. Laminin-5 is essential for the adhesion of basal keratinocytes to the underlying basement membrane as demonstrated by the fact that the genes coding for the \( \alpha_3, \beta_3 \) or \( \gamma_2 \) chains have been associated with a severe skin blistering disease termed Herlitz’s junctional epidermolysis bullosa (Pulkkinen et al. 1994a, Pulkkinen et al. 1994b, Kivirikko et al. 1995). The main symptom of this disease is disruption of the epidermis from its supporting basement membrane. Laminin-5, or at least some of its chains are also expressed in healing skin wounds, suggesting a possible biological role in cell proliferation and migration.

The present study focused on investigations of the role of laminin-5, especially its \( \gamma_2 \) chain, in cell adhesion and migration, and the localization of a migration-related element in the gene. In addition, it was shown that the \( \gamma_2 \) chain protein is expressed in a number of different human carcinomas, with its expression being characteristic of some cancer cells with a budding cell phenotype. Because \( \gamma_2 \) was expressed in invasive tumor cells of epithelial origin, but only marginally in mature human tissues, the question was raised whether or not antibodies against this chain could potentially be used as an \textit{in vivo} marker.
of some epithelial tumors. This idea was also evaluated using gamma camera imaging of radioactively labeled antibodies.
2. Review of the literature

2.1. Basement Membranes

Epithelial and endothelial cells are in close contact with highly specialized extracellular matrices called basement membranes that are widely distributed within the body. Basement membranes also surround most muscle and fat cells, as well as peripheral nerve axons. The basement membranes affect cell phenotypes and tissue compartmentalization in many ways, starting from early embryonic development. During development, cells attach and move along the basement membrane. It is needed for the polarization of cells, both in the embryo and the adult, and it serves as a substratum for cell adhesion and locomotion during wound healing and nerve regeneration (Engvall 1995, Timpl 1996).

The basement membrane is composed of several types of collagens, laminins, proteoglycans, calcium-binding proteins, as well as some other structural or adhesive proteins. These proteins form a thin sheet-like structure of 50-100 nm in thickness through specific self-assembly mechanisms. Although electron microscopy shows a tight structure of lamina lucida and lamina densa layers, the real structure may be more variable. In addition to the traditional basement membrane proteins, other components may also be selectively incorporated conferring additional biologically important qualities to the membranes (Engvall 1995, Timpl 1996).

Major architectural features of basement membranes are two independent networks, one formed from type IV collagen and the other from laminin. The covalently cross-linked collagen network is considered to maintain the mechanical stability, while the laminin network is more dynamic (Timpl & Brown 1996). The most widespread basement membrane component is type IV collagen with the chain composition [α1(IV)]2α2(IV). Three less frequent type IV collagen isoforms, [α3(IV)]2α4(IV), [α5(IV)]2α6(IV), and α3(IV)α4(IV)α5(IV) have also been identified and shown to be involved in some inherited or autoimmune disorders (Hudson et al. 1993). Collagen VII, which consists of three α1(VII) chains, is a constituent of anchoring fibrils which connect the basement membranes of squamous epithelia to the underlying stroma (Timpl 1996, Timpl & Brown 1996). Proteoglycans, of which perlecan is the most abundant, are further important basement membrane constituents. Some other proteoglycans exist in basement membranes,
but they are less well characterized. Quite recently the 250 kDa protein agrin was shown to be a proteoglycan. It was identified in basement membranes where it is able to cluster acetylcholine receptors at neuromuscular synapses. Agrin also exists in other basement membranes. There is also another group of basement membrane proteins, which includes nidogen, BM-40 (osteonectin/SPARC), fibulin-1 and fibulin-2 (Timpl & Brown 1996). The 150 kDa protein nidogen connects collagen and laminin meshworks, thus stabilizing the network structure (Fox et al. 1991, Mayer et al. 1993). Nidogen also binds to perlecain, fibulin-1 and fibulin-2 and it has been shown to exist in at least two significantly distinct forms (Timpl & Brown 1996, Kohfeldt et al. 1998).

2.2. Laminins

The first laminin isoform, laminin-1, was isolated from the mouse Engelbreht-Holm-Swarm (EHS) tumor in 1979 (Timpl et al. 1979), but currently at least 12 different isoforms are known. The number of both chains and isoforms can be expected to increase further, and alternative splicing of some laminin gene transcripts may also raise the number of laminin variants. Laminins exhibit a complex variety of biological functions and they are expressed as early as the two cell stage of embryonic development (Timpl 1996). However, because of the increasing number of laminin chains and isoforms, it remains to be shown if the tissue distributions and biological functions linked earlier e.g. to laminin-1 or the α1 chain are really true or do they belong to other isoforms or chains.

2.2.1. Isoforms and chains

Five genetically distinct α, three β and three γ chains have been cloned and characterized and they can assemble into at least 12 different isoforms. The isomers and chains known so far are summarized in Table 1.
<table>
<thead>
<tr>
<th>Isoform</th>
<th>Chain composition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin-1</td>
<td>α1β1γ1</td>
<td>Beck et al. 1990</td>
</tr>
<tr>
<td>Laminin-2</td>
<td>α2β1γ1</td>
<td>Engvall et al. 1990</td>
</tr>
<tr>
<td>Laminin-3</td>
<td>α1β2γ1</td>
<td>Engvall et al. 1990</td>
</tr>
<tr>
<td>Laminin-4</td>
<td>α2β2γ1</td>
<td>Engvall et al. 1990</td>
</tr>
<tr>
<td>Laminin-5</td>
<td>α3β3γ2</td>
<td>Carter et al. 1991</td>
</tr>
<tr>
<td>Laminin-6</td>
<td>α3β1γ1</td>
<td>Rousselle et al. 1991</td>
</tr>
<tr>
<td>Laminin-7</td>
<td>α3β2γ1</td>
<td>Champliaud et al. 1996</td>
</tr>
<tr>
<td>Laminin-8</td>
<td>α4β1γ1</td>
<td>Miner et al. 1997</td>
</tr>
<tr>
<td>Laminin-9</td>
<td>α4β2γ1</td>
<td>Miner et al. 1997</td>
</tr>
<tr>
<td>Laminin-10</td>
<td>α5β1γ1</td>
<td>Miner et al. 1997</td>
</tr>
<tr>
<td>Laminin-11</td>
<td>α5β2γ1</td>
<td>Miner et al. 1997</td>
</tr>
<tr>
<td>Laminin-12</td>
<td>α2β1γ3</td>
<td>Koch et al. 1999</td>
</tr>
</tbody>
</table>

The primary structure of the α1, α2, α3, α4, β1, β2, β3, γ1, γ2 and γ3 chains has been reported from man (Pikkarainen et al. 1987, Pikkarainen et al. 1988, Carter et al. 1991, Haaparanta et al. 1991, Nissinen et al. 1991, Kallunki et al. 1992, Marinkovich et al. 1992, Gerecke et al. 1994, Ryan et al. 1994, Vuolteenaho et al. 1994, Wewer et al. 1994, Iivanainen et al. 1995a, Iivanainen et al. 1995b, Miner et al. 1997, Koch et al. 1999) and those of the α1, α3, α4, α5, β1, γ1, γ2 and γ3 chains from mouse (Sasaki et al. 1987, Sasaki et al. 1988, Miner et al. 1995, Sugiyama et al. 1995, Iivanainen et al. 1997, Iivanainen et al. 1999) and α1, β1 and γ1 chains from Drosophila as well (Montell & Goodman 1988, Chi & Hui 1989, Garrison et al. 1991, Kusche-Gullberg et al. 1992). In addition, the primary structure of the β1 variant from rat has been determined (Hunter et al. 1989a, Hunter et al. 1989b). Apart from the number of possible chain associations, it is quite obvious that only certain chains can combine with each other to form trimers. The α1 chain can be found in laminin-1 and laminin-3 isoforms, which are composed of α1β1γ1 and α1β2γ1 chains. Laminin-1 represents the previously known EHS-type laminin, and laminin-3 was known earlier as s-laminin (Beck et al. 1990, Engvall et al. 1990, Burgeson et al. 1994). The α2 chain can associate with β1 and γ1 chains into the laminin-2 isoform (merosin), and also with β2 and γ1 chains into the laminin-4 isoform, which was previously also known as s-merosin (Engvall et al. 1990). Laminin-5 (epiligrin, kalinin, nicein, ladsin) has a chain composition of α3β3γ2 (Verrando et al. 1988, Carter et al. 1991, Marinkovich et al. 1992b, Mizushima et al. 1996). The α3 chain is also found in laminin-6 (α3β2γ1) and laminin-7 (α3β2γ1), which can covalently associate with laminin-5 in epidermal anchoring structures (Champliaud et al. 1996). Both α4 and α5 chains can assemble with β1 and γ1 chains thus forming isoforms laminin-8 (α4β1γ1), laminin-9 (α4β2γ1), laminin-10 α5β1γ1) and laminin-11 (α5β2γ1) (Miner et al. 1997). Koch et al.
have suggested that α2 and β1 could be the partner chains of the recently described γ3 chain.

Laminin-5 is initially synthesized as a 460 kD molecule which undergoes specific proteolytic processing to a smaller form after secretion into the extracellular matrix. So far, no other laminin isoform has been shown to be proteolytically modified after translation. The reduction of size is a result of cleaving the α3 subunit from 190-200 kDa to 160 kDa and γ2 subunit from 155 to 105 kD, respectively (Marinkovich et al. 1992b, Matsui et al. 1995). Recently, processing of the α3 subunit of laminin-5 was shown to be mediated by a plasmin-dependent mechanism involving tissue-type plasminogen activator (tPA)-catalyzed plasminogen activation (Goldfinger et al. 1998). The processing of the γ2 chain and the components taking part in it remain to be characterized.

Some laminin chain transcripts have been shown to be alternatively spliced, which may increase the number of laminin variants. Nevertheless, it is not known if all transcript variants also exist as proteins, how they assemble in trimers, and what are their functions in living animals. Ryan et al. reported in 1994 two distinct human α3 transcripts that display variability within domain IIIα. The α3EpA transcript has a smaller version of domain IIIα and is missing the 4th, 5th, and 6th cysteine residues that are typically found in the beginning of domain IIIα. The α3EpB transcript has maintained similarity to the α1 chain throughout domain IIIα and into domain IV and thus has a larger amino-terminal domain (Ryan et al. 1994). Two alternatively spliced α3 isoforms have also been reported for mouse (Galliano et al. 1995) and these isoform chains are generated by usage of two promoters (Ferrigno et al. 1997). The longer mouse α3B transcript shows 77% homology to the available sequence of the human α3B and the shorter α3A has 67.4% identity to the homologous 5'-amino acid sequence of the human α3A counterpart. According to in situ performed on sections of tissue obtained from 13.5- and 17.5-day mouse embryos these two transcripts also have distinct expression patterns (Galliano et al. 1995). Miner and coworkers have presented a third mouse α3 sequence encoded by the LAMA3 gene (Miner et al. 1997). This isoform extends the sequence reported by Galliano et al. (1995) at the 5’end by the about 2.2 kb. This α3 isoform has the NH2-terminal portion of domain IVb, a complete domain V, and almost all of a domain V1 (Miner et al. 1997). The γ2 chain of the laminin-5 isoform also has two alternatively spliced transcripts for which a functional role at the protein level remains to be shown. Kallunki et al. (Kallunki et al. 1992) demonstrated two transcripts with differences at their 3’ends. The longer γ2 transcript is encoded by 23 exons, which represent 5200 bp cDNA. The shorter transcript is encoded by 22 exons together with part of the 5’end of intron 22, resulting in a cDNA of 4316 bp. The longer transcript encodes a protein of 140 kDa, assuming that the protein is fully glycosylated, and the shorter codes for a protein of 130 kDa (Kallunki et al. 1992). Later, it has been shown in in situ experiments that these transcripts have different tissue distributions (Airenne et al. 1996). The consequence of the alternative splicing of the γ2 transcript at the protein level could be that the shorter polypeptide lacks a C-terminal cysteine residue that is thought to form a disulfide bond with a cysteine residue at the same location in the β3 chain (Airenne et al. 1996, Gerecke et al. 1994). It is, therefore, possible that this γ2 chain would not be able to form stable long-arm structures with β3 chain. Until now only human β2 chain has been shown to have alternative splicing at the 5’-untranslated region of the mRNA (Durkin et al. 1996).
2.2.2. Structure, self-assembly and polymerization

Laminins have a cruciform, T- or Y-shape structure as visualized by electron microscopy after rotary shadowing. The structure of the laminin-1 molecule is presented in Fig 1. The understanding of laminin structure is mostly based on electron microscopy and sequence analysis, as the flexibility and size of laminins have prevented crystallization and NMR studies. Fragmentation by limited proteolysis, circular dichroism spectroscopy, hydrodynamic methods and atomic force microscopy have also been used for structural characterization (Engel 1992, Maurer 1995, Chen et al. 1998).

Fig 1. Structural model of a laminin-1 molecule (adapted and modified from Timpl & Brown 1994). The domain structure is divided into regions I to VI according to the sequence, and each domain is further divided into smaller modular units that are presented here with LN, LE, L, LG and CC.
The overall structure of different isoforms is quite similar among the vertebrate laminins studied thus far. Domains I and II, that take part in the chain assembly, are quite conserved and differences in shape are mainly caused by the missing N-terminal regions of \( \alpha_3, \beta_3, \) and \( \gamma_2 \) chains (Engel 1992, Maurer 1995). The best studied example of invertebrate laminins is Drosophila laminin, whose component chains have been sequenced to completion (Montell & Goodman 1988, Chi & Hui 1989, Garrison et al. 1991, Kusche-Gullberg et al. 1992).

The laminin polypeptide chains are folded into a large number of structurally and often functionally autonomous protein units. These units are divided according to their sequences into domains I to VI (fig.1 and 2). The domains are further divided into smaller units, since laminins belong to a class of multifunctional proteins designated as mosaic or modular proteins. In mosaic proteins, domains occur as modular units in several different extracellular matrix proteins and also in proteins of non-extracellular matrix origin. In laminins, an \( \alpha \)-helical coiled-coil structure (CC) in region I and II of all three chains has a heptad repeat of nonpolar amino acid residues. Rod-like regions III and V in \( \beta \) and \( \gamma \) chains and IIIa, IIIb, and V in \( \alpha \) chain are composed of many repeating EGF-like (epidermal growth factor) domains (LE). Regions IVa and IVb of the \( \alpha \) chain and IV of the \( \gamma \) chain have been proposed to be EGF-like domains (L4). Region IV in the \( \beta \) chain (LF) has a unique sequence motif and occurs only in laminin-1. Regions VI (LN) are homologous between chains and do not show homology to the other domains in laminin. The C-terminal globular region has been defined at the \( \alpha \) chain and it consists of five LG repeats (Engel 1992, Maurer 1995).
The three different chains of the laminin molecule are held together by an $\alpha$-helical coiled-coil structure. The $\beta$ and $\gamma$ chains both contain a cysteine residue at their C-termini and are disulfide bonded, which further stabilizes the trimer structure. The coiled-coil structure is formed via domains I and II where different chains have little sequence homology. All polypeptide chains contain a heptad pattern of residues of the form $(a,b,c,d,e,f,g)^n$, where nonpolar hydrophobic amino acids are located preferentially in positions a and d, charged residues in positions e and g, and polar residues in b, c and f (Beck et al. 1990). The hydrophobic residues in positions a and d are located in the center of the trimer structure protecting them from the aqueous environment, whereas hydrophilic amino acids are exposed on the surface. The coiled-coil is further stabilized by ionic interactions between residues e and g (Beck et al. 1990, Beck et al. 1993). Nomizu and coworkers (Nomizu et al. 1996) have further concluded from experiments with laminin-2 ($\alpha2\beta1\gamma1$) peptides that the $\beta$–$\gamma$ dimer forms an acidic pocket because of the negatively charged ionic interactions. The basic residues of a short peptide representing the C-terminal part of the $\alpha$-chain then specifically interact with the acidic pocket of the

Fig. 2. Schematic representation of the domain organization and motifs in various laminin chains (adapted from Engvall 1995). The domains or their parts with homology in different chain variants are presented with similar patterns.
dimer to form a stable triple-stranded coiled-coil structure. In particular, the conformational instability of γ1 chain is a driving force for this interaction (Nomizu et al. 1996). Although the triple-stranded coiled-coil region of laminin consists of about 570 amino acids of each chain only a short C-terminal sequence of 25-amino acids of each chain is active for efficient initiation of trimer formation (Utani et al. 1994, Utani et al. 1995). This sequence can also function as a nucleation site to initiate chain interactions which lead to the completion of assembly in a C- to N-terminal direction (Nomizu et al. 1994, Nomizu et al. 1996).

Laminins are glycoproteins with varying numbers of N-glycosylation sites. The structural studies of laminin carbohydrates suggest that laminins, or at least laminin-1, contain only N-linked oligosaccharides (Arumugham et al. 1986, Fujiwara et al. 1988, Knibbs et al. 1989, Tanzer et al. 1993). The function of laminin carbohydrates has not yet been definitely determined, although a functional role of glycosylation of laminin-1 has been reported for tumor cell adhesion, cell spreading, neurite outgrowth and integrin-laminin interactions (Engel 1992). The glycosylation of laminins does not seem to take part in the stability against proteases, heparin binding or chain assembly (Howe 1984, Wu et al. 1988).

In most basement membranes, laminin forms a double polymer with type IV collagen and these polymers are connected via a bridging molecule nidogen (Aumailley et al. 1989, Fox et al. 1991, Yurchenco et al. 1992). A three-arm interaction model has been proposed for laminin polymerization (Yurchenco & Cheng 1993, Cheng et al. 1997). According to this model, laminin-1 self-assembles through interactions between N-terminal short arm domains forming a meshwork polymer. The assembly is reversible and calcium-dependent and calcium binding is believed to confer the correct conformation for favorable binding to the other short arms. The flexible long arm of each monomer would then be free to interact with cells or heparin or heparan sulfates out of the plane of the polymer (Yurchenco & Cheng 1993, Cheng et al. 1997). Because of the large number and variety in the structures of laminin isoforms, it has been found that there are also differences in the ability to polymerize. It has also been suggested that only laminins with three “full-sized” short arms (i.e. laminins 1, 2 and 4) are able to polymerize (Cheng et al. 1997) as presented in Fig. 3. Laminin-5, which is a non-polymerizing laminin, does not bind to nidogen, but it may have other matrix binding interactions through type VII collagen, laminin-6 and 7 or fibulin-2 (Cheng et al. 1997, Rousselle et al. 1997, Utani et al. 1997).
Fig. 3. A model for the polymerization of different types of laminin (Cheng et al. 1997). Laminins with three full-length short-arms can polymerize via NH₂-terminal parts of the chains (a), while isoforms with truncated short-arm structures cannot. Nidogen links laminins with full-length β or γ chain to the type IV collagen network, but the laminin-5 isoform which has truncated short-arms has to be linked via other proteins, i.e. type VII collagen (b).

Neither homotrimers nor single laminin chains have been found in the form of separate proteins. β-γ dimers have been observed as biosynthetic intermediates into which the α chain is added later (Morita et al. 1985, Peters et al. 1985). Alternatively, it has been suggested that the laminin chains are initially assembled randomly (Wu et al. 1988). According to studies done by Yurchenco et al. (1997), expression of β or γ chains alone results in intracellular retention of a non-disulfide linked chain with dimerization of the β chain, but not the γ chain. Coexpression of β and γ chains results in intracellular retention of heterodimers. Expression of the α chain results in secretion of monomeric chains with partial proteolytic cleavage. However, coexpression of all three chains is needed for secretion of intact trimeric laminin. Thus, the addition of the α chain also seems to drive and regulate the secretion of the trimer (Yurchenco et al. 1997).
2.2.3. Location, structure and regulation of genes

The laminin genes can be subdivided into LAMA, LAMB and LAMC families that encode α, β and γ chains, respectively. With the exception of mouse LAMC3, all laminin genes have been localized in the chromosomes and some gene structures have also been reported. Table 2. summarizes the chromosomal locations of laminin genes of man, mouse and Drosophila.

Table 2. Chromosomal location of laminin genes.

<table>
<thead>
<tr>
<th>Chain</th>
<th>Gene</th>
<th>Species</th>
<th>Locus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>LAMA1</td>
<td>man</td>
<td>18p11.3</td>
<td>Nagayoshi et al. 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>17</td>
<td>Weber-Kaye et al.1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Okazaki et al. 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Doyle et al. 1996</td>
</tr>
<tr>
<td>α2</td>
<td>LAMA2</td>
<td>man</td>
<td>6q22-23</td>
<td>Vuolteenaho et al .1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>10</td>
<td>Sunada et al. 1994</td>
</tr>
<tr>
<td>α3</td>
<td>LAMA3</td>
<td>man</td>
<td>18q11.2</td>
<td>Ryan et al. 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>18, band A</td>
<td>Aberdam et al. 1994b</td>
</tr>
<tr>
<td>α4</td>
<td>LAMA4</td>
<td>man</td>
<td>6q21</td>
<td>Richards et al. 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>10</td>
<td>Miner et al. 1997</td>
</tr>
<tr>
<td>α5</td>
<td>LAMA5</td>
<td>man</td>
<td>20q13.2-13.3</td>
<td>Durkin et al. 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>2</td>
<td>Miner et al. 1997</td>
</tr>
<tr>
<td>β1</td>
<td>LAMB1</td>
<td>man</td>
<td>7q22</td>
<td>Pikkarainen et al. 1987</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>12</td>
<td>Seldin et al. 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Drosophila</td>
<td>28D(2L)</td>
<td>Montell &amp;Goodman 1998</td>
</tr>
<tr>
<td>β2</td>
<td>LAMB2</td>
<td>man</td>
<td>3p21</td>
<td>livanainen et al. 1995b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>9</td>
<td>Wewer et al. 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Porter et al. 1993</td>
</tr>
<tr>
<td>β3</td>
<td>LAMB3</td>
<td>man</td>
<td>1q32</td>
<td>Vailly et al. 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>1, band H2-6</td>
<td>Aberdam et al. 1994b</td>
</tr>
<tr>
<td>γ1</td>
<td>LAMC1</td>
<td>man</td>
<td>1q25-q31</td>
<td>Fukushima et al. 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>1</td>
<td>Weber-Kaye et al. 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Drosophila</td>
<td>67C(3L)</td>
<td>Montell &amp;Goodman 1998</td>
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<tr>
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<td>LAMC2</td>
<td>man</td>
<td>1q25-q31</td>
<td>Kallunki et al. 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>1, band H1</td>
<td>Aberdam et al. 1994b</td>
</tr>
<tr>
<td>γ3</td>
<td>LAMC3</td>
<td>man</td>
<td>9q31-34</td>
<td>Koch et al. 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mouse</td>
<td>unknown</td>
<td>livanainen et al. 1999</td>
</tr>
</tbody>
</table>

Although five α chains have been identified, only the structures of LAMA2 and LAMA4 have been determined thus far (Zhang et al. 1996, Richards et al. 1997). The human LAMA2 gene was the first structure determined as a mammalian laminin α-chain gene, and it has been shown to be affected in congenital muscular dystrophy (Helbling-

All known LAMB gene structures have been determined (Vuolteenaho et al. 1990, Pulkkinen et al. 1995a, Durkin et al. 1996), as well as, the structures for LAMC1 and LAMC2 (Kallunki et al. 1991, Airenne et al. 1996). The entire human β1chain gene (LAMB1) has a size of more than 80 kb and contains 34 exons (Vuolteenaho et al. 1990). The human and mouse LAMB2 have been found to consist of 33 exons that occupy 12 kb or less of genomic DNA (Durkin et al. 1996). LAMB3 consists of 23 exons accounting for the full-length cDNA with an open reading frame of 3516 bp encoding 1172 amino acids (Pulkkinen et al. 1995a). LAMC1 is over 58 kb in size and has 28 exons (Kallunki et al. 1991). LAMC2 has 23 exons that covers about 55 kb and 16 of them have the same size as exons in the LAMC1 gene (Kallunki et al. 1992, Airenne et al. 1996). The two genes are located close to each other on chromosome 1q25-31 suggesting that they have evolved through duplication of a common ancestral gene (Fukushima et al. 1988, Kallunki et al. 1991). Mutations identified in the LAMC2 gene in junctional epidermolysis bullosa were the first description of a genetic laminin disease (Aberdam et al. 1994b, Pulkkinen et al. 1994a). Later, the LAMA3 and LAMB3 genes were found to be mutated in junctional epidermolysis bullosa (Pulkkinen et al. 1994b, Kivirikko et al. 1995). Recently, the human LAMC3 gene has been located on chromosome 9q31-34 (Koch et al. 1999).

Laminin genes are expressed at different stages during development and in a tissue specific manner. It is apparent that this complexity demands tight control of gene regulation. At present, knowledge of the regulatory patterns is still quite limited. The sequences of the promoter regions are known and have, at least partially, been characterized for human LAMB1, LAMB2 and LAMC1 (Vuolteenaho et al. 1990, Kallunki et al. 1991, Durkin et al. 1996), as well as, for the mouse LAMA3 and LAMA4 (Ferrigno et al. 1997, Richards et al. 1997) and LAMB2 and LAMC1 genes (Ogawa et al. 1988, Durkin et al. 1996). Many of these promoter regions lack a TATA or CAAT box needed for the initiation of transcription (Ogawa et al. 1988, Vuolteenaho et al. 1990, Kallunki et al. 1991, Durkin et al. 1996, Ferrigno et al. 1997, Richards et al. 1997). However, the LAMA3A and LAMC2 genes have putative TATA box sites (Airenne et al. 1996, Ferrigno et al. 1997) and human LAMA4 and LAMB2 have AT-rich regions which could fulfill this function (Durkin et al. 1996, Richards et al. 1997). The large sizes of laminin genes have made it difficult to identify the regulatory elements of the genes and to date, they remain largely uncharacterized. Potential Sp1, AP-1, AP-2 binding sites or GC-boxes have been localized in the mouse LAMA3, human LAMC1, LAMB1 and LAMB2 genes (Kallunki et al. 1991, Vuolteenaho et al. 1990, Durkin et al. 1996, Ferrigno et al. 1997). The Sp1 factor is thought to have a role in the initiation of transcription of genes
that contain no TATA boxes in their promoters. Recently, two reports dealing with the transcriptional regulation of the LAMC1 and LAMA3 genes were published. Suzuki and coworkers characterized a highly conserved enhancer element, bcn-1, in both human and mouse LAMC1 gene promoters in mesangial cells (Suzuki et al. 1996). This motif recognizes an inducible nuclear protein(s) BCN-1, which might regulate laminin γ1 chain gene transcription. The murine laminin-5 α3A and α3B isoform chains are generated by usage of two promoters, and transcription results in distinct expression patterns of these two polypeptides (Galliano et al. 1995, Ferrigno et al. 1997, Miner et al. 1997). Virolle et al. (1998) studied the transcriptional regulation of the α3A gene by transforming growth factor-β (TGF-β), which is a prototype member in a large family of morfogens and differentiation factors, and which is an important regulator of connective tissue during healing processes (Roberts & Sporn 1996, Virolle et al. 1998). They characterized three activator protein-1 (AP-1) binding sites between nucleotides –297 and –54 relative to the transcription start site of which one seems to be essential for gene expression. They also demonstrated a specific binding of Fra-2 and JunD to the AP-1 sites, suggesting a possible regulatory function for this complex in a basal keratinocyte-specific gene (Virolle et al. 1998).

2.2.4. Tissue distribution

Laminins are expressed in a tissue-specific manner in the embryo and adult, and the expression changes during development (Engvall et al. 1990, Sanes et al. 1990, Nissinen et al. 1991, Kallunki et al. 1992, Aberdam et al. 1994a, Vuolleenaho et al. 1994, Galliano et al. 1995, Ivainainen et al. 1995a, Miner et al. 1995, Champiaux et al. 1996, Orian-Rousseau et al. 1996, Durkin et al. 1997, Frieser et al. 1997, Ivainainen et al. 1997, Miner et al. 1997). However, in many cases the expression patterns are overlapping. The α5 and α4 chains seem to be the most widely expressed α chains both in man and mouse, while expression of the α1 chain is the most restricted, only being detected in the placenta and kidney (Nissinen et al. 1991, Ivainainen et al. 1995a, Durkin et al. 1997, Frieser et al. 1997, Ivainainen et al. 1997, Miner et al. 1997). Northern blot and in situ studies have shown expression of the α5 chain in the mouse intestine, heart, lung, skeletal muscle, skin and placenta, with low levels also in kidney, and the α4 chain almost as widely expressed as the α5 chain (Miner et al. 1997). A more restricted expression pattern for the α4 chain has also been presented (Ivainainen et al. 1997). In situ hybridization revealed expression in mesenchymal cells of the developing (branching) lung epithelia, in the villi and submucosa of the intestine and in the external root sheet of vibrissae follicles. The expression in the developing kidney was transient. At the protein level the α4 subunit of embryonic and postnatal mouse tissues showed it to be generally located in mesenchymal tissues (skeletal and heart muscles), lung septa, as well as, in subendothelial basement membranes of capillaries (Ivainainen et al. 1997). Expression levels of the α2 chain is highest in tissues with mesodermally derived components, such as skeletal and cardiac muscle, with some expression also in kidney, whereas α3 chain is found in organs rich in epithelia (skin, lung, low levels in kidney) (Nissinen et al. 1991, Kallunki et al. 1992,
Aberdam et al. 1994a, Vuolteenaho et al. 1994, Galliano et al. 1995, Miner et al. 1997). The main pattern of expression for each α chain seems to be established before birth, but some individual basement membranes change the α chain composition as development proceeds. For example, in the developing kidney, forming glomeruli express three different α chains in dynamic progression. Distinct regions of a continuous basement membrane, as found in the nephron, can contain distinct combinations of α chains, and change these during development (Miner et al. 1997).

The human β1 and β2 chains are expressed in the brain, lung, heart, muscle, adipose tissue, blood vessels, skin and kidney according to Northern and in situ analysis of human chains. Expression of the β2 chain can also be detected in liver (Nissinen et al. 1991, Kallunki et al. 1992, Iivanainen et al. 1995b). When the expression patterns of these chains were compared with each other, it appeared that cell types expressing the β1 chain also expressed the β2 chain, but that certain cell types expressed only the β2 chain. For example, in kidney the β2 chain is expressed in the glomeruli, while the β1 chain is not. In the skin, strong expression of β2 has been seen in cells of both epidermis and dermis, but the expression of β1 chain was prominent in stromal cells of the dermis and adnexes (Iivanainen et al. 1995b). The major difference between the γ1 and γ2 chain expression is that the γ1 chain is expressed in both epithelial and vascular endothelial cells, while the γ2 chain is restricted to epithelial cells (Kallunki et al. 1992, Aberdam et al. 1994a, Sugiyama et al. 1995). When the γ1 chain is expressed throughout most tissues, the γ2 chain is strongly expressed in epithelial cells of skin and lung as well as in the collecting tubes in kidney, although some expression can be seen also in the thymus, choroid plexus, cerebellum and the brain intermediate zone (Kallunki et al. 1992, Airenne et al. 1996). The recently characterized murine γ3 chain has highly restricted distribution, being mainly expressed in blood vessels and Leydig cells (Iivanainen 1999).

Laminin-5 is a unique isoform in that expression of its subunits is restricted to epithelial tissues (Kallunki et al. 1992, Aberdam et al. 1994a). Alternatively spliced variants have been described for the α3 chain (Galliano et al. 1995, Miner et al. 1997), the γ2 chain (Kallunki et al. 1992, Airenne et al. 1996) and the β2 chain (Durkin et al. 1996) and they have some differences in their distribution. Polypeptides encoded by LAMA3 are known but it remains to be shown if γ2 and β2 chain variants also exist at the protein level. The two α3 chain transcripts have different 5’ ends. The longer α3B chain was exclusively found in the bronchi and alveoli, the stomach and intestinal crypts, and the whisker beds and central nervous system (Galliano et al. 1995). The longer transcript of α3B chain was exclusively found in the bronchi and alveoli, the stomach and intestinal crypts, and the whisker beds and central nervous system (Galliano et al. 1995).

The primary transcript of LAMC2 also undergoes an alternative splicing, resulting a shorter 3’ end (Kallunki et al. 1992, Airenne et al. 1996). The shorter chain has an even more restricted expression pattern than that of the longer γ2 chain. According to Northern analysis and in situ experiments, the expression of the longer γ2 chain is seen in most epithelial cell types except for glomeruli. It is absent in embryonic and neonatal brain,
endothelia and skeletal muscle. The shorter transcript of γ2 chain can be seen, however, in the periventricular layer of the brain, perialveolar mesenchyme of the lung, and distal tubules of the kidney (Kallunki et al. 1992, Airenne et al. 1996). Nevertheless, it remains to be shown if this is true also at the protein level and if so, how these chains are assembled in laminin-5 isoform or isoforms.

### 2.2.5. Interactions with other basement membrane ligands

Basement membrane components have been shown to interact and self-assemble to form a supramolecular network. Laminin and type IV collagen are connected to each other via nidogen, which is probably a central connecting element within basement membrane structures since it also binds perlecan (Timpl & Brown 1996). The data accumulated thus far on the interactions of laminin with extracellular matrix proteins are mainly based on studies with laminin-1 extracted from the EHS-tumor (see Fig. 4). The lack of purified proteins in large quantities has restricted studies with other isoforms, but something is known about the binding activities of laminins 2, 4, 5 and 7 to basement membrane ligands (Timpl et al. 1995).

The laminin-1-nidogen complex has been purified in large quantities and in native form from the EHS-tumor. Analysis of the purified complex has demonstrated that the two proteins occur in an equimolar ratio and that nidogen specifically interacts with the center of the cross-shaped laminin molecule (Paulsson et al. 1987). Electron microscopy of recombinant nidogen shows three globular domains (G1, G2 and G3) which are connected by a long rod and a flexible linker region. High binding to laminin-1 was assigned to the C-terminal domain, G3 (Fox et al. 1991). Later, the exact binding site in laminin-1 was mapped by proteolytic and recombinant protein studies to a single EGF-like motif, γIΙΙ4, in the short arm of the γ1 chain (Gerl et al. 1991, Mayer et al. 1993). Laminin-1, 2 and 4 share the γ1 chain and high affinity nidogen binding (Mann et al. 1988, Fox et al. 1991, Brown et al. 1994). Recombinant nidogen, as well as the laminin-nidogen complex purified from tissues, have also been shown to specifically bind to type IV collagen. The major collagen binding site in nidogen has been localized to domain G2 and in type IV collagen two nidogen binding sites have been localized to the triple-helix of type IV collagen about 80 and 200 nm away from its C-terminal globular domain, NC1 (Aumailley et al. 1989, Fox et al. 1991, Reinhardt et al. 1993). Nidogen also binds to the core protein of the BM proteoclycan, perlecan, and to fibulin-1 and fibulin-2 (Battaglia et al. 1992, Sasaki et al. 1995a, Sasaki et al. 1995b). Recently, a new laminin-1-binding human nidogen, nidogen-2, was described (Kohfeldt et al. 1998). It seems to share some of the functions of nidogen-1, since it binds to collagen IV, perlecan and collagen I, but it fails to bind fibulins. Furthermore, a new mouse gene for entactin/nidogen, entactin-2, has been reported (Kimura et al. 1998).

Laminins have been shown to interact directly with heparin, perlecan, fibulin-1 and fibulin-2 (Timpl 1996). Heparin and heparan sulfate binding have been attributed to the distal domains, G1 to G3 (Ott et al. 1982, Yurchenco et al. 1990, Yurchenco et al. 1993). The most abundant heparan sulfate proteoglycan, perlecan, has also been shown to bind
the proteolytic long-arm fragment E3 of laminin-1 as strongly as intact laminin (Battaglia et al. 1992). In contrast to laminin-1, which can bind perlecan either directly or via nidogen G2 domain, laminin-2 and 4 seem to depend entirely on nidogen for perlecan association (Brown et al. 1994). Agrin, which is also a heparan sulfate proteoglycan, is required for the formation and maintenance of neuromuscular junctions (Tsen et al. 1995, Gautam et al. 1996). The binding of an NH₂-terminal fragment of agrin to laminin-1 is confined to a particular region in the upper part of the triple coiled-coil domain of laminin-1 (Denzer et al. 1997).

Fibulin-1 (BM-90) and fibulin-2 compose an extracellular protein family that interacts with the laminin-1-nidogen complex, type IV collagen and fibronectin and they are suggested to function as mediators of the assembly of basement membranes (Balbona et al. 1992, Pan et al. 1993, Sasaki et al. 1995a, Sasaki et al. 1995b, Utani et al. 1997). Binding studies with laminin-1 have demonstrated no affinity for fibulin-2, but rather a distinct affinity for fibulin-1, which is mediated through the laminin long-arm fragment E3 (Sasaki et al. 1995a). Fibulin-1 has also been proposed to bind to laminin-4 but not to laminin-2 (Brown et al. 1994). However, recently it has been demonstrated that fibulin-2 binds to the short-arms of laminin-5 and laminin-1, suggesting that it could function as a bridge between the laminin-1 and laminin-5 molecules, along with other extracellular matrix proteins, providing a link between the cell surface and the basement membrane (Utani et al. 1997).

Laminin-5, a component of the epithelial anchoring system, has truncated short-arms as compared with laminin-1 (Rousselle et al. 1991, Marinkovich et al. 1992b). The γ2 chain cannot bind nidogen (Mayer et al. 1995) and, therefore, laminin-5 cannot associate with perlecan or the type IV collagen network as do the other isoforms. Laminin-5 also lacks the short-arm structures believed to be required to promote network assembly (Schittny & Yurchenco 1990, Yurchenco et al. 1992). Nevertheless, it can form a disulfide-bonded complex with laminin-6 and laminin-7 (Champliaud et al. 1996), and through this mechanism, be incorporated into the basement membrane. A second important mechanism has been proposed by Rousselle et al. (1997) by which laminin-5 interacts with a basement membrane or stromal component. According to this model, monomeric laminin is the primary link between hemidesmosomal components α6β4 integrin and type VII collagen, with the laminin 5-6/7 complex present within the interhemidesmosomal spaces. The binding of laminin-5 has been localized to the NC-1 domain of type VII collagen and the binding site of type VII collagen in laminin-5 probably occurs within the short arm of the β3 or γ2 chains (Rousselle et al. 1995, Rousselle et al. 1997).

**2.2.6. Interactions with cellular receptors**

Laminins interact with cells via cell surface receptors, such as integrins, membrane-bound proteoglycans (e.g. dystroglycan), and other membrane-bound glycoproteins. Integrins are a large family of transmembrane adhesion proteins that are composed of two subunit chains α and β. They can bind laminins, as well as many other extracellular ligands and modulate intracellular signalling pathways in response to this binding. Nine different
integrins (α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, α9β1, αvβ3, αvβ8) have been suggested to be receptors for laminins (Hynes 1992, Mercurio 1995). As an example the specific binding sites of known integrin receptors in laminin-1 molecule are presented in Fig. 4. Laminin-1 has been shown to bind all known integrin-type laminin receptors (Sonnenberg et al. 1988, Elices & Hemler 1989, Gehlsen et al. 1989, Ignatius et al. 1990, Kramer et al. 1990, Kramer et al. 1991, Lee et al. 1992, Forsberg et al. 1994, Venstrom & Reichardt 1995). Laminin-2 has been found to interact with α3β1, α6β1, α6β4, and α7β1 integrins (Pfaff et al. 1994, Chang et al. 1995, Yao et al. 1996), but only α6β1 binds to laminin-3 (Delwel et al. 1993) and α6β4 to laminin-4 (Spinardi et al. 1995). Laminin-5 interacts with three laminin receptors, namely, α3β1 (Carter et al. 1991), α6β1 (Delwel et al. 1993, Rousselle & Aumailley 1994) and α6β4 (Niessen et al. 1994). Recently, cell adhesion onto laminin-10/11 was found to be mediated by integrin α3β1 (Kikkawa et al. 1998).

Fig. 4. Interactions of laminin-1 with other basement membrane components and integrin-type laminin receptors (adapted and modified from Timpl et al. 1994). The proteolytic fragments taking part in interactions of laminin-1 with integrins are presented as E1, P1, and E8.

Some integrins can bind more than one laminin while cells can use multiple integrins to interact with one laminin. Within laminins, some specific domains have been identified as
integrin-binding regions. The α1β1 integrin has been shown to bind to domain VI of the α-chain short arm of laminin-1 (Colognato-Pyke et al. 1995). The binding site for the α6β1 integrin in laminin-1 is located in the long-arm G domain (Sung et al. 1993). The α2 chain of laminin-2/4 has recently been reported to contain two distinct integrin binding sites within its amino-terminal domain, recognizing both the α1β1 and α2β1 integrins. Both of these recognition sites are conserved in domain VI of the laminin α1 chain isoform (Colognato et al. 1997).

Laminin-5 has been demonstrated to be an adhesion ligand for integrins α3β1, α6β1 and α6β4 (Carter et al. 1991, Delwel et al. 1993, Niessen et al. 1994, Rousselle & Aumailley 1994). The α6β4 integrin is present in hemidesmosomes, while α3β1 is recruited into focal contacts in cultured cells (Carter et al. 1990, Stepp et al. 1990, Jones et al. 1991, Grenz et al. 1993, DiPersio et al. 1995). These differences in localization between α3β1 and α6β4 integrins appear to reflect differences in adhesion-related functions. It has been suggested that in skin the α6β4 integrin mediates stable anchorage of keratinocytes to the substrate, while the α3β1 integrin appears to function in cell spreading and migration (Carter et al. 1990, Ryan et al. 1994, Xia et al. 1996, DiPersio et al. 1997). The importance of the α6β4 integrin for attachment of epithelial cells and formation of hemidesmosomes is well demonstrated, since mice lacking the α6 or β4 integrins die at or shortly after birth with severe blistering of the skin and other squamous epithelia reminding the phenotype of the human disorder Epidermolysis bullosa (Georges-Labouesse et al. 1996, van der Neut et al. 1996). Studies of α3β1 deficient mice have shown that the α3β1 integrin is required for postattachment spreading of keratinocytes on laminin-5 (DiPersio et al. 1997).

α-dystroglycan is another ubiquitous laminin receptor in addition to integrins and, at the moment, also the best characterized non-integrin laminin receptor. It is a 156 kDa cell surface protein that is part of the dystrophin-receptor complex in muscle, so it provides a linkage of the basement membrane to the dystrophin-actin cytoskeleton (Campbell 1995, Timpl & Brown 1996). Dystroglycan is encoded by a single gene and cleaved into α- and β-dystroglycans by post-translational processing (Ibraghimov-Beskrovnaya et al. 1992). Skeletal muscle α-dystroglycan is a laminin-binding extracellular peripheral membrane glycoprotein that is anchored to the sarcolemma by a transmembrane glycoprotein, β-dystroglycan (Ibraghimov-Beskrovnaya et al. 1992, Ervasti & Campbell 1993). α-dystroglycan is also a Schwann cell receptor of laminin-2 in peripheral nerves (Matsumura et al. 1993, Yamada et al. 1994).

Some other non-integrin laminin receptors have been reported, but they are still quite poorly characterized, and their functions in vivo are still unclear. However, carbohydrate-binding proteins that can bind specific oligosaccharide structures on the laminins and a family of galactose-specific lectins, the galectins, that bind the lactosamine-type sugars on laminin-1 have been discovered (Mercurio 1995). The structure and functional significance of the 67 kDa laminin-receptor is still an open question. The cDNA encoding a cytoplasmic precursor protein of 37 kDa has been identified (Rao et al. 1989), but the structure of this molecule has not yet been elucidated. Also the post-translational mechanism of processing the 67 kDa laminin receptor from the precursor is still unknown (Castronovo et al. 1991a, Castronovo et al. 1991b).
2.2.7. Examples of biological functions

The biological functions of different laminin isoforms have been investigated in hundreds of in vitro studies. Laminins have been shown to have effect on cell attachment, proliferation, differentiation and migration (Timpl 1996). However, only recently have the first laminin diseases and transgenic mice been introduced that demonstrated definitive and distinct functional roles for some laminin chains and isoforms also in vivo.

2.2.7.1. Laminin-5 as a component of the epithelial anchoring system

Basement membranes separate epithelial cells from the underlying stroma. In many epithelia, including skin, specific electron-dense points of connection can be seen by electron microscopy. These transmembrane cell-matrix junctions between the epithelial cells and the basement membrane are called hemidesmosomes and they have an important role in stabilizing and maintaining the epithelial structures (Jones et al. 1998). Outside the cell, laminin-5 is suggested to connect the hemidesmosomal complex to the underlying stroma via anchoring fibrils formed from type VII collagen (Champliaud et al. 1996, Chen et al. 1997, Rousselle et al. 1997). In addition to laminin-5, the major components of hemidesmosomes are α6β4 integrin, plectin, a 300-kD intermediate filament-associated protein (IFAP300), HD1, a bullous pemphigoid antigen II (BPAG2, BP180 or type XVII collagen) and a bullous pemphigoid antigen I (BPAG1 or BP230) (for review see (Jones et al. 1998). As shown in the model of the hemidesmosomal complex presented in Fig. 4, the β4 integrin subunit binds the cytoplasmic protein called plectin which, in turn, mediates the interaction between the hemidesmosome and the keratin cytoskeleton (Rezniczek et al. 1998). IFAP300 and HD1 may also bind the same integrin chain participating in keratin anchorage, but it is not sure, however, if plectin, IFAP300 and HD1 are actually the same or closely related proteins (Herrmann & Wiche 1987, Gache et al. 1996). A transmembrane component BPAG2 may also associate with the β4 integrin (Hopkinson et al. 1995, Borradori et al. 1997), while the BPAG1, another bullous pemphigoid antigen, seems to also be involved in linkage of the keratin cytoskeleton to the hemidesmosome (Clarke et al. 1995, Guo et al. 1995).
Fig. 5. The structure of hemidesmosomes (Jones et al. 1998). α6β4 integrin links the interaction of cytoskeleton and extracellular matrix (ECM) at the site of the hemidesmosome. In the cytoplasm, the β4 integrin subunit binds the cytoplasmic protein plectin, which mediates the association between the hemidesmosome and keratin cytoskeleton and intermediate filaments (IF). Another transmembrane protein BP180 (type XVII collagen) associates with integrin α6 and maybe also with integrin β4 in the cytoplasm. The BP230 component is involved in the linkage of the keratin cytoskeleton and intermediate filaments to the hemidesmosome via the BP180 protein. Outside the cell, integrin α6β4 associates with laminin-5, further mediating the cell attachment to the underlying ECM.

A number of skin diseases have been reported in which cell-basement membrane detachment and blister formation are characteristic symptoms, with the main cause being perturbations in hemidesmosomal integrity. Epidermolysis bullosa is a group of skin disorders characterized by the fragility of the skin and the mucous membranes (Epstein 1992, Uitto et al. 1997). In the simplex form of epidermolysis bullosa, mutated genes encode intermediate filament components of the epidermal cytoskeleton. In the dystrophic form, the type VII collagen gene is mutated, affecting the anchoring fibrils, while in the junctional form, the split in the skin is within the epidermal-dermal basement membrane zone (Epstein 1992, Uitto et al. 1997). The first demonstration of a genetic laminin disease came out when Pulkkinen et al. demonstrated mutations in the γ2 chain gene (LAMC2) in Epidermolysis bullosa junctionalis (Pulkkinen et al. 1994a). This disease was first attributed to a keratinocyte protein termed nicein (Verrando et al. 1991, Domloge-Hultsch et al. 1992). Subsequently, cloning of its component chains showed this protein to contain the epithelium-specific laminin γ2 chain (Kallunki et al. 1992, Vailly et al. 1994b). Later, it has been shown that mutations in all three laminin-5 chain genes underlie
cause the same disease (Pulkkinen et al. 1994a, Pulkkinen et al. 1994b, Kivirikko et al. 1995, Vaillly et al. 1995a). As a consequence of mutations, the laminin-5 trimer assembly or connections to other extracellular proteins are probably disturbed explaining why normal attachment of keratinocytes to the underlying stroma cannot be formed. Based on this, it is very obvious that laminin-5 has an important role as a structural attachment component of epithelial cells.

In addition to that seen in epidermal-dermal structures, there are also epithelial tissues that do not form hemidesmosomes. For example, the anchoring system of epithelial cells and basement membrane in the gut is different from that seen in skin. Epithelial cells in the human intestine do not possess type VII collagen. However, laminin-5 and HD1 are expressed, suggesting that some kind of hemidesmosome-like complexes may exist in the intestine. Laminin-5 and HD1 are located mostly at the basal pole of epithelial cells migrating up the villi, suggesting a role for these molecules in intestinal epithelial cell proliferation, differentiation and migration, in addition to cell anchorage (Virtanen et al. 1995, Orian-Rousseau et al. 1996).

2.2.7.2. Laminin-2/laminin-4 and attachment of muscle cells

The dystrophin-glycoprotein complex is composed of dystroglycan and sarcoglycan complexes and it mediates attachment of muscle cells to the surrounding tissue compartments. The dystroglycan complex contains α- and β-dystroglycans of which α-dystroglycan links the sarcolemmal membrane to the extracellular matrix by binding the G domain of the α chain in laminin-2 (for review see Campbell 1995, Worton 1995). α-dystroglycan is linked to the sarcolemma by a complex which is composed of five integral membrane proteins, namely α-dystroglycan, β-dystroglycan, and α-, β- and γ-sarcoglycans (Ervasti et al. 1990, Yoshida & Ozawa 1990, Ibraghimov-Beskrovnaya et al. 1992, Suzuki et al. 1994).

Congenital muscular dystrophies are autosomal recessive muscle diseases and symptoms exhibited are muscle weakness, hypotonia, delayed motor development, severe and early contractures and often joint deformities (Campbell 1995). Specific absence of the α2 chain of laminin-2 was observed in patients affected by a classical non-Fukuyama type of CMD (Tome et al. 1994) and the analysis of laminin-2 deficient CMD families by homozygosity mapping localized the CMD gene to chromosome 6q2 (Hillaire et al. 1994). Later, splice site and nonsense mutations were found that lead, presumably, to a truncated α2 chain protein lacking domains I and II and the C-terminal G domain (Helbling-Leclerc et al. 1995). A homozygous missense mutation has also been identified that affects one of the conserved EGF-like repeats in the short arm of the α2 chain (Nissinen et al. 1996).

In addition to the dystrophin-glycoprotein complex, muscle cells and myofibers can attach to the surrounding basement membrane via integrin α7β1D-based attachment system. Integrin α7β1D is a specific receptor for laminin-1 (von der Mark et al. 1991) as well as laminin-2 and –4 (Yao et al. 1996). The absence of the integrin α7 causes a novel form of muscular dystrophy demonstrating distinct roles for dystroglycan-sarcoglycan complex and integrin-mediated complex of muscle cells and basement membrane (Mayer
et al. 1997). Analysis of integrins have shown an abnormal expression and localization of α7β1 integrin in myofibers of laminin-2/4-deficient human patients and mice, but not in dystrophin-deficient or sarcoglycan-deficient humans or mice suggesting that accurate expression and membrane localization of α7β1 integrins in myofibers, as well as myofiber survival, depend on laminin-2/4 (Vachon et al. 1997).

2.2.7.3. β2 chain of laminin-3 in neuromuscular junctions and kidney

Skeletal muscle fibers are surrounded by basement membrane and at neuromuscular junctions basement membrane passes between the pre- and postsynaptic membranes. The synaptic and extrasynaptic regions contain different laminin β chains. The β2 chain is located at synapses, whereas the β1 chain can be found extrasynaptically (Hunter et al. 1989b). In in vitro experiments, the β2 chain has been shown to arrest the growth of axons promoted by the β1-containing laminin trimers (Hunter et al. 1989b, Hunter et al. 1991). In mutant mice that lack the β2 chain, neuromuscular junctions have defects in structure, function and molecular architecture (Noakes et al. 1995). Thus, the restriction of the β2 chain to the synaptic cleft and the β1 chain to the extrasynaptic area suggests a role for these chains in normal neuronal development. What is known of synaptic specialization, it has been proposed that the β2 chain could be a link in the signalling pathway in assuring the alignment of presynaptic and postsynaptic structures (Martin et al. 1995).

The glomerular basement membrane (GBM) also has variations in laminin β1 and β2 chainlocalizations. The β1 chain is a component of the immature GBM, but is replaced with the β2 chain as development proceeds with β2 being the major component in the adult (Miner & Sanes 1994). In mice that lack the β2 chain, the GBM is structurally intact and obviously the β1 chain compensates for the missing chain. However, mutant mice develop a massive proteinuria due to failure of the glomerular filtration barrier, which strongly suggest the idea that these chains are functionally different despite of their structural similarity (Noakes et al. 1995).

2.2.7.4. Importance of the α5 and γ1 chains in embryogenesis

The laminin α5 chain associates with the γ1 chain and either the β1 or β2 chains to form laminins 10 and 11 (Miner et al. 1997). It is widely expressed in fetal and adult tissues, including kidney, skeletal muscle, lung and intestine (Miner et al. 1997, Patton et al. 1997, Sorokin et al. 1997). Embryos lacking this chain die late in embryogenesis because of several localized structural and developmental defects. Laminin α5 may be important in placental endothelial cell migration, blood vessel branching, trophoblast adhesion to basement membranes, and in formation of a proper basement membrane. At least some basement membranes are ultrastructurally defective in α5 deficient mice despite ectopic deposition of other α chains, suggesting that the compensation is functionally inadequate (Miner et al. 1998).
The LAMC1-null mutation causes early embryonic lethality, as well (Smyth et al. 1999). The γ1 chain is the most widely expressed laminin chain and it assembles in all other isoforms except laminin-5 (Timpl 1996). Studies of knock-out mice revealed that it is necessary for laminin assembly and that laminin is, in turn, essential for the organization of other basement membrane components in vivo and in vitro (Smyth et al. 1999).

2.3. Interactions of tumor cells with ECM and basement membrane

The basement membranes coordinate and regulate the behaviour of cells of organized tissues in many ways. Their composition and three-dimensional structure, as well as proteolytic remodelling are involved in the complex processes of microenvironmental signalling that affect cell shape, locomotion, growth, differentiation and apoptosis. The regulatory effects exhibited by the basement membranes are, therefore, essential for the normal function of tissues and cells associated with these matrices while disturbances in these structures can contribute to many diseases, including cancer (Liotta & Stetler-Stevenson 1991, Werb 1997, Lukashev & Werb 1998).

2.3.1. General aspects of tumor progression

The hallmark of cancer cells is their ability to invade other tissues and locomote throughout the body to distant organs to form new tumors, referred to as metastases. Uncontrollable growth of metastases is the major single cause of death of cancer patients. Formation of metastasis requires the disruption of local cell-cell contacts, invasion, penetration of blood or lymphatic vessels (intravasation), escape from those vessels (extravasation), migration and growth (Liotta et al. 1991, King 1996, Ziober 1996). Although uncontrolled proliferation and growth of cells and cell colonies can result from some genetic changes, it does not, by itself, result in invasion and metastases. During the development of metastases, tumor cells have to pass cellular and extracellular boundaries including basement membranes, survive the circulatory or lymphatic system and cross into foreign tissues (Liotta & Stetler-Stevenson 1991, Ziober et al. 1996). Thus, a complex mix of consecutive processes of cell attachment, detachment and migration are needed for tumor progression, as well as the degradation of temporary complexes formed during these processes (Liotta & Stetler-Stevenson 1991, King 1996).

The interactions of tumor cells with basement membranes include cell adhesion via cell surface receptors, matrix dissolution by degradative enzymes, and migration (Liotta & Stetler-Stevenson 1991). Integrins are the main cell surface receptors that tumor cells use in binding to the extracellular matrix (ECM) or the basement membrane proteins type IV collagen, laminin and fibronectin (Hynes et al. 1992, Martin et al. 1995, Ziober et al. 1996), but there are also numerous non-integrin receptors (Aznavorian et al. 1990, Mercurio 1991, Martin et al. 1995, Mercurio 1995). The main enzymes degrading ECM and basement membrane components include matrix metalloproteinases, the adamalysin-
related membrane proteinases, the bone morphogenetic protein 1 family of
metalloproteinases, and tissue serine proteinases, such as thrombin, tissue plasminogen
activator (tPA), urokinase (uPA), and plasmin (Werb 1997). The third step of invasion is
migration of tumor cell across the basement membrane and stroma through the zone of
matrix proteolysis. The induction of pseudopodial protrusion at the leading edge of the
migrating cell is directional and regulated by cell surface ligand binding and it involves
coordinated mobilization of cytoskeletal elements that interact with the inner membrane

2.3.2. Laminins and cancer

Considering the important role of basement membranes for maintaining the architecture of
organized tissues, abnormality in the function of basement membrane components, such as
laminin, could affect tumor cell behavior. This is particularly the case for epithelial
basement membranes and the epithelium specific laminin-5, as 85 % of all cancers are of
epithelial origin (King 1996). However, the overall picture of the interactions of basement
membrane proteins, such as laminins and/or laminin-binding receptors with tumor cells is
incomplete. The interaction of laminin-1 with cancer cells has been investigated
extensively (Martin et al. 1995), but not much is known concerning other isomers, except
laminin-5. The effects of laminin-1 on tumor cells are mediated through cell surface
receptors (Hynes 1992, Mecham 1991, Mercurio & Shaw 1991) and, at least integrins
α1β1, α2β1, α3β1, α6β1, α6β4, and α7β1 have been shown to be differentially
expressed in a number of tumor cells that are believed to interact with laminins during
invasion. However, their exact role in tumor progression is still under speculation (Martin
et al 1995, Ziober et al. 1996). Among the non-integrin receptors, the 67 kDa laminin
receptor has been found to be elevated in malignant cells (Wewet et al. 1986). Laminin-1
has been shown to promote tumor cell adhesion and growth (Terranova et al. 1980,
Kubota et al. 1992, Terranova et al. 1982, Terranova et al. 1984) and the level of laminin-
1 has also been shown to correlate with malignancy in some biopsy specimens (Mafune et
al. 1990). Studies using proteolytic fragments, synthetic peptides and recombinant regions
of this molecule have revealed some active sites. A pentapeptide sequence, YIGSR of the
β1 chain of laminin-1, has been reported to bind to the 67 kDa metastasis associated
laminin receptor (Graf et al. 1987a, Graf et al. 1987b, Mafune et al. 1990, Massia et al.
1993). More recently, a synthetic radioiodinated peptide, YIGSR, was used in imaging of
Lewis Lung carcinoma (Koliakos et al. 1997). Three more peptides in laminin-1, namely,
SIKVAV in the α1 chain, PDSGR and RLVPRL, have been connected with tumor cell
adhesion, growth, lung colonization, collagenase IV secretion, and angiogenesis by
promoting or reducing these activities, but they appear to be much less active that YIGSR
3. Outlines of the present study

Laminin-5 is a highly epithelium-specific basement membrane protein that is essential for anchoring epithelial cells to the ECM. Considering the fact that about 85% of all malignant tumors are of epithelial origin (carcinomas), it was considered possible that abnormal expression and turnover of laminin-5 might be altered in carcinomas. Such alterations might involve altered epithelial adhesion or migration properties. The purpose of this study was to obtain more detailed information about the function of laminin-5, especially its γ2 chain, in adhesion and migration of epithelial cells. In addition, it was examined if expression of the γ2 chain becomes affected in malignant epithelial tissues, and if so whether applications of laminin-5 analysis might be useful in cancer diagnosis and therapy. The specific aims of the study were as follows:

1. To generate antibodies against the laminin γ2 chain.
2. To study expression of the γ2 chain in human tissues and different cancers.
3. To explore the involvement of the γ2 chain in cell attachment and migration of epithelial and tumor cells.
4. To characterize a potential migration-related element in the gene (LAMC2).
5. To evaluate the potential of the γ2 chain as a marker for the detection of epithelial tumor cells using antibodies in immunohistochemical staining and for \textit{in vivo} imaging.
4. Materials and methods

The details of materials and methods used in this study are described in the original articles I–IV.

4.1. Preparation and characterization of polyclonal antibodies (I, II, III, IV)

Polyclonal antibodies against domain III (anti-LNγ2-III) and the C-terminus (anti-LNγ2-I/II) of laminin γ2 were prepared in rabbits using γ2-GST fusion proteins as antigens. The domain III antigen contained 177 amino acid residues (res. #391-567) and the domain I/II antigen 161 amino acid residues (res. #1017-1178) (Kallunki et al. 1992). Antibodies against the GST-epitopes were removed from the antisera by negative immunoadsorption with GST-Sepharose made by coupling E.coli expressed GST protein to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden).

4.2. Expression of recombinant laminin γ2 chain (I, II, III)

The γ2 chain of laminin-5 was expressed as a recombinant protein using the baculovirus system. A full-length human laminin γ2 chain cDNA containing 6 bp of the 5’ UTR and 822 bp of the 3’ UTR was constructed from four overlapping cDNA clones L52, HT2-7, L15 and L61 (Kallunki et al. 1992). The resulting 4,402 bp cDNA was cloned into the pVL1393 recombinant transfer plasmid prior to transfer into the AcNPV-γ2 baculovirus vector. For expression of the recombinant protein, High Five (H5) cells were infected with the recombinant pVL1393-γ2 virus at a multiplicity of infection (MOI) of 5 – 10 pfu per cell by using the standard protocol (Summers & Smith 1987). The recombinant γ2 chain was partially purified by first resuspending the cells in 10 volumes of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM EDTA, 1 % Triton X-100, 1 mM PMSF and 1 mM NEM followed by homogenization in a Dounce homogenizer. The solubilized proteins were removed by centrifugation and the pellet was extracted again with buffer containing 1 – 3 M urea. The recombinant γ2 chain was extracted with a buffer containing 5 M urea, and renatured by dialysis against 50 mM Tris-HCl, pH 7.4, 100 mM NaCl.
4.3. Immunohistochemistry and in situ hybridization (I, II, III, IV)

Five μm thick paraffin sections were stained with polyclonal antibodies against laminin-1 (a kind gift from Dr. Foidart, Liege, Belgium) or the γ2 chain of laminin-5. The paraffin sections were deparaffinized in xylene, then in some cases heated to 90°C in a microwave oven in 10 mM citrate buffer (pH 6.0) for 10 min, and were then allowed to cool in the buffer at room temperature for 20 min. To properly expose the laminin γ2 chain in the highly cross-linked native basement membrane the sections were digested either with 0.03 % trypsin for 10 min at 37°C or with 0.4 % pepsin in 1 mM HCl at 37°C for 20 min, blocked for nonspecific binding with 5 % newborn rabbit serum, 0.1 % BSA, and then incubated for 1 h at 37°C with the polyclonal IgG diluted in TBS to 2 – 10 μg/ml. Subsequently, a biotinylated swine-anti-rabbit antibody was applied, followed by horseradish peroxidase-avidin-biotin complex diluted as recommended by the manufacturer (DAKO, Copenhagen, Denmark). The color was developed in diaminobentsamidine (DAB), followed by counterstaining of the slides with hematoxylin. In study IV, when visualization of newly synthesized γ2 chain was important, the staining was carried out without digestion.

In situ hybridization was performed with 35S-labeled RNA probes as described previously (Pyke et al. 1991, Pyke et al. 1994). Plasmid constructs for the γ2 chain of human laminin-5 and for human urokinase-type plasminogen activator receptor were used as described for preparation of RNA probes (Pyke et al. 1991, Pyke et al. 1994). The β3 chain of human laminin-5 cDNA was made using reverse transcriptase-PCR. A pool of cDNAs from human cultured cells was made from RNA purified from cells and primed with a poly(dT) primer with reverse transcriptase (Pyke et al. 1994). This pool was used for PCR amplification of a β3 cDNA fragment with two primers designed to amplify 2897-3506 of the coding sequence of the laminin β3 chain (Gerecke et al. 1994). Sense and antisense RNA transcripts were made by in vitro transcription using T3 and T7 polymerase and 35S-labeled UTP (Pyke et al. 1994).

4.4. Cells (II, III)

Mouse squamous cell carcinoma cells, KLN-205 (cat. No. ATCC CRL-1453), were maintained as monolayer cultures in Eagle’s minimum essential medium (MEM) containing non-essential amino acids and Earle’s BSS, supplemented with 10 % fetal calf serum (FCS). The HaCat human keratinocyte cells (a kind gift from Dr. N. Fusenig, Heidelberg, Germany) were cultured in Dulbecco’s MEM supplemented with 10 % FCS.

4.5. Cell adhesion and migration assays (II)

Microtiter plates (96 wells; Nunc, Copenhagen, Denmark) were coated with 100 μl/well of laminin-1 (10 μg/ml, Gibco BRL), or recombinant laminin γ2 chain (10 μg/ml) in PBS or
50 mM Tris-HCl, pH 7.4. Control wells were uncoated or coated with the same amounts of BSA. In some experiments, the proteins were first denatured by dialysis overnight against 5 M urea, 50 mM Tris-HCl, pH 7.4 and then renatured by dialysis against 50 mM Tris-HCl, pH 7.4. Remaining active sites on the plates were blocked with 150 μl of 10 mg/ml BSA in PBS for 2 hours at room temperature. The wells were washed with PBS, and 100 μl of Eagle’s MEM containing 5 mg/ml BSA was added. A total of 20 000 KLN-205 cells were added to each well and allowed to attach for 90 min at 37°C. The extent of cell adhesion was determined by measuring color yields at 600 nm, following fixation with 3 % paraformaldehyde and staining with 0.1 % crystal violet (Gillies et al. 1986). For inhibition assays with the anti-γ2 antibody, the substrate coated wells were incubated with 20 μg/ml of anti-LNγ2-III in PBS for 60 minutes prior to incubation with the cells.

The effect of endogenous laminin-5 on migration of KLN-205 cells was determined by using a modified Boyden chamber assay (Hujanen & Terranova 1985), and the effect of exogenous laminin-5, by using a modified Transwell assay (Pelletier et al. 1996). Briefly, polycarbonate filters (pore size 10 μm, diameter 12 mm; Costar, Cambridge, MA) were coated with 2.5 μg of EHS type IV collagen, and used to separate the upper and lower compartments of the 50 μl chamber. A total of 1 x 10^5 cells in Eagle’s MEM containing 0.1 % BSA were placed in the upper compartment, and the lower compartment was filled with medium, with or without chemoattractants (50 μg/ml laminin-1 or fibronectin). To study the effect of the laminin γ2 chain antibodies on cell migration, anti-LNγ2–III or anti-LNγ2–I/II was added to the upper compartment together with the cells at a concentration of 20 μg/ml. Normal rabbit IgG was used as a negative control. After an 8 hour incubation at 37°C in a humidified atmosphere, the filters were removed, fixed and stained (Diff-Quick, Baxter Diagnostics, Tübingen, Germany). The cells that had not migrated were removed from the upper surface of the filter with cotton swabs. Migration of cells was quantified by counting the cells on the lower surface of each filter in 10 randomly selected high power fields (400x). All assays were performed in triplicate.

The Transwell plate assay was used to determine the effect of exogenous laminin-5 on cell migration. The membranes of the Transwell plates had pore sizes of 12 μm. The lower side of the membrane was coated with 2.5 μg of EHS type IV collagen for 3 hours at room temperature. Both side were blocked with 1 % bovine serum albumin for 1 hour. 1 x 10^5 cells were added per well to the upper compartment in Eagle’s MEM containing 10 % FCS and the lower compartment was filled with 2.5 μg/ml laminin-5 as a chemoattractant. Antibodies against either the γ2 chain or normal rabbit IgG were added to the upper compartment, together with the cells, at a concentration of 20 μg/ml. Following a 16 hour incubation at 37°C, the cells were fixed and stained with Diff-Quick. Cells from the top surface of the membrane were removed with cotton swabs and cells that had migrated to the lower side of the membrane were counted (12 fields +/- S.D.).

4.6. Generation and analyses of transgenic mice (II)

Two different segments of the LAMC2 5’flanking regions were subcloned into a pKK2480 vector containing the β-galactosidase gene and the SV40 polyadenylation signal (kind gift from Mikkel Rohde, Aarhus, Denmark) for expression in transgenic mice. The
longer construct, pHH-1, contained 5946 bp (-5,900 to +46) and the shorter construct, pHH-2, contained 668 bp (-613 to +55). The 5’ end of the pHH-1 insert was made by ligating a 3,900 bp HindIII-PstI fragment and a 1,150 bp PstI fragment of the genomic clone, P14 (Airenne et al. 1996) The 3’ end of the construct was a PstI-SalI fragment (-699 to +46) made by PCR and ligated to the 5’ end 5,050 bp fragment. The full-length fragment was blunt ended with Klenow and subcloned into pKK2480 vector. The shorter construct was made by PCR from the genomic clone P14, digested with SalI and XbaI and subcloned into pKK2480. The plasmids, pHH-1 and pHH-2, were digested with EagI and XhoI to release the inserts from the vectors. Transgenic mice were produced by pronuclear microinjection of (C57B1/6+DBA/2)F1 fertilized oocytes as described by Hogan et al. (1986). Expression of the \textit{lacZ} gene was detected by staining with X-gal (5-bromo-4-chloro-3-indolyl-\textbeta-D-galactopyranoside) as a substrate (Behringer et al. 1993).

4.7. Tumor imaging with unlabeled and radioactively labeled antibodies (III)

To estimate the specific targeting of the \( \gamma_2 \) chain antibody into tumors, 1 x 10^5 KLN-205 cells were injected i.m. or i.v. into four DBA/2 adult male mice and 3 or 5 weeks later, anti-LN\( \gamma_2 \)-III antibodies or normal rabbit IgG (20 – 40 \( \mu \)g/mouse) were injected into the tail vein. Mice were killed 6, 24 or 48 hours after injection and tumor, muscle, lung, liver, kidney and spleen were prepared and examined histologically after staining with FITC-conjugated anti-rabbit IgG secondary antibody (Cappel, Durham, NC).

\( ^{99m} \)Tc-labeling was carried out according to the instructions of MAP Medical Technologies (Tikkakoski, Finland). 1 mg of either anti-LN\( \gamma_2 \)-III or normal rabbit IgG was dissolved in 200 \( \mu \)l of PBS and mixed with 200 \( \mu \)l of ascorbic acid solution, pH 5.5 – 6.0. After 30 min incubation at room temperature, 150 \( \mu \)l of freshly prepared Na-dithionite solution was added (30 mg/ml Na-dithionite in 0.15 M Na-acetate biffer, pH 6). 8 mCi of \( ^{99m} \)Tc (MAP Medical Technologies OY, Tikkakoski, Finland) was added and the incubation was continued for 30 min at room temperature. The labeling efficiency was determined with instant thin layer chromatography (ITLC) using 0.15 M NaCl as the solvent.

Biodistribution studies and radioimmunoimaging were performed in KLN-205 male mice having primary tumors in their left legs. 20 \( \mu \)g of either \( ^{99m} \)Tc-labelled anti-LN\( \gamma_2 \)-III IgG or normal rabbit IgG were administered into the tail vein, and groups of three mice were perfused 24 or 48 h after injection. Mice were imaged with an Elscint Apex 409ECT gamma scintillation camera (Elscint Ltd, Israel) using a low-energy, high-resolution collimator or a low-energy all-purpose collimator. Afterwards, the tissues (tumor, muscle, lung, liver, kidney and spleen) were dissected out, weighed and their radioactivity counted. The experiment was repeated twice.
5. Results

5.1. Antisera against recombinant γ2

Polyclonal antibodies against domain III and the C-terminus of the γ2 chain were prepared using γ2-GST fusion proteins as antigens (original articles I and II). The recombinant γ2-GST fusion proteins were purified on a glutathione-Sepharose 4B column and the purified proteins were used to immunize rabbits. Antibodies against the GST-epitopes were removed from the antisera by negative immunoadsorption with GST-Sepharose made by coupling E. Coli expressed GST protein to CNBr-activated Sepharose. The antisera were tested for immunoreactivity against purified human laminin γ chains, as well as, purified laminin-5 and commercial mouse laminin-1 by Western blotting. Purified full-length recombinant human γ1 and γ2 chains, laminin-5 and laminin-1 were electrophoresed by SDS-PAGE, transferred to nylon membranes and immunoreacted with the antisera. Both antisera reacted with the full-length γ2 chain, while no reactivity was observed with the closely related γ1 chain or laminin-1 chains. In addition, both antibodies reacted with the laminin-5 chains, corresponding to the processed and unprocessed (full-length) γ2 chains. The specificity of the antibodies was also tested by ELISA. The removal of anti-GST IgG was verified by Western analysis.

5.2. Expression of the γ2 chain in human cancers

The presence of the laminin γ2 chain in normal human tissues was verified by immunohistochemical analysis of a variety of adult human tissues. The γ2 chain protein was exclusively seen in basement membranes under the basal epithelial cells in the endocervix, endometrium, intestine, stomach, lung, skin, tongue and esophagus. The staining in the lung was strongest in the bronchial epithelia, although a weak signal was also seen in some alveolar epithelia. In the kidney, expression of the γ2 chain was localized to the collecting tubules. These results were confirmed in a recent study (Mizushima et al. 1998).
Under pathological conditions, expression and immunoreactivity of laminin-5 chains have been found to be upregulated in migrating keratinocytes of healing skin wounds (Larjava et al. 1993, Pyke et al. 1994, Ryan et al. 1994). γ2 chain mRNA has also been shown to be expressed in a number of cancer cells of epithelial origin, and in colon adenocarcinomas, the expression appears to be a characteristic of cancer cells showing a budding cell phenotype (Pyke et al. 1994). In the present study a variety of human tumor samples were studied to further explore whether expression of laminin-5 is deregulated in carcinomas. A total of 72 human cancer biopsies were stained with the polyclonal antibody produced against domain III of the γ2 chain to examine the localization of this chain (original article I). These biopsies included 23 cases of colon adenocarcinomas, 16 ductal breast carcinomas, 9 malignant melanomas, 14 squamous cell carcinomas of the skin and cervix, and 10 sarcomas. In situ hybridization of adjacent sections was used as a control for the immunostaining results. All colon adenocarcinoma specimens were positive for the γ2 chain, and the staining was almost exclusively confined to the cancer cells. The staining was most prominent at the invasive front of the tumors. Eleven out of the 16 cases of mammary cancer were positive for the γ2 mRNA and protein staining, and 10 of the cases contained positive cells immediately surrounding nests of cancer cells. Six out of nine malignant melanomas showed moderate to strong staining in apparently nonmalignant keratinocytes, which, in all cases, were judged to be engaged in wound healing responses. All cases of squamous cell carcinomas originating from the cervix, vulva, and skin were γ2 chain immunoreactive, and the staining was seen only in the cancerous cells. In most of the cases, cancer cells located immediately adjacent to the surrounding stromal tissue showed the most intense labeling. None of the sarcoma specimens studied showed positive staining. In situ hybridization of both the γ2 chain and urokinase receptor (uPAR) on adjacent sections established that the cancer cell population containing γ2 chain mRNA and protein also contained uPAR mRNA.

To evaluate in more detail if the laminin-5 γ2 chain can be used as a marker of invasiveness in carcinomas, an extensive systematic study was carried out on uterine cervical tumors. Ninety paraffin-embedded cervical lesions containing mild dysplasias to invasive cancers were analyzed with the polyclonal anti-LNγ2-III antibody (original article IV). The tissues were not pretreated with proteases or strong denaturing agents, in order to minimize staining of the native basement membrane. With one exception, all lesions of mild and moderate dysplasia did not show any positivity for the anti-LNγ2-III antibody. A total of 21 out of 32 samples diagnosed with cancer in situ (CIS), were negative, whereas all lesions with invasive and microinvasive cancers were positive for the antibody with the staining confined to the cancer cells at the invasive front of the tumor. Interestingly, one of the lesions diagnosed as moderate dysplasia was strongly positive for the γ2 chain antibody, suggesting that the synthesis of γ2 is not always indicative of malignant transformation of epithelial cells. Histopathologically, the squamous epithelia was judged as of metaplastic type with considerable degenerative changes and with an atypia difficult to grade. Importantly, however, one month later a cervical cone from the same patient was diagnosed being microinvasive, or alternatively, a moderately differentiated invasive cancer. This specific case showed a strong cytoplasmic γ2 chain immunostaining and also a tendency to stain extracellularly. Thus, this study strongly supports the use of laminin γ2 as a marker for microinvasive cervical cancers.
5.3. The effect of the \( \gamma_2 \) chain laminin-5 on cell attachment and migration of epithelial cells

Having observed increased expression of the \( \gamma_2 \) chain in carcinomas, we wished to examine the role of laminin-5 and its \( \gamma_2 \) chain in epithelial cell adhesion and migration. Laminin-5 isolated from human keratinocyte cell culture medium and commercial laminin-1 were used as substrata in attachment assays (original article II). Adherence of two epithelium-derived cell lines, HaCat and KLN-205, to laminin-5 was about 2.5 times higher than to plastic, and was slightly higher than that to laminin-1. The attachment to laminin-5 was not significantly decreased in the presence of two different polyclonal antibodies raised against the short and long arms of the \( \gamma_2 \) chain or by non-immune sera. When the cells were plated on the recombinant \( \gamma_2 \) chain alone, the number of cells attached was not significantly higher than to plastic, and this attachment was not influenced by the polyclonal antibodies mentioned above. Thus, these results confirm the earlier results suggesting that trimeric laminin-5 promotes adhesion of epithelial cells, but further strongly suggest that this adhesion is not mediated by the \( \gamma_2 \) chain.

The potential role of the \( \gamma_2 \) chain of laminin-5 in the cell migration process was examined for the KLN-205 cells in vitro using Boyden chamber and Transwell assays. Migration of cells in the presence of preimmune IgG was arbitrarily set as 100 %. When laminin-1 and fibronectin were used as chemoattractants the migration of cells through the filter was decreased to about 35 to 45 % of that observed with the preimmune IgG when polyclonal antibody against the domain III of the \( \gamma_2 \) chain was added. In contrast, the polyclonal IgG against the C-terminal domain I/II did not affect migration. When native laminin-5 was used as a chemoattractant in the lower compartment of the chamber, the results were essentially the same as above. The addition of IgG raised against domain III inhibited migration to about 50 % as compared to the preimmune IgG. The polyclonal IgG against domain I/II did not have any effect. These in vitro results demonstrated that laminin-5 can have a role in the locomotion of epithelium-derived cells, and that this function can be inhibited by antibodies directed against domain III of the \( \gamma_2 \) chain.

5.4. Identification of a migration-related element in the LAMC2 gene

In order to search for potential epithelium-specific enhancer elements in the LAMC2 gene, transgenic mice containing the LAMC2 promoter and a reporter gene were produced. DNA constructs containing varying lengths of the 5’ flanking region of the LAMC2 gene were cloned with a downstream LacZ reporter gene (original article II). This reporter gene codes for bacterial \( \beta \)-galactosidase. The first construct, HH-1, contained about 5900 bp, including 55 bp from the 5’ untranslated region and the 5’ flanking region. The second construct, HH-2, contained 55 bp of the 5’ untranslated region and 613 bp of the 5’ flanking region. In mouse embryos, very little expression was observed with either
construct, demonstrating that the reporter gene constructs did not contain the cis-acting elements needed for epithelial expression. Previous results had shown strong expression of the LAMC2 gene in epithelial layers of skin, respiratory tract and kidney in human embryos, as determined by in situ hybridization (Kallunki et al. 1992). When tissues of adult transgenic mice were studied, both constructs also yielded as highly restricted and as temporary an expression as in mouse embryos, although in adult tissues the distribution of expression was slightly more extensive. The limited expression was confined to epithelial cells, but, for example, in skin, discontinuous expression was observed in some keratinocytes of the epidermis and the epithelial cells of some hair follicles. In the stomach, intense expression was seen in some villi of the gastric mucosa, but it was absent in most areas.

To examine if the lacZ gene was expressed in migrating keratinocytes during wound healing, small incision wounds were made in the dorsal skin and tails of mice made transgenic with these two constructs. No color reaction could be seen in keratinocytes resting on a normal epithelial basement membrane. However, intense staining was seen in several layers of keratinocytes migrating over the healing wound, indicating that the enhancer element(s) directs expression in proliferating and migrating keratinocytes. Identical patterns were obtained with both constructs. Thus, the element necessary for driving the expression is probably located within the 613 bp region immediately upstream of the transcription site of the LAMC2 gene.

5.5. Targeting of polyclonal antibody to tumors in KLN-205 mice

Having observed high association of laminin γ2 chain expression with carcinomas, we wished to explore whether anti-γ2 antibodies target to carcinomas in vivo, and thus might serve as a potential markers for in vivo imaging. Such a study was then carried out in a KLN-205 mouse model (original article III). Following intramuscular or subcutaneous inoculations of KLN-205 cells, large γ2 chain positive primary tumors developed in 4 weeks with numerous lung metastases after 4 to 6 weeks. Cells injected into the tail vein produce multiple lung tumors (experimental metastases) in 3 to 4 weeks. When injected into tail vein of KLN-205 mice the unlabeled anti-LNγ2-III targeted specifically to primary tumors and lung metastases as examined by histochemical staining of normal and tumor tissues with FITC-conjugated anti-rabbit IgG secondary antibody. No staining was observed in epithelial basement membranes that do contain some amount of laminin-5. Normal rabbit IgG revealed negative results. The results clearly demonstrated that intravenously administered anti-γ2 antibodies bind newly synthesized laminin-5 in vivo but poorly the protein in native basement membranes.

The above results indicated that radioactively labeled anti-γ2 antibodies might be used for in vivo imaging of carcinomas. For example, antibodies labeled with γ-rays emitting isotopes could be detected by a gamma camera. The biodistribution of 99mTc-labeled anti-LNγ2-III antibodies and normal rabbit IgG were first studied in mice carrying KLN-205 tumors. Samples containing 20 μg of specific antibody or normal rabbit IgG were injected intravenously and the mice were perfused 24 or 48 hours after injection. The mice were
first imaged using a gamma camera, after which the tissues (tumor, muscle, lung, liver, kidney and spleen) were excised, separated, and their radioactivity was counted. The accumulation of the $^{99m}\text{Tc}$-labeled anti-LN$\gamma$2-III antibody in the tumor was about 4.5 %ID/g tissue, or about twice as much as that of labeled normal rabbit IgG. Fine contrast between tumors and normal could also be obtained with the gamma camera using a $^{99m}\text{Tc}$-labeled anti-LN$\gamma$2-III antibody and normal rabbit IgG. However, perfusion of the animal was necessary for imaging with the gamma camera, because the background could not be removed by diminishing the amount of injected polyclonal antibody. The staining of histological sections of tumors with FITC-conjugated anti-rabbit IgG secondary antibody confirmed further the specificity of biodistribution of the radioactively labeled antibody in these animals.
6. Discussion

The present work provided new information on the role of laminin-5 in epithelial cell adhesion and, particularly, on the role of its γ2 chain in cell migration. The role in migration was emphasized by identifying the presence of gene elements induced in cell migration using transgenic mice as a model system. The present work further demonstrated increased levels of γ2 chain protein in cells of epithelial cancers and, notably, that such antibodies can be used for both histochemical and in vivo imaging of malignant epithelial tumors.

6.1. Expression of the γ2 chain of laminin-5 in human epithelial tissues and malignancies

The results of this study demonstrated a strong increase amount of the laminin γ2 chain in invading tumor cells of most epithelial cancers, supporting a previous finding of the upregulation of laminin-5 expression in such tumors (Pyke et al. 1994). It was also shown in this study that in some human carcinomas, upregulated expression of laminin-5 or its subunit chains is characteristic of budding cancer cells located at the tip of invading malignant epithelia. The results of this work also demonstrated that the basement membranes of normal human skin, lung, stomach, small intestine, kidney, and germinal epithelium were positive for γ2 chain staining and that the staining was always continuous under the basal epithelial cells, except in the stomach and small intestine where the γ2 chain localized only to the microvilli tips. Several reports have described the expression of laminin-5 also in malignancies (Pyke et al. 1994, Hao et al. 1996, Tani et al. 1996, Tani et al. 1997, Fukushima et al. 1998, Martin 1998, Orian-Rousseau et al. 1998, Sordat et al. 1998). There is, however, some differences in the expression patterns of laminin-5 in epithelial cancers. Expression of laminin-5 chains is up-regulated quite generally in epithelial cancers, such as squamous cell carcinomas and gastric carcinoma, but not in mesenchymally derived cancers (Larjava et al.1993, Pyke et al. 1994, Tani et al. 1996, Orian-Rousseau et al. 1998, Sordat et al. 1998). Down-regulation has been reported in epithelial prostate and breast carcinomas (Hao et al. 1996, Martin et al. 1998). In this study the cancer cells of ductal mammary carcinomas and malignant melanomas were
shown to be negative for \( \gamma 2 \) and the weak or moderate staining was judged to be engaged in wound healing responses. Similar kind of down-regulation of laminin-5 in breast carcinoma cells has been reported recently by others (Martin et al. 1998). Urokinase plasminogen activator (uPA) is a protease involved in cancer invasion and metastasis. The proteolytic enzyme plasmin is generated by the action of uPA that binds to cell membrane receptor urokinase plasminogen activator (uPAR). The activation is proposed to lead to extracellular matrix degradation and to facilitate cancer cell invasion (King 1996). In this study uPAR was shown to be coexpressed with the \( \gamma 2 \) chain of laminin-5 in the budding cancer cells at the invasion front in colon cancer. uPAR has previously been shown to be expressed in stromal cells and in cancer cells at invasive foci being a marker of invasiveness (Grøndahl-Hansen et al. 1991, Pyke et al. 1991). The \( \gamma 2 \) chain was not expressed in cancer cells of ductal mammary carcinomas and malignant melanomas. Interestingly, recently it has been shown that in breast carcinomas uPA is located predominantly also in the stromal cells, although some expression can be detected in malignant cells, as well. uPAR localized principally to stromal cells, especially when these cells surrounded invasive breast cancer (Kennedy et al. 1998). Malignant epithelial cells do not seem to express uPA, either (Grøndahl-Hansen et al. 1991). Altogether, these results suggest that probably stromal cells collaborate with malignant cells to mediate metastases. However, the reason for the differences in the up- or down-regulation of laminin-5 in some epithelial cancers, as well as the possible biological function of the expression of laminin-5 in stromal cell in breast carcinoma remains to be solved. Nevertheless, because almost 85% of all cancers are of epithelial origin (King 1996) the connection between the early expression of laminin-5 in most carcinomas and adhesion and migration of carcinoma cells raises interesting questions about the role of this protein in these processes and the potential of this protein as a marker of epithelial malignancy.

This study also provided the first example of a specific type of cancer in which antibodies against laminin-5 or its subunit chains can be used for histopathological diagnosis. Localization of the \( \gamma 2 \) chain was examined in 90 specimens of both precancerous stages and manifest cancer from the uterine cervix. The high incidence of cervical cancer worldwide, about half a million women each year, constitutes a major problem in clinical diagnostics (Pisani et al. 1993). The precursor stages of invasive cervical carcinoma are defined as different types of atypia; mild cervical intraepithelial neoplasia, moderate and severe or cancer in situ (Richard et al. 1973). Many clinical questions and issues concern whether the cancer is in situ, microinvasive or frank invasive and, depending on the diagnosis, the patient will have different treatments. Markers of cellular proliferation, including such as Ki-67 and PCNA, and more recently other biomarkers such as nm23-H1, MN protein and matrix metalloproteinase-9 have been used in diagnostics of cervical disease (Bulten et al. 1996, Marone et al. 1996, Resnick et al. 1996, Davidson et al. 1998). However, so far no single marker has been found to have outstanding prognostic or diagnostic significance. In this study, all microinvasive and frank invasive cancers showed \( \gamma 2 \) chain immunopositivity located almost exclusively to the cytoplasm of the cancer cells at the invasion front of the carcinous area. In 11 out of 32 carcinomas in situ, the \( \gamma 2 \) chain was localized to the cells close to the basement membrane. The investigation of the material with the Ki-67 and cyclin A proliferation markers, showed an increasing proliferative activity with increasing advancement in lesion with cancer in situ and invasive cancer. The degree of proliferative activity was independent of
laminin-5 positivity in both lesion with cancer in situ and invasive cancer. It has been suggested that approximately 30 % of all CIS, when left untreated, develop later into invasive cancer (Petersen 1956), although later it has been reported that 12.2 % of cases of new cancer in situ progress to invasive cancer (Gustafsson et al. 1989). Interestingly, in this study it was shown that the proportion of laminin γ2 positive CIS samples was roughly 30 %. Thus, it could be speculated that these cases will progress to invasive cancer or are, possibly, already invasive, and, thus, need therapy. All mild and moderate dysplasia lesions were negative for the γ2 chain in immunohistology, except one which was, one month later, associated with a microinvasive cancer. By means of hematoxylin staining, the invasive cells could easily be missed because of the numerous inflammatory cells, but they were clearly indentified by positive staining for the γ2 chain. Thus, the results showed that antibodies against the γ2 chain of laminin-5 are able to distinguish lesions with or without invasive capacity and could become a useful help in the histopathological diagnostics of cervical cancer. Naturally, one of the future goals is to do a statistically relevant study with hundreds of samples to validate whether this marker has real clinical utility.

6.2. The γ2 chain of laminin-5 in epithelial cell adhesion and migration

The results of this study showed that the short arm of the laminin-5 γ2 chain does not promote epithelial cell adhesion, but that it does have an effect on epithelial cell migration. Adhesion of human keratinocytes and KLN-205 mouse squamous cell carcinoma cells to laminin-5 was similar to that of laminin-1, but the full-length recombinant γ2 chain produced in insect cells, did not show any significant effect on cell adhesion. In addition, the polyclonal antibody raised against domain III of the γ2 chain did not inhibit the adhesion, which further supported the idea that the short arm of the γ2 chain does not interact with cellular receptors.

All genes coding for the chains of laminin-5 have been shown to be mutated in an inherited skin disease called Epidermolysis bullosa junctionalis (Aberdam et al. 1994b, Pulkkinen et al. 1994a, Pulkkinen et al. 1994b, Kivirikko et al. 1995, Vailly et al. 1995b, Vailly et al. 1995a, Vidal et al. 1995) demonstrating the essential role of this protein in both epidermal, as well as, general epithelial cell attachment. The G-domain in the α3 chain has been proposed to be the major cell attachment site (Rousselle & Aumaillé 1994, Rousselle et al. 1995), and the integrins, α6β4 and α3β1, mediating the adhesion properties, both bind to the long-arm of this molecule (Carter et al. 1991, Niessen et al. 1994, Rousselle & Aumaillé 1994, Pulkkinen et al. 1995b). However, there has also been indications that the short arm domains are involved in the anchorage of epithelial cells, as well. An in-frame mutation removing 73 amino acid residues from domains III and IV of the short-arm of the γ2 chain results in lethal Herlitz’s junctional epidermolysis bullosa (Pulkkinen et al. 1994a). It remains to be shown if the important binding site of the short-arm, lost in Epidermolysis bullosa junctionalis, interacts with some extracellular matrix proteins. Laminin-5 has been shown to bind type VII collagen, as well as fibulin-2.
The NC-1 domain of type VII collagen binds to laminin-5, but not to laminin-6, and because these isoforms share the α3 chain, it is suggested that the binding activity is mediated by the β3 or γ2 chain (Rousselle et al. 1997). Recently, the NC-1 domain of type VII collagen was reported to bind predominantly to the β3 chain, but also to the γ2 chain, when examined by a protein overlay assay (Chen et al. 1999). Fibulin-2 has been demonstrated to bind to laminin-1 and laminin-5 through the α1 and γ2 chain short arms. The 9 amino acid sequence in domain IV of the γ2 chain was defined as a critical site for fibulin-2 binding (Utani et al. 1997).

Because the processing of the γ2 chain removes most of the short-arm of this chain, including domain IV, it raises questions if there are laminin-5 or other laminin isoform with differently processed γ2 chains that could have different functions. So far, only the α3 and γ2 chains of laminin-5 have been shown to be proteolytically processed (Marinkovich et al. 1992b, Goldfinger et al. 1998).

The present results suggest that the γ2 chain has a role in epithelial cell migration. Polyclonal antibodies against domain III of the γ2 chain inhibited the migration of KLN-205 squamous cell carcinoma cells by about 55 to 65%. The antibody used was raised against the residues mutated in Epidermolysis bullosa junctionalis. The results with transgenic animals further supported the suggested role of laminin-5 and the γ2 chain in cell locomotion. The reporter gene was strongly expressed in healing skin wounds and the migration-related element was located in the 613 bp upstream region flanking the LAMC2 gene. However, the results also demonstrated that all the enhancer elements necessary for normal tissue specific expression in epithelial cells are not present in a sequence reaching as far as 5900 bp upstream of the transcription site.

Already the first reports characterizing laminin-5 demonstrated that it appeared to be made primarily by dividing and migrating cells and not by stationary keratinocytes (Marinkovich et al. 1992a, Ryan et al. 1994). In normal skin, laminin-5 is expressed as a part of the basement membrane that connects the epidermal cells to the underlying stroma (Carter et al. 1991). The wounding of skin results in a transition of keratinocytes from a stationary to a migratory state and these proliferating and migrating cells start to strongly express the subunit chains of laminin-5 (Carter et al. 1991, Larjava et al. 1993, Pyke et al. 1994, Ryan et al. 1994). Also in the mature cut the differentiating epithelial cells migrating from crypt to villus tip express actively laminin-5 (Virtanen et al. 1995, Leivo et al. 1996, Orian-Rousseau et al. 1996, Orian-Rousseau et al. 1998). The involvement of laminin-5 or some of its chains in the process of cell migration has been demonstrated in several in vitro and immunohistological studies, but they have revealed, in some cases, controversial results (Kikkawa et al. 1994, Verrando et al. 1994, Zhang & Kramer 1996, Tani et al. 1997, O’Toole et al. 1997a, Giannelli et al. 1997). Some studies have suggested that exogenous laminin-5 inhibits cell locomotion (O’Toole et al. 1997a, O’Toole et al. 1997b), while others propose that endogenous or newly secreted laminin-5 promotes motility of keratinocytes or carcinoma cells (Tani et al. 1996, Zhang & Kramer 1996, Tani et al. 1997). It has also been suggested that the optimal migration occurs at intermediate adhesion strengths (Verrando et al. 1994) which could partially explain the opposing results. Another explanation to the controversial results could be that cells used in different in vitro studies express different laminin-5 receptors (i.e. either α6β4, α3β1 or both), which might have different functional roles in cell attachment, spreading and
migration (Carter et al. 1990, Ryan et al. 1994, Xia et al. 1996, DiPersio et al. 1997). Cell migration has been reported to be induced by the cleavage of laminin-5 by matrix metalloproteinase-2 which cleaves the γ2 subunit of rat laminin-5 at residue 587, removing the short arm (Giannelli et al. 1997). Nevertheless, it is not known if the cleavage also occurs with the human protein because the sequence of the cleavage area is different between the rat and human. This study, however, support the hypotheses that the newly deposited γ2 chain of laminin-5 is important for the interaction of this laminin isoform with the extracellular environment and that laminin-5 could be one of the first ECM anchorage proteins of epithelial cells expressed during cellular movement. However, it remains to be confirmed if the migration promoting activity is based on the interactions between the γ2 chain and some extracellular ligands or the possible different functions of the processed and unprocessed (full-length) γ2 chain.

6.3. Potential of the γ2 chain of laminin-5 as an in vivo marker of epithelial tumors

To estimate the potential of this isomer for in vivo imaging, the detection of epithelial tumors was studied using polyclonal antibodies raised against the γ2 chain of laminin-5. Both the unlabeled and radioactively labeled antibodies produced against the short arm domain III of the γ2 chain targeted specifically into primary tumors and lung tumors in KLN-205 mouse model when injected into the tail vein. Accumulation of the radioactively labeled antibody in the primary tumor was about 4.5 % (ID%/g tissue), being almost twice as high as that of radioactively labeled normal rabbit IgG. A fine contrast could also be detected by gamma camera in tumor tissue of the KLN-205 mice. Thus, the results of this study demonstrated the potential of the γ2 chain molecule in in vivo imaging.

More than a decade ago antibodies were thought to provide a specific and accurate tool for tumor imaging and to bring help for different therapies by carrying drugs to target sites. A lot of setbacks have come out since then mainly because of the problems with background, specificity, unstability, toxicity and poor penetration into tissues (Bischor-Delaloye et al. 1996). In recent years the delivery of antibodies to tumors have, however, become more promising because antibody fragments Fab, Fv, scFv or bispecific diabodies have been used (Holliger and Hoogenboom 1998). New detection techniques using e.g. magnetic resonance imaging and new contrast agents for that (Sipkins et al. 1998) may also improve the results of tumor imaging with antibodies.

A mix of the consecutive processes of cell attachment, detachment, and migration, as well as, the degradation of the temporary complexes formed for these processes are needed for the locomotion of cancer cells to distant locations (Liotta 1991, Liotta & Stetler-Stevenson 1991). The degree of differentiation is a significant factor in predicting the behavior of tumor cells. Because laminin-5 or its subunit chains seem to be a marker of differentiation stage or malignant growth of epithelial cells, it could have prognostic or diagnostic value. Early and accurate detection of malignant growth is still important for good results in cancer therapy. Although the effects of laminin-1 on cancer cells has been investigated quite efficiently (Martin et al. 1995), not much is known so far concerning
other laminin isomers, except laminin-5. In addition, the overall picture of the links between the expression of laminin-1 and/or some of its receptors and malignant growth is still very much incomplete. Laminin-5 appears to be a very promising protein for tumor imaging because of its restricted and clear tissue specificity and aberrant expression in invasive carcinomas, as in the cervical cancers presented in this study. Monoclonal antibodies or antibody fragments need to be produced for future studies and for optimization of the critical parameters of in vivo visualization. Probably another animal model is also needed to investigate the potential in vivo detection of metastasis. In a mouse model the detection of at least lung metastasis may be impossible, if the background in kidney, liver and spleen cannot be removed. In addition, the expression patterns of laminin-5 in different human carcinomas need to be investigated more accurately to identify the most potential cancers that could benefit from this laminin isomer as a marker protein in detection.

6.4. Conclusion

The present study demonstrated the role of laminin-5 for cell locomotion in vitro and confirmed its role as an attachment component of epithelial cells. The short arm of the $\gamma_2$ chain is thought to serve as a bridging molecule to the extracellular environment, because it does not have any cell binding activity by itself. An important challenge for future studies is to determine molecules that interact with the short-arms of the trimer and the $\gamma_2$ chain. As a migration-associated molecule, laminin-5 may also be an interesting target in pathological processes where cell locomotion plays a role. The $\gamma_2$ chain of laminin-5 is, so far, known to assemble only in laminin-5 isoforms and is, therefore, a good marker for this epithelium cell specific protein. In this study, the $\gamma_2$ chain was shown to be expressed in a highly restricted manner in human epithelial tissues and in carcinomas and shown to be a predictive marker for microinvasive epithelial cancer. The gene and protein expression in some human carcinomas, especially in colon adenocarcinomas, appeared to be characteristic of cancer cells showing a budding cell phenotype, suggesting that this molecule could be useful as a marker for these type of invasive cells. Importantly, in uterine cervical tumors, antibodies against the $\gamma_2$ chain of laminin-5 were able to distinguish between lesions with or without invasive capacity and, thus, laminin-5 may have the potential to become useful help in histopathological diagnostics of cervix squamous carcinomas. Unlabeled and radioactively labeled polyclonal antibodies raised against domain III of the $\gamma_2$ chain targeted specifically to primary tumors and metastases of epithelial origin, demonstrating the potential of this molecule in in vivo imaging. In conclusion, since the differentiation stage is an important criterium in diagnosis of malignancy and invasiveness of carcinoma cells, laminin-5, as a marker of differentiation of epithelial cells, might be useful as a marker, not only in immunohistology, but also as a target for in vivo imaging.
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