The Assembly of Laminin-5 Subunits*

(Received for publication, May 8, 1995, and in revised form, July 20, 1995)

Chihiro Matsui, C. Kathy Wang, Charlotte F. Nelson, Eugene A. Bauer, and Warren K. Hoeffler§

From the Department of Dermatology, Stanford University School of Medicine, Stanford, California 94305

Laminin-5 is a heterotrimer composed of α3, β3, and γ2 chains, produced by keratinocytes and the human squamous cell carcinoma line (SCC-25), and is one of the candidate proteins for the genetic lesion in junctional epidermolysis bullosa. Two-dimensional SDS-polyacrylamide gel electrophoresis (first dimension, nonreducing conditions; second dimension, reducing conditions) revealed that the immunoprecipitated laminin-5 from a SCC-25 cell fraction consisted of α3, β3, and γ2 monomers, a β3γ2 heterodimer, and an α3β3γ2 heterotrimer. The presence of the β3γ2 heterodimer, but not heterodimers containing an α3 chain and any of the other chains, was suggestive of assembly of laminin-5 proceeding from a β3γ2 heterodimer to an α3β3γ2 heterotrimer. We showed, by cotransfection experiments using full-length recombinant β3 and γ2 chains in a human cell line devoid of endogenous laminin-5, that stable heterodimers can be formed in the absence of α3 chain expression. In the SCC-25 cell fraction, the α3 monomer pool was the smallest of the monomers. Pulse-chase experiments using the cell fraction also indicated that the heterotrimer was assembled after a 10-min pulse and was nearly absent after a 24-h chase. These results are consistent with the synthesis of α3 being limiting for heterotrimer assembly, with rapid association of the α3 chain with β3γ2 heterodimers to form complete heterotrimers. Treatment with tunicamycin reduced the size of each of the laminin-5 subunits, indicating that all chains are glycosylated, but that N-linked glycosylation is not necessary for chain assembly and secretion.

Laminin-5 (kalinin/nicein) is an epithelial-specific laminin subtype and is a component of the anchoring filament of the lamina lucida in the basement membrane of the skin (1). The anchoring filament is the bridge between hemidesmosomes and the lamina densa region of the basement membrane and is believed to mediate the adhesion of the epithelium to the basement membrane. Laminin-5 is therefore one of the primary adhesion proteins holding together the epidermis and the dermis. Laminin-5 is initially synthesized in a cell-associated form, estimated to be 460 kDa, and is composed of three polypeptides: the 200-kDa (α3), 145-kDa (β3), and 155-kDa (γ2) chains (2). The three chains are presumed to form a cruciform structure where the chains are bound by disulfide linkages (2). The two heterotrimers composed of a γ2 chain to 105 kDa (2). Processing of the 200-kDa α3 chain to 165 kDa, while the 400-kDa form is derived from the 440-kDa form by extracellular processing of the 155-kDa γ2 chain to 105 kDa (2).

A number of variant laminin subunits, in addition to those of laminin-5, are assembled in the rough endoplasmic reticulum. N-Linked oligosaccharide processing of some of these has been shown to occur within the Golgi apparatus prior to secretion (3, 4). These variant chains are produced in different cell types (5, 6); for example, α1, α2, α3, α4, β1, β2, β3, γ1, and γ2 chains are produced, but only the following combinations of chains have thus far been observed: α1-β1-γ1 (laminin), α2-β1-γ1 (merosin), α1-β2-γ1 (s-laminin), α2-β2-γ1 (s-merosin), α3-β3-γ2 (kalinin/nicein), and α4-β1-γ1 (k-laminin).

Two models of laminin-1 chain assembly, possibly relevant to the steps in laminin-5 assembly, have been proposed. Peters et al. (3) and Morita et al. (4, 5) observed a disulfide-linked β1γ1 heterodimer as a presumed intermediate and therefore suggested that the α1 chain was added at a later stage. Alternatively, Wu et al. (7) reported that initially laminin chains are assembled randomly.

Defects in laminin-5 result in defective anchoring filaments, causing blistering of the skin, and are now known to cause Herlitz junctional epidermolysis bullosa (HJ EB), an autosomal recessive disorder characterized by generalized blister formation at the level of the lamina lucida within the epidermal basement membrane (8, 9). Recently, mutations in the γ2 chain (10, 11) and β3 chain (12) genes of laminin-5 have been reported in HJ EB patients. Since laminin-5 is a heterotrimer, knowledge of the steps in the assembly of the complete protein may be important in future attempts to correlate specific chain mutations with laminin-5 dysfunction and ultimately with clinical phenotype. As a first step in understanding the pathophysiology of HJ EB, we have characterized the subunit assembly of laminin-5.

Our assembly study was conducted in a squamous cell carcinoma line (SCC-25). This cell line produces laminin-5 that is indistinguishable from that produced by normal human keratinocytes (2). We determined the steps in laminin-5 chain assembly using endogenous laminin-5 and checked our conclusions using exogenous chains expressed from full-length cDNAs. We conducted immunoprecipitation reactions with general and chain-specific antibodies and used two-dimensional gels to resolve subunit association.

**EXPERIMENTAL PROCEDURES**

Antisera—Fusion proteins derived from cDNAs encoding the central domains of the γ2 (13) and β3 (14) proteins was used to raise anti-γ2 and anti-β3 antisera in rabbits. The cDNA clones were subcloned into the pGEX prokaryotic expression vector (Pharmacia Biotech Inc.). The fusion protein was prepared upon induction, purified according to manufacturer's instructions, and used as an antigen for immunization of rabbits. Polyclonal anti-laminin-5 antiserum was kindly provided by

---

*This work was supported by NIAMS Grants AR41045-03 and AR19537. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Partially supported by a scholarship from the Uehara Memorial Foundation.

§ To whom correspondence should be addressed. Tel.: 415-723-7843; Fax: 415-723-8762.
Assembly of Laminin-5 Subunits

Biosynthetic Labeling Study and Immunoprecipitation—Squamous cell carcinoma line SCC-25 (ATCC CRL1628) was cultured in 50% Ham’s F-12 medium, 50% DMEM supplemented with 0.5 μg/ml hydrocortisone, and 10% fetal bovine serum. Cells grown to subconfluency in 60-mm plastic dishes were incubated in methionine- and cystine-deficient DMEM for 2 h and then cultured in deficient DMEM with 100 μCi/ml protein labeling mixture ([35S]methionine and [35S]cystine) (DuPont NEN) for 2 h (cell) or 24 h (medium). After the first 6 h of the total 24-h incubation used for the preparation of the medium fraction, 0.1 volume of fresh DMEM was added to the deficient DMEM to prevent amino acid exhaustion. Cell and medium fractions were processed for immunoprecipitation, which was performed as described previously (15). Briefly, cell layers were harvested with a cell scraper and ice-cold radiimmunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 250 μM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, 2 mM l-methionine, 2 mM l-cysteine, 0.3% Nonidet P-40, 0.05% Triton X-300, 0.3% sodium deoxycholate, 0.1% bovine serum albumin) containing 0.1% SDS and 0.1% iodoacetamide. 250 mM phenylmethylsulfonyl fluoride and 0.1% iodoacetamide were added to the culture medium and centrifuged at 2000 rpm to remove cells and debris. Medium and cell lysate were cleaned by a mixture of preimmunized rabbit serum, anti-rabbit IgG-conjugated agarose beads (Sigma), and rabbit serum, anti-rabbit IgG-conjugated agarose beads (Sigma), and 15% Briefly, cell layers were harvested with a cell scraper and ice-cold radiimmunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 250 μM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, 2 mM l-methionine, 2 mM l-cysteine, 0.3% Nonidet P-40, 0.05% Triton X-300, 0.3% sodium deoxycholate, 0.1% bovine serum albumin) containing 0.1% SDS and 0.1% iodoacetamide. 250 mM phenylmethylsulfonyl fluoride and 0.1% iodoacetamide were added to the culture medium and centrifuged at 2000 rpm to remove cells and debris. Medium and cell lysate were cleaned by a mixture of preimmunized rabbit serum, anti-rabbit IgG-conjugated agarose beads (Sigma), and gelatin-Sepharose (Pharmacia Biotech Inc.) for 1 h. Immunologically reactive laminin-5 was precipitated from preclarred medium and cell fraction by incubation for 16-h incubation with a mixture of anti-rabbit precipitation buffer (medium sample) or radiimmunoprecipitation buffer containing 0.1% SDS (cells). After five washes, the pellets were mixed with SDS sample buffer, heated to 95°C for 3 min, and analyzed by SDS-PAGE.

Pulse-Chase Kinetic Analysis of Laminin-5 Biosynthesis—Dissociated SCC-25 cells were seeded at 10^6 cells/60-mm dish and allowed to attach in complete growth medium for 24 h and then incubated in methionine- and cystine-deficient DMEM for 2 h. Cells were pulsed with 100 μCi/ml protein labeling mixture in deficient DMEM for 10 min and then chased by incubation in complete growth medium. In these experiments, cells and conditioned medium were removed after 0, 0.5, 1.5, 3, and 24 h of chase in complete medium and then processed for immunoprecipitation. The precipitates of immunoreactive laminin-5 forms were fractionated by SDS-PAGE.

Interaction between Recombinant β3 and γ2 Subunits in 293 Cells—We constructed full-length β3 and γ2 chain cDNAs that encode the gene product of the human laminin-5 molecule, the 440-kDa (aβγ) trimer, the 350-kDa (aβγ) monomer, and the 250-kDa fibronectin band (FN) is also visible.

**RESULTS**

Biosynthesis and Assembly of the Laminin-5 Protein in SCC-25 Cells—SCC-25 cells were labeled with [35S]methionine and [35S]cystine. The immunoreactive laminin-5 forms were precipitated from both the cell lysate and the tissue culture medium with either anti-laminin-5 antiserum or antiserum raised against individual subunits and examined by SDS-PAGE.

In the cell fraction under nonreducing conditions, five forms of immunoreactive laminin-5 were observed (Fig. 1a, lane L). The slowest migrating band is a cellular form (aβγ) with an estimated molecular mass of 460 kDa. Based on molecular mass and immunoprecipitation by antisubunit antibodies (Fig. 1a, lanes β and γ), the other forms in the cell fraction were apparently dimers (aβγ) and monomers (a, β, and γ) of laminin-5 subunits. Consistent with this conclusion, aβγ chain-specific antisera precipitated the aβγ heterotrimer, aβγ dimer, aβγ monomer, but not the γ chain (Fig. 1a, lane β), whereas γ-specific antisera recognized the γ monomer as well as the β γ dimer and aβγ trimer, but not the β monomer. Under reducing conditions, the same samples showed three bands corresponding to the 200-kDa a3 (α), 145-kDa β3 (β), and 155-kDa γ2 (γ) subunits (Fig. 1b, lanes L, β, and γ).

Using nonreducing conditions to analyze the conditioned medium (Fig. 1c, lanes L, β, and γ), two secreted forms of the laminin-5 molecule, the 440-kDa (aβγ) and 400-kDa (aβγ) forms, were observed (α and γ) as the processed forms of the α and γ chains, respectively, whereas the β chain is not processed. Monomers or dimers are probably not secreted since no bands are seen even after longer exposures of the autoradiograms corresponding to the molecular masses of either monomers or dimers. Under reducing conditions, these same samples containing both forms of the heterotrimer resolved into four bands, corresponding to 165-kDa processed a3 (α), 145-kDa β3 (β), 155-kDa γ2 (γ), and 105-kDa processed γ2 (γ) (Fig. 1d, lanes L, β, and γ). All three antibodies also precipitated an additional band of 250 kDa under reducing conditions (Fig. 1d).
FN). The intensity of this band was diminished by preincubation of the samples with gelatin-Sepharose, previously found to bind fibronectin. The 250-kDa band was present in greater amounts when the preincubation step was deleted (data not shown).

The extent of association of the laminin-5 subunits with each other was assessed in cell lysates and culture medium by two-dimensional SDS-PAGE under nonreducing conditions in the first dimension and under reducing conditions in the second dimension. In the cell lysate, the 460-kDa complex (αβγ) (Fig. 2a, right-most band in the top panel) observed under nonreducing conditions dissociated under reducing conditions into an equimolar mixture of α3, β3, and γ2 subunits (lower left panel, spots directly below band). The second band (βγ) from the origin under nonreducing conditions dissociated into two forms of 145 and 155 kDa under reducing conditions, indicative of a disulfide-linked heterodimer of β3 and γ2. Monomers of the three subunits run under reducing conditions are shown as markers in Fig. 2a (lower right panel). The signal intensity of the monomers under nonreducing conditions was α < γ < β. A weaker signal for α3 was reproducibly observed as compared with β3 (β) or γ2 (γ) monomers, perhaps indicating that the intracellular pool of uncombined α3 is smaller than that of the other chains. We cannot rule out the possibility of preferential precipitation of the β or γ chain by our general antisera against laminin-5.

In the medium (Fig. 2b), the two forms of extracellularly processed laminin-5, 440 kDa (αβγ) and 400 kDa (αβγ'), were observed under nonreducing conditions. Under reducing conditions, the 440-kDa form (right-most band in the top panel) dissociated into a mixture of processed 165-kDa α3 and 140-kDa β3 and unprocessed 155-kDa γ2 subunits. The 400-kDa laminin-5 (the second band from the origin under nonreducing conditions) also dissociated into processed α3 and β3, but displayed a complete substitution of the processed 105-kDa γ2 chain for unprocessed γ2. The nontspecific 250-kDa fibronectin band was again present under reducing conditions.

**Kinetics of Laminin-5 Biosynthesis, Subunit Assembly, and Secretion—** SCC-25 cells were pulse-labeled with [35S]methionine and [35S]cysteine for 10 min and then incubated in complete growth medium as a chase. After 0, 0.5, 1.5, 3, 6, and 24 h of chase in unlabeled complete medium, either cell lysates or medium samples were removed and then processed for immunoprecipitation with anti-laminin-5 antisera. The precipitates of immunoreactive laminin-5 forms were fractionated by SDS-PAGE after preparation under either nonreducing or reducing conditions, a, cell fraction, nonreducing conditions; b, cell fraction, reducing conditions; c, medium fraction, nonreducing conditions; d, medium fraction, reducing conditions. Laminin-5 subunits visible are the αβγ heterotrimer; the βγ heterodimer; the α, β, and γ monomers; the αβγ 440-kDa medium form; and the αβγ' 400-kDa medium form. A 250-kDa fibronectin band (FN) is also visible.
Fig. 4. Expression of recombinant \( \beta 3 \) and \( \gamma 2 \) subunits in 293 cells. 293 cells were not transfected (lanes 1, 7, and 13), transfected with the pCPS vector alone (lanes 2, 8, and 14), or cotransfected with pCPS and pCPS/3 (lanes 3, 9, and 15), pCPS and pCPS-\( \gamma 2 \) (lanes 4, 10, and 16), or pCPS/\( \beta 3 \) and pCPS-\( \gamma 2 \) (lanes 5, 11, and 17). Two days later, the transfected cells were labeled with \[^{35}S\]methionine and \[^{35}S\]cysteine for 2 h as described under "Experimental Procedures." Labeled SCC-25 cells were included as a marker for the positions of the laminin-5 chains (lanes 6, 12, and 18). Cell extracts were immunoprecipitated with anti-\( \beta 3 \) antisera (lanes 1–6), anti-\( \gamma 2 \) antisera (lanes 7–12), or anti-laminin-5 antisera (Anti-lam5; lanes 13–18) and resolved by SDS-PAGE after denaturation in SDS sample buffer either under reducing (a) or nonreducing (b) conditions. \(-\), sample from untransfected 293 cells; \( V \), \( \beta \), and \( \gamma \), pCPS, pCPS/3, and pCPS-\( \gamma 2 \) used for transfection, respectively.

also seen in the 3-h chase lane and increased in intensity after the 24-h chase.

Formation of a \( \beta \gamma \) Heterodimer Using Recombinant \( \beta 3 \) and \( \gamma 2 \) Chains—We used full-length cDNA expression vector constructs for \( \beta 3 \) and \( \gamma 2 \) to determine if the \( \beta \gamma \) dimer is readily assembled as an intermediate in the formation of laminin-5. The target cell for these experiments was the human embryonic kidney cell line 293, which does not express laminin-5 as determined by immunoblotting of the cell fraction with anti-laminin-5 antibody (data not shown; Fig. 4a, lane 13). The assembly of recombinant \( \beta 3 \) (\( \beta \gamma \)) and \( \gamma 2 \) (\( \gamma \gamma \)) chains was conducted in the complete absence of the \( \alpha 3 \) chain.

The recombinant \( \beta 3 \) chain was immunoprecipitated from \( \beta 3 \) cDNA-transfected 293 cells using anti-\( \beta 3 \) antibody (Fig. 4a, lane 3), but not from untransfected cells or from cells transfected with the vector alone (Fig. 4a, lanes 1 and 2). As expected, anti-\( \beta 3 \) antibody did not recognize \( \gamma 2 \) in \( \gamma 2 \) cDNA-transfected 293 cells (Fig. 4a, lane 4); however, under reducing conditions, immunoprecipitates from \( \beta 3 \)- and \( \gamma 2 \)-co-transfected 293 cells using the same antibody showed both \( \beta \gamma \) and \( \gamma \gamma \) chains (Fig. 4a, lane 5), providing evidence for the formation of a \( \beta \gamma \) heterodimer. The recombinant chains showed the same mobility on SDS-PAGE under reducing conditions as endogenous \( \beta 3 \) and \( \gamma 2 \) from SCC-25 cells (Fig. 4a, lane 6). Similar results were obtained when the same set of transfection experiments were immunoprecipitated with anti-\( \gamma 2 \) antibody. As expected, \( \gamma 2 \) antisera did not react with \( \beta 3 \), only with \( \gamma 2 \) (Fig. 4a, lanes 9 and 10), and coprecipitated \( \beta 3 \) and \( \gamma 2 \) from cocranscribed 293 cells (lane 11), providing further evidence of \( \beta \gamma \) heterodimer formation.

To further clarify the identity of the presumed \( \beta \gamma \) heterodimer, we performed two-dimensional SDS-PAGE analysis with the samples from cotransfected cells. The heterodimers of \( \beta 3 \) and \( \gamma 2 \) have a similar molecular mass (300 kDa) to the homodimers of either \( \beta 3 \) (290 kDa) or \( \gamma 2 \) (310 kDa). We resolved the presumed heterodimer on a second dimension under reducing conditions into its component \( \beta 3 \) and \( \gamma 2 \) chains. Anti-\( \beta 3 \) antibody reacted with the \( \beta 3 \) monomer (Fig. 5a, black asterisk) and heterodimer (black and white arrows). The high molecular mass multimers dissociated into \( \beta 3 \) and \( \gamma 2 \) chains (Fig. 5a, black arrowheads) and \( \gamma 2 \) chains (Fig. 5a, white arrowheads).

Anti-\( \gamma 2 \) antibody recognized both the \( \gamma 2 \) monomer, which resolved into two bands (Fig. 5b, white asterisks), and heterodimer (black and white arrows). The \( \gamma 2 \) monomer resolved...
into two bands in the first dimension under nonreducing conditions, probably due to the presence of an intramolecular disulfide bond in the \(\gamma\) chain. In the second dimension (reducing conditions), the high molecular mass multimers dissociated into only \(\gamma\) (Fig. 5b, white arrowheads), with no \(\beta\) containing species visible. Although the two antibodies showed different reactivity to the multimers, cotransfected 293 cells definitely contain monomers (\(\beta\) and \(\gamma\)), multimers, and the heterodimer (\(\beta\gamma\)).

Glycosylation of Laminin-5, Subunit Assembly, and Secretion—Tunicamycin inhibits the synthesis of dolichol phosphate N-acetylglucosamine (an intermediate required for the synthesis of N-glycosylated glycoprotein) and has been used to study the role of oligosaccharide side chains in protein assembly (18). For the purposes of the discussion below, we labeled the tunicamycin-treated unglycosylated subunit precursors with a “p.”

In the presence of tunicamycin, monomers (\(\alpha\)p, \(\beta\)p, and \(\gamma\)p), dimers (\(\alpha\beta\)pp), and heterotrimer (\(\alpha\beta\beta\)p) migrated as lower molecular mass species as compared with the untreated normal cell lysate samples under nonreducing conditions (Fig. 6a, compare lanes N and T in the top panel). These presumably correspond to unglycosylated precursors as a result of tunicamycin treatment. A similar observation was made under reducing conditions, where each individual chain migrated as a lower molecular mass species as compared with the untreated normal sample (Fig. 6a, compare lanes N and T in the lower right panel). The assembly of unglycosylated laminin-5 subunits was assessed by two-dimensional SDS-PAGE. The heterotrimer (\(\alpha\beta\beta\)p), under nonreducing conditions, dissociated upon reduction into a mixture of the unglycosylated subunits (\(\alpha\)p, \(\beta\)p, and \(\gamma\)p), as did the heterodimer (\(\beta\beta\)p). These results suggest that in the absence of glycosylation, the assembly of laminin-5 subunits occurs in the same way as in untreated normal cells.

Finally, we assessed the role of glycosylation in the secretion and subsequent processing of laminin-5 by conducting a similar analysis as described above using the medium of tunicamycin-treated cells. In SDS-PAGE of the medium from the tunicamycin-treated cells under nonreducing conditions (Fig. 6b, lane T in the top panel), two bands migrating as lower molecular mass species as compared with the untreated normal medium fractions were observed, corresponding to heterotrimer (\(\alpha\beta\beta\)pp and \(\alpha\beta\beta\beta\)p’). Even after overexposure of this gel, no dimer and monomer bands were seen. Under reducing conditions, the same samples showed four fast migrating bands of unglycosylated proteins (Fig. 6b, \(\alpha\gamma\), \(\beta\gamma\), \(\gamma\gamma\), and \(\gamma\gamma\)’ in the lower right panel). On two-dimensional SDS-PAGE analysis, the larger band of first dimension electrophoresis dissociated into three proteins, processed \(\alpha\) (\(\alpha\gamma\)), unprocessed \(\beta\) (\(\beta\beta\)), and unprocessed \(\gamma\) (\(\gamma\gamma\)’) precursors. The lower signal dissociated into processed \(\alpha\) (\(\alpha\gamma\)), unprocessed \(\beta\) (\(\beta\beta\)), processed \(\gamma\) (\(\gamma\gamma\)’) precursors. Both the tunicamycin-treated cell and medium fractions showed a small band estimated to be 80 kDa under reducing conditions (Fig. 6, a and b, lane T in the lower right panel). Since both anti-\(\beta\) and anti-\(\gamma\) reacted with this band on immunoprecipitation (data not shown), it may be a product of degradation of the heterotrimer, due to an increased instability of unglycosylated precursors as compared with the normally glycosylated chains.

**DISCUSSION**

We have characterized the subunit assembly of a novel tissue-specific laminin variant, laminin-5, formerly known as nicenin or kalinin (1, 2). Laminin-5 is present at epithelial-stromal interfaces and is the primary component of the anchoring filaments associated with hemidesmosomes and therefore is crucial to the attachment of basal epithelial cells to the basement membrane zone. Defects in laminin-5 have been shown to be a cause of a lethal skin disease, Herlitz junctional epidermolysis bullosa (10–12). We utilized the squamous cell carcinoma line SCC-25 as a source of constitutively high levels of laminin-5 rather than cultured primary keratinocytes because of the tendency of the keratinocytes to differentiate, resulting in a down-regulation of laminin-5. As is the case for a variety of multimeric proteins, we observed a specific order of addition for the assembly of individual chains into heterotrimeric laminin-5. In the cell fraction containing laminin-5 prior to secretion, we observed monomers of each of the three subunits (\(\alpha\), \(\beta\), and \(\gamma\)), a \(\beta\gamma\) heterodimer, and an \(\alpha\gamma\) heterotrimer. The presence of a \(\beta\gamma\) heterodimer, but the absence of any other heterodimeric species, was suggestive of an ordered assembly
of laminin-5 proceeding from the $\beta\gamma$ heterodimer to the heterotrimer by the addition of the $\alpha_3$ chain.

We employed a eukaryotic expression vector to further test the possibility of a stable association of the $\beta$ and $\gamma$ chains into a heterodimer as an intermediate step in the assembly of the laminin-5 heterotrimer. Earlier use of recombinant proteins to study assembly of other laminin isotypes has been restricted to the use of the E8 fragment of mouse Engelbreth-Holm-Swarm laminin (19), prokaryotic expressed Engelbreth-Holm-Swarm laminin (20), and synthetic peptides (21) in vitro. Expression of recombinant proteins has been an important tool in the study of subunit assembly of a variety of proteins, however, including the interaction of fibronectin subunits (22, 23). Here we utilized complete cDNA expression vectors for the $\beta$ and $\gamma$ chains to establish that the two chains stably heterodimerize in the absence of the $\alpha_3$ chain. Our experiment also rules out the possibility that the $\beta\gamma$ heterodimer is a breakdown product of the heterotrimer since assembly occurs in the absence of the $\alpha_3$ chain. We used 293 cells for our transfection experiments because they contain no endogenous laminin-5 chains and are transfectable at high efficiencies and yield high levels of protein expression when exogenously added genes are driven by the cytomegalovirus promoter. The fact that the human embryonic kidney cell line 293 will express the cytoskeletal proteins was previously identified as a $300$ kDa precursor (20), and synthetic peptides (21)

Assembly of Laminin-5 Subunits

The $\alpha_3$ monomer pool was observed to be the smallest of the monomers (Figs. 1a (lane L), 2a, and 6 (a–c)). Pulse-chase experiments revealed that the $\alpha_3$ chain signal seemed to decay faster than the other two subunits (Fig. 3b). We postulate that the $\alpha_3$ chain is limiting for heterotrimer assembly and that the association of the $\alpha_3$ chain with $\beta\gamma$ heterodimers occurs rapidly to yield complete heterotrimers. A precedent in laminin-1 from murine teratocarcinoma cell lines has been shown in which the $\alpha_1$ chain is limiting for assembly at the protein level, in accordance with its low mRNA levels (24, 25). One possible rationale for the synthesis of the $\alpha_3$ chain being limiting in the synthesis of laminin-5 is that the cell uses $\alpha_3$ chain synthesis as a determining step in controlling the type of laminin to be assembled. Thus, expression levels of either $\alpha_3$ or $\alpha_2$ may determine whether laminin-1 ($\alpha_1\beta_1\gamma_1$) or merosin ($\alpha_2\beta_1\gamma_1$) is to be assembled or whether $\alpha_3$-laminin ($\alpha_1\beta_2\gamma_2$) or $\alpha_2$-merosin ($\alpha_2\beta_2\gamma_1$) is assembled. Although no laminin variant containing $\beta\gamma$ other than laminin-5 ($\alpha_3\beta_3\gamma_2$) has yet been described, the possibility of additional laminin variants seems likely. One circumstance in which laminin-5 production would be crucial is in activated keratinocytes regenerating a wound bed. Keratinocytes in tissue culture display markers for the activated phenotype as well, and the $\alpha_3$ chain pool typically exceeds the other monomer pools in these cells (data not shown).

Following assembly of the heterotrimer, processing of two out of the three chains occurs, $\alpha_3$ and $\gamma_2$. The cytoplasmic form of laminin-5 was previously identified as a $460$-kDa precursor that contains unprocessed forms of each chain (2). Processing occurs after secretion, with two predominant forms of 440 and 400 kDa present in the medium fraction. Processing of the $\alpha_3$ chain to 165 kDa has already occurred in both of these forms. However, in our pulse-chase experiments, faint bands of 200 and 180 kDa were also visible (Fig. 3d, 3e, 6f, and 24-h chase samples). The 180-kDa species may be an intermediate in the processing of the 200-kDa $\alpha_3$ chain to its final processed form of 165 kDa. Since these bands were minor components, they presumably belong to a minor fraction of heterotrimers secreted into the medium that were not yet processed at the moment of sampling. Resolution of a small amount of the unprocessed heterotrimer, and the presumed processing intermediate, may not be readily resolvable from the major form of 440 kDa in these gels.

We resolved the 440- and 400-kDa forms of heterotrimeric laminin-5 in the medium fraction into the component chains in our SCC-25 cell model system, as was previously done in keratinocytes (2) (Fig. 1c, lanes L, $\beta$, and $\gamma$). The larger species consists of 165-kDa $\alpha_3$, 145-kDa $\beta_3$, and 155-kDa $\gamma_2$ chains, while the smaller one consists of 165-kDa $\alpha_3$, 145-kDa $\beta_3$, and 105-kDa $\gamma_2$ chains (Fig. 2b). Furthermore, in pulse-chase experiments of the nonreduced medium samples (Fig. 3c), the 440-kDa form of laminin-5 first appeared after the 1.5-h chase and was clearly evident after the 3.0-h chase, whereas the 400-kDa form appeared later, only after a 3-h chase, clearly visible after a 6-h chase. Consistent with the 440- and 400-kDa forms being composed of the same chains but with processed $\gamma_2$ (105 kDa) substituted for unprocessed $\gamma_2$, the processed form of $\gamma_2$ (105 kDa) appears with the same kinetics as the 400-kDa form. These results support a precursor-product relationship between the 440- and 400-kDa forms, due to processing of the $\gamma_2$ chain from 155 to 105 kDa. Dimers or monomers were not detected in SCC-25 medium, consistent with a lack of secretion in the absence of heterotrimer assembly, although these forms might be present at steady-state concentrations below the detection limit of the assay. A 250-kDa protein was co-precipitated with laminin-5. Since the intensity of this signal was reduced by preincubation with gelatin, this protein may be fibronectin. Coprecipitation of fibronectin with laminin-5 was previously reported in normal and HJ EB human keratinocyte systems (26).

Multimers of individual chains, such as a $\beta_1$ chain multimer as reported for mouse laminin-1 (7), were not detected in laminin-5 from SCC-25 cells (Figs. 1a (lanes L, $\beta$, and $\gamma$) and 2a). It is possible that these forms might be present at steady-state concentrations below the detection limits of the assay or are immunologically unreactive. The latter possibility is less likely since $\beta$ and $\gamma$ multimers were detected in transfected 293 cells by the same antibodies. Multimer bands have previously been noted when other proteins have been expressed in heterologous systems, for example the expression of human fibrinogens $\alpha$, $\beta$, and $\gamma$ in baby hamster kidney cells (22). In a study on the influenza hemagglutinin protein, only properly folded multimeric proteins were transported out of the rough endoplasmic reticulum, whereas incompletely folded proteins either accumulated or were degraded in the endoplasmic reticulum (27).

One characteristic of the 293 cells used to conduct our transfection experiments may be a reduced capacity to deal with improperly folded proteins. We have shown that the assembly of recombinant $\beta_3$ and $\gamma_2$ chains is achieved without the presence of the $\alpha_3$ chain (Fig. 4a, lanes 5 and 11). In a further analysis of the cotransfection products on two-dimensional SDS gels, cells cotransfected with $\beta_3$ and $\gamma_2$ expression vectors produced both monomers (Fig. 5, a and b, black and white arrows) and the $\beta\gamma$ heterodimer (Fig. 5, a and b, black and white arrows). When anti-$\beta_3$ antibody was used, the major bands were $\beta_3$ monomers (145 kDa) and $\beta\gamma$ dimers (300 kDa). The coexpression of $\beta$ and $\gamma$ leads mainly to the formation of a $\beta\gamma$ dimer held together by disulfide bond(s) as well as free $\beta$ chain. When anti-$\gamma_2$ antibody was used, a $\beta\gamma$ dimer signal was also detected. Since this antibody does not recognize the $\beta\gamma$ heteromultimer (Fig. 5a, black and white arrows), heteromultimerization might obstruct binding to antibody recognition sites. We conclude that the $\alpha_3$ chain is not necessary for $\beta\gamma$ heterodimer formation, but that it is an intermediate in heterotrimer formation in SCC-25 cells. Considering that laminin-1 assembly probably proceeds via a $\beta\gamma$ dimer, and from our results with laminin-5, it seems a reasonable
prediction that other laminin isoforms also will be shown to assemble via a \( \beta \gamma \) dimer intermediate.

The pulse-chase study demonstrated that newly synthesized laminin-5 subunits appear in the cells immediately following a 10-min biosynthetic pulse with \( [35S] \)methionine and [35S]cysteine. Since other related proteins are known to undergo addition of N-linked glycochains after a 1-h chase, as reported for laminin-1 of human choriocarcinoma cells (3) and mouse embryonic carcinoma F9 cells (4), we expected to observe an increased mobility of the laminin-5 chains on SDS-PAGE due to the lack of glycosylation of these intracellular precursors. However, precursors with altered electrophoretic mobility were not detected on two-dimensional SDS-PAGE (Fig. 2a) and in the pulse-chase study (Fig. 3b). These results suggest that the high mannose chains on the subunits are not processed into complex forms.

To further investigate the role of glycosylation in laminin-5 chain assembly, SCC-25 cells were treated with tunicamycin to inhibit the addition of asparagine-linked carbohydrates, and cell and medium fractions were analyzed by SDS-PAGE and two-dimensional SDS-PAGE. The heterotrimer, heterodimer, and monomers were present in the cell fraction, but had slightly increased mobility on the gels due to the inhibition of glycosylation. The presence of the heterotrimer suggested that the assembly of unglycosylated subunits also proceeds through a \( \beta \gamma \) heterodimer to the \( \alpha \beta \gamma \) heterotrimer. Since protein disulfide isomerase is present at the luminal side of the rough endoplasmic reticulum (28), disulfide bond formation between laminin-5 subunits is expected to be completed before they leave the rough endoplasmic reticulum. Additionally, it is well established for N-glycosylated proteins that transfer of high mannose-type oligosaccharide side chains on polypeptides occurs cotranslationally (29). Dimer and trimer formation in the presence of tunicamycin demonstrates that the inhibition of this oligosaccharide transfer by tunicamycin does not affect subsequent disulfide bond formation. However, high mannose oligosaccharide chains can have a profound effect on the stability of proteins (30, 31), as has also been shown for laminin-1 (3). The amount of laminin-5 in the medium fraction was extremely small, so we postulate that N-glycosylation protects laminin-5 polypeptides from nonspecific proteolytic degradation.

Fig. 7 presents a summary diagram of our conclusions concerning post-transcriptional assembly and glycosylation of laminin-5 subunits. Laminin-5 chain mRNAs are translated into individual chain unglycosylated (thin lines) monomers, which are then glycosylated (thick lines). Heterodimers (\( \beta \gamma \)) assemble before the addition of the \( \alpha \) chain. Only heterotrimers are secreted regardless of whether N-linked glycosylation is blocked by tunicamycin. Proteolytic processing of the \( \alpha \) and \( \gamma \) chain occurs after secretion. Laminin-5 intermediates observed in our experiments are as follows: the \( \alpha \beta \gamma \) unglycosylated cellular form of laminin-5; the \( \beta \gamma \) unglycosylated heterodimer; the \( \alpha \beta \) and \( \gamma \) unglycosylated monomer precursors; the \( \beta \gamma \) unglycosylated larger medium form of laminin-5; and the \( \alpha \beta \gamma \) unglycosylated smaller medium form of laminin-5. (CHO)\( \alpha \) core oligosaccharide side chain transferred en bloc.

**Fig. 7. Model of post-translational assembly and glycosylation of laminin-5 subunits.** Laminin-5 chain mRNAs are translated into individual chain unglycosylated (thin lines) monomers, which are then glycosylated (thick lines). Heterodimers (\( \beta \gamma \)) assemble before the addition of the \( \alpha \) chain. Only heterotrimers are secreted regardless of whether N-linked glycosylation is blocked by tunicamycin. Proteolytic processing of the \( \alpha \) and \( \gamma \) chain occurs after secretion. Laminin-5 intermediates observed in our experiments are as follows: the \( \alpha \beta \gamma \) unglycosylated cellular form of laminin-5; the \( \beta \gamma \) unglycosylated heterodimer; the \( \alpha \beta \) and \( \gamma \) unglycosylated monomer precursors; the \( \beta \gamma \) unglycosylated larger medium form of laminin-5; and the \( \alpha \beta \gamma \) unglycosylated smaller medium form of laminin-5. (CHO)\( \alpha \) core oligosaccharide side chain transferred en bloc.

Agreement with experimental observations and that may allow predictions of the stability of distinct laminin isoforms (32, 33). Now that we have noted the appearance of a \( \beta \gamma \) heterodimer as a likely assembly intermediate for laminin-5, we would expect some of the mutations contained in HJ EB patients to effect \( \beta \gamma \) dimerization, while another class of mutations might interfere with a \( \alpha \) association with the heterodimer to form a functional heterotrimer. We are currently correlating HJ EB patient phenotypes with characterization of molecular defects to ascertain the extent to which we are able to use this knowledge to predict clinical features and ultimately the course of the disease.

**Acknowledgments**—We thank Dr. Peter Marinkovich for providing polyclonal anti-laminin-5 antibody. We thank Drs. Robert Burgeson and Donald Gerecke for partial cDNA clones for the laminin-5 (\( \beta \) chain, from which we constructed full-length cDNA clones.

**REFERENCES**

1. Verrando, P., Hsi, B. L., Yeh, C. J., Pisani, A., Seriyes, N., and Ortonne, J. P. (1987) Exp. Cell Res. 170, 116–128
2. Marinkovich, M. P., Lunstrum, G. P., and Burgeson, R. E. (1992) J. Biol. Chem. 267, 17900–17906
3. Peters, B. P., Hartlie, R. J., Kreuzek, R. F., Kroll, T. G., Perini, F., Balun, J. E., Goldstein, I. J., and Ruddon, R. W. (1985) J. Biol. Chem. 260, 14732–14742
4. Morita, A., Sugimoto, E., and Kitagawa, Y. (1985) Biochem. J. 229, 259–264
5. Tokida, Y., Arafita, Y., Morita, A., and Kitagawa, Y. (1990) J. Biol. Chem. 265, 18123–18129
6. Green, T. L., Hunter, D. D., Chan, W., Merlie, J. P., and Sanes, J. R. (1992) J. Biol. Chem. 267, 2034–2042
7. Wu, C., Friedman, R., and Chung, A. E. (1988) Biochemistry 27, 8780–8787
8. Verrando, P., Blanchard-Bardon, C., and Pisani, A. (1993) Lab. Invest. 64, 85–92
9. Smith, L. T. (1993) Arch. Dermatol. 129, 1358–1364
10. Pulkkinen, L., Christiano, A. M., Airenne, T., Haakanen, H., Tryggvason, K., and Uitto, J. (1994) Nature Genetics 6, 293–298
11. Abderam, D., Galiana, M. F., Vailly, J., Pulkkinen, L., Bonifas, J., Christiano, A. M., Tryggvason, K., Uitto, J., Epstein, E. H., Jr., Ortonne, J. P., and Meineguzzi, G. (1995) Nature Genetics 6, 1578–1584
12. Vailly, J., Pulkkinen, L., Miége, C., Christiano, A. M., Gerecke, D., Burgeson, R. E., Uitto, J., Ortonne, J. P., and Meineguzzi, G. (1995) J. Invest. Dermatol. 104, 462–466
13. Vailly, J., Verrando, P., Champallaud, M. F., Gerecke, D., Wagner, D. W., Baudon, C., Abderam, D., Burgeson, R. E., Bauer, E. A., and Ortonne, J. P. (1994) Eur. J. Biochem. 219, 209–218
14. Gerecke, D. R., Wagman, D. W., Champliaud, M. F., and Burgeson, R. E. (1994) J. Biol. Chem. 269, 11073–11080
15. Marinkovich, M. P., Lunstrum, G. P., Keene, D. R., and Burgeson, R. E. (1992) J. Cell Biol. 119, 695–703
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Laemmli, U. K. (1970) Nature 227, 680–685
18. Elbein, A. D. (1981) Trends Biochem. Sci. 6, 219–221
19. Hunter, I., Schultz, T., and Engel, J. (1992) J. Biol. Chem. 267, 6006–6011
20. Utani, A., Nomizu, M., Timpl, R., Roller, P. P., and Yamada, Y. (1994) J. Biol. Chem. 269, 19167–19175
21. Nomizu, M., Otaka, A., Utani, A., Roller, P. P., and Yamada, Y. (1994) J. Biol. Chem. 269, 30386–30392
22. Hartwig, R., and Danishefsky, K. J. (1991) J. Biol. Chem. 266, 6578–6585
23. Huang, S., Mulvihill, E. R., Farrell, D. H., Chung, D. W., and Davie, E. W. (1993) J. Biol. Chem. 268, 8919–8926
24. Speth, C., and Oberbäumer, I. (1993) Exp. Cell Res. 204, 302–310
25. Gottschling, C., Huber, J., and Oberbäumer, I. (1993) Eur. J. Biochem. 216, 293–299
26. Baudouin, C., Miquel, C., Blanchet-Bardon, C., Gambini, C., and Ortonne, J. P. (1994) J. Clin. Invest. 93, 862–869
27. Copeland, C. S., Zimmer, K. P., Wagner, K. R., Healey, G. A., Melman, I., and Helenius, A. (1988) Cell 53, 197–209
28. Ohba, H., Harano, T., and Omura, T. (1981) J. Biochem. (Tokyo) 89, 901–907
29. Hanover, J. A., and Lennarz, W. J. (1981) Arch. Biochem. Biophys. 211, 1–19
30. Olden, K., Pratt, R. M., and Yamada, K. M. (1978) Cell 13, 461–473
31. Dullis, B. H., Klöppel, T. M., Grey, H. M., and Kubo, R. T. (1982) J. Biol. Chem. 257, 4369–4374
32. Conway, J. F., and Parry, D. A. D. (1991) Int. J. Biol. Macromol. 13, 14–16
33. Beck, K., Dixon, T. W., Engel, J., and Parry, D. A. D. (1993) J. Mol. Biol. 231, 311–323