BeWo choriocarcinoma cells produce laminin 10

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BeWo is a choriocarcinoma cell line that generates an extracellular matrix (ECM) rich in laminin and is a useful model for human trophoblast. Immunofluorescence with monoclonal antibodies demonstrates that BeWo ECM contains laminin subunits $\beta 1$ and $\gamma 1$. Immunoprecipitation from conditioned medium shows that the cells secrete two distinct laminin trimers both containing $\beta 1$ and $\gamma 1$ but with α subunits of approx. 400 and 450 kDa. The culture medium also contains a species thought to be $\beta 1\gamma 1$ dimer. Immunoprecipitation with monoclonal antibody 4C7, previously thought to recognize the $\alpha 1$ subunit, isolates complexes containing only the smaller α subunit. A second complex containing the larger α subunit along with $\beta 1$, $\gamma 1$ and a 150 kDa polypeptide is precipitated from 4C7-depleted medium with an anti-(laminin 1) polyclonal antibody. Peptide sequencing demonstrates that the 4C7-reactive species is $\alpha 5$, which is present as two similarly sized polypeptides. mRNA species encoding laminin subunits $\alpha 1$, $\alpha 5$, $\beta 1$, $\beta 2$ and $\gamma 1$ are all present in the cells. These results demonstrate the secretion of a novel laminin isoform, laminin 10, the subunit composition of which is $\alpha 5\beta 1\gamma 1$. Laminin 1 is also produced. No evidence for the secretion of $\beta 2$-containing laminin isoforms could be derived despite the presence of $\beta 2$ mRNA. Analysis with reverse transcriptase-mediated PCR also showed the presence of laminin $\alpha 5$ in first-trimester placenta and decidua.

INTRODUCTION

The laminins are a family of heterotrimeric extracellular matrix (ECM) glycoproteins associated with basement membranes [1–4]. Each member of the family comprises a heterotrimer of α, β and γ subunits linked by disulphide bonds to yield a cruciform molecule. So far five α subunits, three β subunits and two γ subunits have been identified; different combinations result in as many as 11 different isoforms that have been shown or predicted to exist [4–6]. Laminins interact with integrins at the cell surface to promote adhesion, migration and differentiation in diverse cell types [7,8].

Laminins have been shown to be localized at the implantation site [4,9,10]. Up-regulation of laminins 2 and 4 ($\alpha 2\beta 1\gamma 1$ and $\alpha 2\beta 2\gamma 1$) occurs during the decidualization of endometrial stroma in pregnancy [9]; in addition, several laminin subunits have been localized to the trophoblastic and vascular basement membranes of placental villi [4,11]. When cells produce more than one α, β or γ subunit, however, localization studies alone cannot determine subunit association [12].

In previous work we have demonstrated that BeWo choriocarcinoma cells produce a basement membrane-like ECM that contains laminin but not fibronectin [13,14]. Immunochemical studies indicated that the cells produce abundant quantities of the $\beta 1$ and $\gamma 1$ subunits but did not identify the corresponding α subunit. Subunits $\alpha 1$ and $\alpha 2$ have both been shown to associate with $\beta 1$ and $\gamma 1$, producing laminins 1 ($\alpha 1\beta 1\gamma 1$) and 2 ($\alpha 2\beta 1\gamma 1$) respectively [3]. The more recently discovered $\alpha 4$ and $\alpha 5$ subunits also associate with $\beta 1$ and $\gamma 1$ [15,16]. In the present study we characterize the repertoire of laminin mRNA and polypeptide species present in BeWo cells, and isolate and characterize secreted laminin heterotrimers from conditioned medium. We demonstrate the presence of two mature species, one of which is laminin 10 ($\alpha 5\beta 1\gamma 1$) and the other laminin 1 ($\alpha 1\beta 1\gamma 1$). We show that a widely used monoclonal antibody (mAb), 4C7, previously thought to recognize $\alpha 1$, in fact recognizes $\alpha 5$. The results also indicate that these cells contain $\beta 2$ mRNA but do not produce significant amounts of the corresponding translation product.

MATERIALS AND METHODS

Antibodies

mAbs 4C7, 3E5, 2E8 and 5H2 against laminin subunits $\alpha 5$, $\beta 1$, $\gamma 1$ and $\alpha 2$ (light) respectively were a gift from Dr. E. Engvall. mAbs C4 and D18 against laminin subunits $\beta 2$ and $\gamma 1$ were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, U.S.A.). mAb BM165 against laminin subunit $\alpha 3$ was supplied by Dr. P. Rousselle. Polyclonal antiserum YY4 against laminin subunit $\alpha 1$ was provided by Dr H. Kleinman. Polyclonal antiserum P3 against laminin subunit $\alpha 4$ was provided by Dr. A. Richards, polyclonal antiserum KB2 against laminin subunit $\gamma 2$ by Dr. Y. Yamada, and polyclonal antiserum R9 against laminin 1 (EHS) by Professor D. Garrod. Further details of the antibodies are given in [4,9].

Cell culture

The choriocarcinoma cell line BeWo was cultured in Dulbecco's modified Eagle's medium/Ham's F12 (1:1, v/v) containing 10% fetal calf serum and antibiotics, as described previously [13]. Cells were grown on coverslips for 48 h to form confluent monolayers, fixed in ice-cold methanol, washed in PBS and then used for immunostaining.

Immunofluorescence

Indirect immunofluorescence was performed on fixed BeWo cell monolayers. The cells were incubated in primary antibody dilutions for 1 h at room temperature, washed in PBS, incubated

Abbreviations used: ECM, extracellular matrix; mAb, monoclonal antibody; MALDI-MS, matrix-assisted laser desorption ionization MS; RT–PCR, reverse transcriptase-mediated PCR; IP, immunoprecipitation.

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in fluorescein isothiocyanate-conjugated secondary antibody (DAKO, High Wycombe, Bucks., U.K.) for 1 h, washed in PBS and mounted in non-fade aqueous mountant (Immumount; Life Sciences International, Basingstoke, Hants., U.K.).

Immunoprecipitation (IP)

Metabolic labelling and IPs were performed as previously described [4]. In brief, 75 cm^2 flasks were incubated in methionine-free minimal essential medium (Gibco-BRL) (20 ml/flask) for 2 h then overnight in methionine-free medium containing ^{35}S-labelled methionine/cysteine (Trans-label; ICN Pharmaceuticals, Thame, Oxon., U.K.) (250 μCi, 10 ml per flask). The cell layer was washed three times with PBS, scraped into extraction buffer [PBS containing 0.5 % deoxycholate, 5 mg/ml BSA and the protease inhibitors aminoethylbenzylsulphonyl fluoride (ICN) (1 mM), *N*-ethylmaleimide (Sigma) (2 mM) and leupeptin (10 μg/ml), 1 ml per flask] and left to extract on ice for 1 h. The cell extracts were centrifuged to pellet remaining cell debris, and the supernatants were pooled and used for IP. Medium was dialysed into water, freeze-dried and resuspended in PBS containing protease inhibitors. The resulting cell extracts and medium concentrates were precleared with 50 μl of Protein A-agarose beads (Sigma) for 2 h at room temperature and the supernatants were recovered by centrifugation. The supernatants were then divided into aliquots and primary antibody was added and incubated overnight on a rolling mixer at 4 °C. Protein G-agarose beads that had previously been blocked with non-radiolabelled cell extract or conditioned medium were added (20 μl per tube) and incubated at room temperature on a rolling mixer for 2 h. The beads were recovered and washed five times in 0.3 % (v/v) Triton X-100/0.05 % sodium deoxycholate in PBS and then twice in PBS, then heated in reducing or non-reducing sample buffer (30 μl) and supernatants separated by SDS/PAGE on either 5 % (w/v) gels for reducing conditions or 2–8 % (w/v) gels for non-reducing conditions with prestained calibration standards (Bio-Rad, Hemel Hempstead, Herts., U.K.). The gel was dried and autoradiographed. The immunoprecipitates illustrated in each Figure originated from a single experiment and the results have been found to be fully reproducible.

Immunoaffinity chromatography

An anti-laminin immunoaffinity column was generated by conjugating mAb 4C7 to a HiTrap® *N*-hydroxysuccinimide-activated column (Pharmacia Biotech, St. Albans, Herts., U.K.). Conditioned BeWo medium was passed through the column, which was then eluted with 0.1 M glycine/HCl, pH 2.5. Fractions were collected, neutralized with 1 M Tris/HCl, pH 8.5, and assayed for laminin activity by ELISA with the anti-(laminin 1) polyclonal antibody R9 and horseradish peroxidase-conjugated donkey anti-(rabbit IgG) (Jackson Laboratories; supplied by Stratech Scientific, Luton, Beds., U.K.) as a secondary antibody for detection. Immunoreactive fractions were pooled and concentrated for protein analysis.

SDS/PAGE and Western blotting

SDS/PAGE and Western blotting were performed as described previously [9]. BeWo-conditioned serum-free medium was harvested after incubation overnight, dialysed into distilled water and concentrated by freeze-drying. The resulting protein concentrate was resuspended in PBS containing protease inhibitors and quantified with the Bio-Rad protein assay. A total protein loading of 10 μg per well was loaded on a 3–8 % (w/v) gradient gel, separated and then blotted as described previously [9]; the blot was probed with anti-(laminin 1) polyclonal antibody R9. The washing buffer was 0.05 % (v/v) Tween 20 in Tris-buffered saline, the secondary antibody used was alkaline phosphatase-conjugated anti-(rabbit IgG) (Dako, High Wycombe, Bucks., U.K.), and the substrate was 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt/Nitro Blue Tetrazolium chloride (BCIP/NBT; Bio-Rad).

Protein digestion and analysis by MS

Laminins purified by immunoaffinity chromatography were analysed by SDS/PAGE at a loading of 20 μg of total protein per lane and stained with a silver stain. Bands of interest were excised from the gel and subjected to digestion with trypsin in accordance with a published method [17]. Digests were analysed by matrix-assisted laser desorption ionization (MALDI)-MS with a VG Tofspec E mass spectrometer. The resulting peptide masses were mapped with the Internet peptide database search site MS-Fit (URL http://prospector.ucsf.edu/htmlucsf/msfit.htm) against the database Genpept.r102, with relative molecular mass as a limiting parameter.

Reverse transcriptase-mediated PCR (RT–PCR)

Total RNA was extracted from first-trimester placenta and decidua and from BeWo cells by the acid guanidinium thiocyanate/phenol/chloroform method as described previously [18]. First-strand reverse transcription of mRNA was performed as described [9]. PCR amplifications were performed with the following human primers essentially as described previously [9]. The annealing temperature and $MgCl_2$ concentration were optimized for each primer pair as stated below. PCR products were analysed by electrophoresis on 1.2 % (w/v) agarose gels containing ethidium bromide (0.5 % μg/ml). All product sizes were as predicted. Laminin α1 [19]: LAMA1 U1, 5′-GAT GTG GAG GGT TTG TTC TAC-3′; LAMA1 L1, 5′-CAC ATT GGT TAC CAC TAA TGT CAC-3′; product 476 bp; annealing temperature 58 °C; 1.5 mM $MgCl_2$. Laminin α2 [20]: LAMM U2, 5′-AGC TGA GAA ATG GAT TGC CCT ACT-3′; LAMM L2, 5′-GGC CAA CAT GCT TGA GGT CAT CTG-3′; product 783 bp; annealing temperature 55 °C; 2.5 mM $MgCl_2$. Laminin α4 [21]: LAMA4 U1, 5′-TGG TCT CCG AGT CCT AGA AGA AAG-3′; LAMA4 L1, 5′-CTG TTT CCA TGG GGC CTT CAA AGC-3′; product 233 bp; annealing temperature 58 °C; 1.5 mM $MgCl_2$. Laminin α5 [22]: LAMA5 U4, 5′-CCC CCA GCC CCA CAC CCT CTT-3′; LAMA5 L3, 5′-ACA TTC CCG CTT TTC ATC ACC-3′; product 465 bp; annealing temperature 63 °C; 1.5 mM $MgCl_2$. Laminin β1 [23]: LAMB1 U1, 5′-CAC ACA ACG CCT GGA AGA AAG-3′; LAMB1 L1, 5′-GCA CTA TGC TGA AGA ATA ACC-3′; product 385 bp; annealing temperature 58 °C; 1.5 mM $MgCl_2$. Laminin β2 [24]: LAMS U2, 5′-GCT CGG CAG TTG GAT GCT CTC-3′; LAMS L2, 5′-GCC CGC TCA TTT TCC TCA TAG-3′; product 315 bp; annealing temperature 57 °C; 1.5 mM $MgCl_2$. Laminin γ1 [25]: LAMB2 U2, 5′-CAA AGC CAA AGA TGA AAT GAA-3′; LAMB2 L2, 5′-AGA GGA GTG GGG GTC TGA AAA-3′; product 305 bp; annealing temperature 51.5 °C; 2.5 mM $MgCl_2$.

Each primer pair was used to amplify cDNA from BeWo cells, first-trimester placenta and first-trimester decidua. Negative controls were performed by omitting either cDNA from the PCR reaction or reverse transcriptase from the reverse transcription reaction. Both controls failed to amplify a product.

RESULTS

Immunofluorescence of laminin subunits in BeWo cells

Cultured cell monolayers were stained with a panel of subunit-specific anti-laminin antibodies (Figure 1 and Table 1). Antibody against subunit $\alpha 1$ (Figure 1a) stained the monolayer, giving a distribution that was consistent with extracellular localization. Staining with antibody 4C7 is shown in Figure 1(b), where the monolayer displayed weak reactivity and seemed to be at least partly intracellular. Antibodies against subunits $\beta 1$ (Figure 1c) and $\gamma 1$ (Figure 1d) detected strong punctate and short linear deposits, consistent with localization principally in the ECM. Antibodies against $\beta 2$ gave only very weak cellular reactivity (results not shown). These results suggest the presence of laminin(s) of the form $\alpha x \beta 1 \gamma 1$. Staining for $\alpha 1$ showed a distribution in the ECM that was consistent with that of $\beta 1$ and $\gamma 1$, although less intense, but the 4C7 epitope did not co-distribute with subunit $\alpha 1$. Antibodies against subunits $\alpha 2$, $\alpha 3$ and $\alpha 4$ produced no significant staining (Table 1).

Table 1 Expression of laminin subunits by BeWo monolayers

Symbols: + + + E, very strong extracellular staining pattern; + + E, strong extracellular staining; +, weak staining; −, negative

Laminin subunit	Antibody	Staining pattern
$\alpha 1$	YY4	+ + E
$\alpha 2$	5H2	−
$\alpha 3$	BM165	−
$\alpha 4$	P3	−
$\alpha 5$	4C7	+
$\beta 1$	4E10/3E5	+ + + E
$\beta 2$	C4	+
$\gamma 1$	D18/2E8	+ + + E

IP of laminin isoforms from BeWo cells

Cells were metabolically labelled with [^{35}S]methionine, then detergent extracts were prepared. IP was performed with antibodies R9 against laminin 1 ($\alpha 1 \beta 1 \gamma 1$), 2E8 against the $\gamma 1$ subunit, and 4C7, previously thought to recognize $\alpha 1$ but now thought to recognize $\alpha 5$. Figure 2 shows that the intracellular pool of immunoreactive material precipitated by antibody 2E8 (lane b) or R9 (lane c) is enriched in a doublet at approx. 200 kDa, consistent with subunits $\beta 1$ and $\gamma 1$. These subunits could have been co-precipitated by the $\gamma 1$ antibody from intracellular heterotrimer or from the dimeric disulphide-linked intermediate $\beta 1 \gamma 1$. The latter explanation seems likely because there is relatively little signal in the α chain region of the gel (300–500 kDa). In agreement with this, antibody 4C7 precipitated a weak α chain signal at approx. 400 kDa together with small amounts of $\beta 1$ and $\gamma 1$ (Figure 2, lane a). The results suggest the presence of a considerable molar excess of $\beta 1$ and $\gamma 1$ chains relative to α chain in the intracellular pool.

IP analysis was next performed with conditioned medium from [^{35}S]methionine-labelled BeWo cells to analyse the secreted laminins present (Figure 3A). As expected, antibodies against the $\beta 1$ and $\gamma 1$ subunits recognized identical complexes containing closely migrating bands at approx. 200 kDa (Figure 3A, lanes e and g). Their apparent molecular masses are slightly greater than in the respective intracellular pools, probably reflecting increased glycosylation. These lanes each contain two distinct polypeptide species migrating in the α chain region of approx. 400 kDa. Polyclonal antibody against laminin 1 produced essentially identical results (results not shown). Antibody 4C7 precipitated polypeptide species that migrated at the β/γ position along with a single α chain of approx. 400 kDa (Figure 3A, lane a). Antibodies against subunits $\alpha 2$, $\alpha 3$, $\alpha 4$, $\beta 2$ and $\gamma 2$, shown in Figure 3(A), lanes b, c, d, f, and h respectively, precipitated no

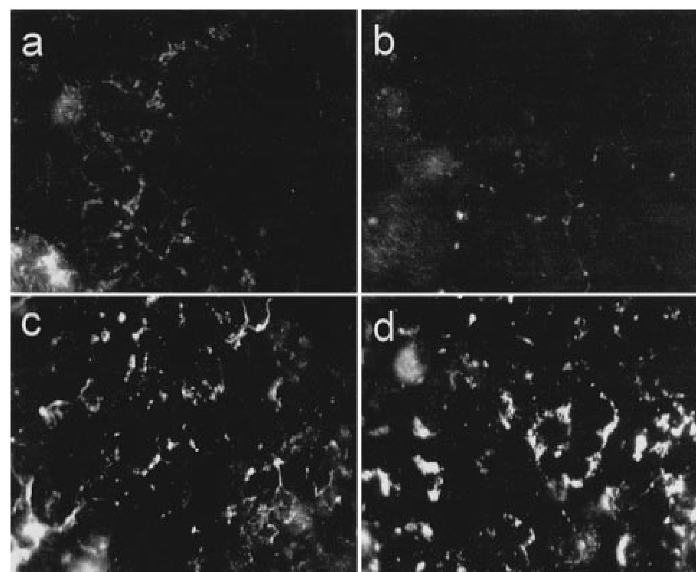


Figure 1 Immunofluorescence of laminin subunits in BeWo cell monolayers

(**a**) $\alpha 1$ (polyclonal antibody YY4); (**b**) $\alpha 5$ (mAb 4C7); (**c**) $\beta 1$ (mAb 3E5); (**d**) $\gamma 1$ (mAb 2E8). All four are present, although the two α subunits are weaker than $\beta 1$ and $\gamma 1$.

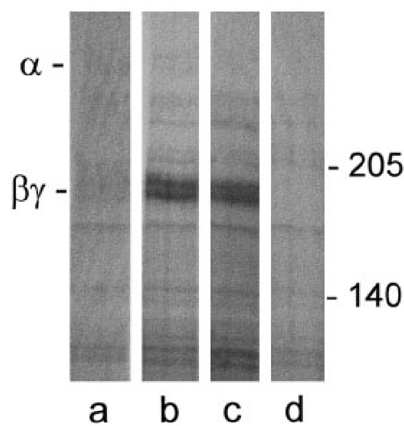


Figure 2 Reducing SDS/PAGE of immunopreciptates from [^{35}S]methionine-labelled BeWo cell extracts

The antibodies used recognized (lane a) α5 (mAb 4C7); (lane b) γ1 (mAb 2E8) and (lane c) $\alpha1\beta1\gamma1$ polyclonal antibody R9. Lane d is a negative control. Lanes b and c show prominent bands at approx. 190 kDa corresponding to the β1 and γ1 polypeptides, but only very restricted amounts of α chain. Lane a shows only faint traces of α, β and γ chains. The positions of marker proteins are indicated (in kDa) at the right.

detectable laminin complex. Lane i in Figure 3(A) shows a negative control.

Non-reducing SDS/PAGE of IPs made from methionine-labelled culture medium with polyclonal anti-(laminin 1) (Figure 3B) indicated the presence in the medium of two large complexes of similar molecular mass (approx. 900 kDa). In addition, bands can be seen at approx. 400 and 150 kDa. A Western blot of conditioned medium with anti-(laminin 1) (Figure 3C) showed the presence of immunoreactive high-molecular mass complexes. In addition the bands at approx. 400 kDa resolved into a doublet. This might represent homodimers or heterodimers of β1 and γ1. There is also some evidence of immunoreactive bands at approx. 200 kDa, perhaps representing monomeric β1 or γ1. The IP and blotting data shown in Figure 3 suggest the presence of two immunologically distinct laminin heterotrimers in medium, both of which contain β1 and γ1. Only one of them contains the α subunit recognized by antibody 4C7.

Immunodepletion experiments with mAb 4C7 were used to confirm the presence of two isoforms (Figure 4). Methionine-labelled conditioned medium was subjected to three sequential IP steps with mAb 4C7 (Figure 4, lanes a–c), then the supernatant was reprecipitated with anti-(laminin 1) (lane d). The control lane, which contained a one-step IP with anti-(laminin 1), showed the presence of two α subunits, a heavy band containing β1 and γ1, and a further polypeptide species at approx. 150 kDa that might be entactin (Figure 4, lane e). The first-step IP with mAb 4C7 (Figure 4, lane a) depleted all immunoreactive material; SDS/PAGE analysis revealed the presence of one major α chain polypeptide. In addition there were trace amounts of a product that might represent a smaller or processed α chain species. Subunits β1 and γ1 were present at the expected molecular mass of approx. 200 kDa. mAb 4C7 precipitated the faster-migrating of the two α chains present. The other α chain appeared slightly more abundant; this observation is consistent with the immunofluorescence findings (Figure 1). Figure 4 (lane f) represents a negative control.

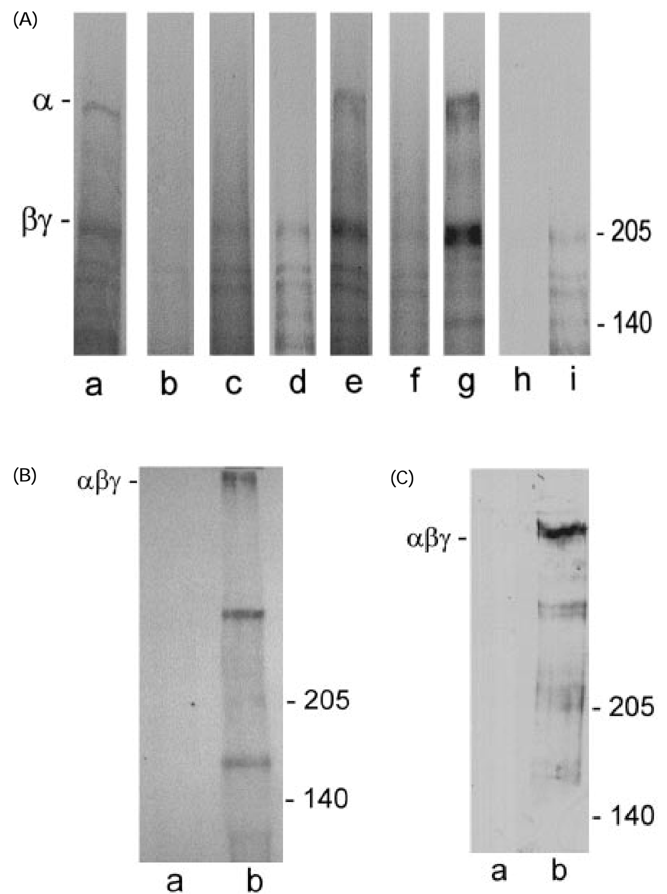


Figure 3 Immunochemical analysis of laminins in BeWo cell medium

(A) Reducing SDS/PAGE of immunoprecipitates from BeWo conditioned medium after labelling with [^{35}S]methionine. Lane a, α5 (mAb4C7); lane b, α2 (mAb 5H2); lane c, α3 (mAb BM165); lane d, α4 polyclonal antibody P3; lane e, β1 (mAb 4E10); lane f, β2 (mAb C4); lane g, γ1 (mAb 2E8); lane h, γ2 (KB2 polyclonal); lane i, control. Trimeric species containing α, β and γ subunits are precipitated with anti-α5 (lane a), anti-β1 (lane e) and anti-γ1 (lane g). Two α-chain polypeptides are present at approx. 400 kDa in the anti-β1 and anti-γ1 precipitates. BeWo cells do not secrete subunits α2, α3, α4, β2 or γ2. (B) Non-reducing SDS/PAGE of BeWo cell culture medium after labelling with [^{35}S]methionine, and IP. Immunoprecipitates were made with control serum (lane a) or polyclonal antibody R9 against $\alpha1\beta1\gamma1$ (lane b). The prominent doublet at high molecular mass represents intact secreted heterotrimers. The medium also contains significant amounts of a product (approx. 400 kDa) whose mobility corresponds to $\beta1\gamma1$ dimer and a product of approx. 150 kDa, possibly entactin. (C) Non-reducing Western blot of BeWo cell culture medium. Control serum (lane a) or polyclonal antibody R9 against $\alpha1\beta1\gamma1$ (lane b). The prominent band at high molecular mass is intact secreted heterotrimer. The doublet at approx. 400 kDa might correspond to dimeric $\beta1\gamma1$ species. In all panels the positions of marker proteins are indicated (in kDa) at the right.

Immunoisolation of laminin 10 from BeWo cell medium

Conditioned medium was passed through an affinity column containing mAb 4C7 and eluted at low pH. The resulting fractions were analysed by ELISA with polyclonal antibody against laminin 1 (Figure 5, top panel) and positive fractions were pooled for SDS/PAGE/silver analysis under both non-reducing (Figure 5, middle panel) and reducing (Figure 5, bottom panel) conditions. The immunopurified laminin under non-reducing conditions (Figure 5, middle panel, lane c) migrated slightly faster than laminin 1 (lane b). On reduction, immunopurified laminin (Figure 5, bottom panel, lane a) contained two α-chain bands (1 and 2) as well as bands 3 and 4 corresponding to β1 and γ1 chains, and a band at approx. 150 kDa (band 5).

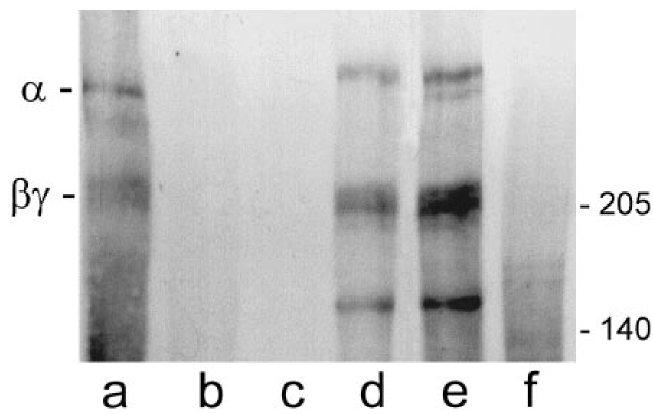


Figure 4 Immunodepletion of laminins containing the subunit recognized by mAb 4C7 from conditioned BeWo medium

The medium was sequentially depleted with mAb 4C7 (lanes a, b and c) and then precipitated with anti-(laminin 1) (lane d). Lane a shows a trimer containing a 400 kDa α subunit co-precipitated with β1 and γ1. Lane d shows a laminin species that contains a 450 kDa α subunit co-precipitated with β1 and γ1, and a 150 kDa species. Precipitation of non-depleted conditioned medium with anti-(laminin 1) shows laminin complexes containing the two different α subunits (lane e). Lane f is a control. The positions of marker proteins are indicated (in kDa) at the right.

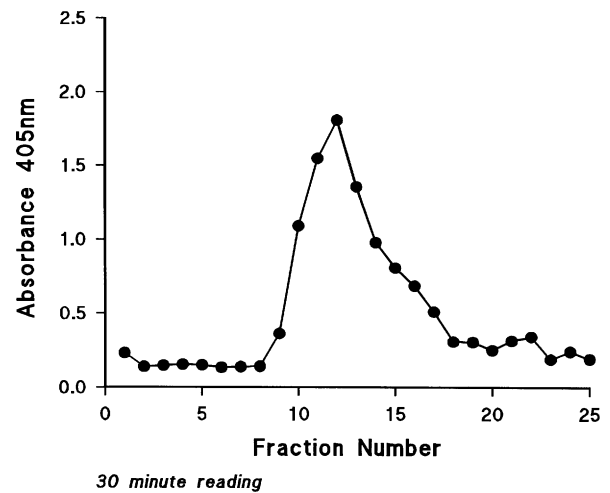
Isolated bands were treated with trypsin and subjected to MALDI-MS analysis (Table 2). This showed the identity of both α-chain bands with laminin α5 subunit, and also confirmed the identities of β1 and γ1. The results demonstrate definitively that one of the two laminin isoforms produced by BeWo cells is laminin 10 ($\alpha5\beta1\gamma1$) and indicates the presence of two α5 polypeptides. It also shows that mAb 4C7, previously reported as an anti-α1 reagent, binds human α5.

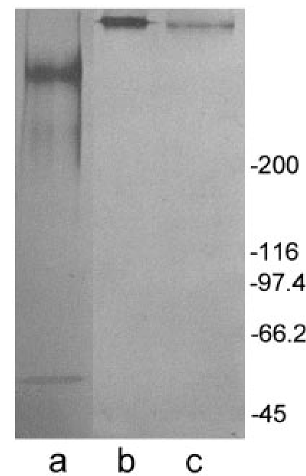
Laminin α5 subunit mRNA in placenta, decidua and BeWo cells

RT–PCR showed that BeWo cells expressed mRNA for laminin subunits α1, α5, β1, β2 and γ1 but not α2 and α4 (Figure 6, upper panel). The expression of mRNA for the laminin α5 subunit by first-trimester placenta and first-trimester decidua was demonstrated by RT–PCR (Figure 6, lower panel). The primers chosen spanned an intron–exon boundary and amplified a product of approx. 1 kb from human genomic DNA (results not shown). The absence of this product from Figure 6 indicates a lack of contaminating DNA in the RNA preparations.

DISCUSSION

The results demonstrate definitively that BeWo cells produce two laminin heterotrimers, one of subunit composition $\alpha5\beta1\gamma1$. This conclusion is supported by co-immunoprecipitation and co-purification of the three disulphide-linked subunits, and their identification by reducing and non-reducing SDS/PAGE of intact polypeptides and MALDI-MS of tryptic peptides. Although the full human α5 subunit cDNA sequence has not been completed, conserved sequences from the published G domain that give a precise match were identified [22]. Overall the human peptides give an 86 % match to the mouse α5 homologue [26]. By the classification system suggested by Burgeson et al. [5] and extended by Timpl [3] and Miner et al. [15], this variant is laminin 10.





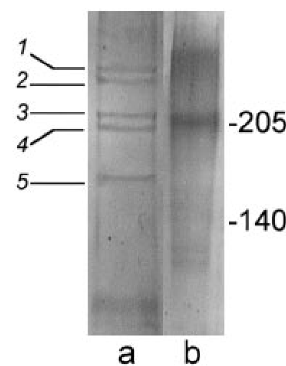


Figure 5 Immunoaffinity purification of laminin 10 ($\alpha5\beta1\gamma1$) on a column containing mAb 4C7

Top panel: fractions were eluted at low pH and monitored by ELISA with antibody against $\alpha1\beta1\gamma1$. Middle panel: SDS/PAGE with silver staining under non-reducing conditions showing fibronectin (lane a), laminin 1 (lane b) and immunopurified laminin 10 (lane c). The positions of marker proteins are indicated (in kDa) at the right. Bottom panel: SDS/PAGE with silver staining of immunopurified laminin 10 (lane a) and laminin 1 (lane b) under reducing conditions. The positions of marker proteins are indicated (in kDa) at the right.

Table 2 Identification of protein bands isolated by immunoaffinity chromatography with mAb 4C7 and analysed by MALDI-MS

Identities reported refer to the published mouse LAMA5 [26], human LAMB1 [23] and human LAMC1 [25] sequences. Abbreviation: AA, amino acids.

Band no.	Apparent molecular mass (kDa)	Masses matched	Matched peptide masses	Protein identification	AA covered
1	450	87% (7 out of 8)	2249	Laminin $\alpha5$ subunit	14% (515 out of 3610 AA)
			2284		
			2822		
			3359		
			3832		
			5349		
			5599		
2	400	86% (20 out of 23)	1472	Laminin $\alpha5$ subunit	31% (1137 out of 3610 AA)
			1645		
			1852		
			1987		
			2205		
			2226		
			2244		
			2278		
			2413		
			2654		
			2673		
			2775		
			2799		
			2807		
			2908		
			2926		
			3086		
			3332		
			3796		
			3806		
3	200	72% (13 out of 18)	1167	Laminin $\beta1$ subunit	24% (438 out of 1786 AA)
			1296		
			1999		
			2297		
			2672		
			2809		
			2815		
			3341		
			3628		
			3797		
			3806		
			5314		
			5559		
4	180	81% (9 out of 11)	1247	Laminin $\gamma1$ subunit	11% (190 out of 1609 AA)
			1482		
			1498		
			1518		
			1589		
			1814		
			2300		
			2345		
			3167		

Two α-chain polypeptides are present in affinity isolates of laminin 10. Both are derived from $\alpha5$, as shown by MALDI-MS of tryptic peptides. The nature of the difference between these two chain variants is not clear, but it might arise from proteolytic processing or glycosylation. Previous studies of the $\alpha5$ subunit also suggest the presence of chain variants [15,16]. Because overnight pulse-labelling–IP studies showed only trace amounts of the smaller $\alpha5$ product, whereas spent medium contained more, the second polypeptide might result from extracellular processing requiring longer periods in culture.

Laminin $\alpha5$, $\beta1$ and $\gamma1$ chains have been shown to be relatively widespread in tissues [15,16] and it is likely that laminin 10 will emerge as one of the most abundant isoforms in adult epithelial and endothelial cells. Human placenta contains all three chains, and immunolocalization studies with mAb 4C7 have detected $\alpha5$ in both the villous and vascular basement membranes [4,11]. Placental villous basement membrane has a particularly rich repertoire of laminin isoforms, because the $\alpha1$, $\alpha2$, $\alpha4$, $\alpha5$, $\beta1$, $\beta2$ and $\gamma1$ subunits are all present [4,10,11]. BeWo cells have a more restricted repertoire; although $\beta2$ mRNA can be detected by RT–PCR, we have not detected $\beta2$ in secreted laminin. This is consistent with results indicating the preferential utilization of $\beta1$ over $\beta2$ [27]. Further, we have not detected $\alpha2$ or $\alpha4$ mRNA. However, $\alpha1$ mRNA and polypeptide are present and so the

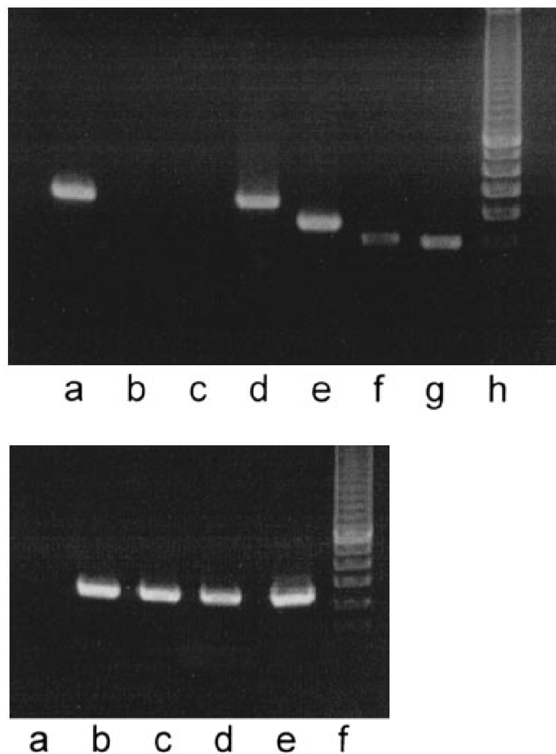


Figure 6 RT–PCR of laminin subunits in BeWo cells, placenta and decidua

Upper panel: RT–PCR amplification of laminin subunit mRNA species in BeWo cells. Lane a, laminin $\alpha1$ (476 bp); lane b, laminin $\alpha2$ (783 bp); lane c, laminin $\alpha4$ (233 bp); lane d, laminin $\alpha5$ (465 bp); lane e, laminin $\beta1$ (385 bp); lane f, laminin $\beta2$ (315 bp); lane g, laminin $\gamma1$ (305 bp); lane h, 100 bp ladder. Each set of primers was also used to amplify mRNA from first-trimester placenta and decidua as positive controls to verify the product size (results not shown). BeWo cells express mRNA for laminin subunits $\alpha1$, $\alpha5$, $\beta1$, $\beta2$ and $\gamma1$ but not $\alpha2$ and $\alpha4$. Lower panel: RT–PCR of first-trimester placenta, first-trimester decidua and BeWo mRNA encoding laminin $\alpha5$. Lane a, control (primers only); lane b, first-trimester placenta; lane c, first-trimester placenta; lane d, first-trimester decidua; lane e, BeWo cells; lane f, 100 bp ladder. The product is 465 bp.

results suggest that BeWo cells produce both laminin 10 and laminin 1 ($\alpha1\beta1\gamma1$). Another choriocarcinoma cell line, JAr, shows a similar phenotype [28,29]. Laminin 1 is associated mainly with developing epithelial tissues, including placenta, kidney and lung [15,30,31].

Previous immunoblotting data on BeWo [14] and other cells (see, for example, [32] and [33]) show that there exists an intracellular pool of $\beta1$ and $\gamma1$ subunits, the quantity of which exceeds total α chain, such that the latter limits the assembly and export of mature heterotrimer. Our pulse–chase experiments suggest that intracellular assembly of trimer precedes secretion, so that the labelled laminin present in culture medium contains all three subunits, as previously observed in JAr cells [30] and other cell culture models [32]. However, laminin identified by Western blotting in spent BeWo medium collected over 3–4 days clearly contains excess $\beta1$ and $\gamma1$ chain as a disulphide-linked dimer (largely) or a monomer. This result has been confirmed by immunoaffinity isolation with antibodies directed against the $\gamma1$ chain (results not shown). The current results do not clarify the source of this dimeric material; it could be shed from the cell layer during remodelling of ECM, or released from intracellular pools as a result of cell death. Its biological significance is uncertain.

mAb 4C7 was originally raised against pepsin fragments of human placental laminin [34] and it was shown by rotary shadowing to recognize the G domain of a large subunit, then thought to be the A chain, later renamed $\alpha1$ [5]. Subsequently the antibody has been very widely used in localization studies (see, for example, [9–11]) Doubt was cast on this assignment [15] on the basis of the observation of widespread cross-reactivity in adult tissues, compared with the relatively restricted distribution of the $\alpha1$ subunit. Unequivocal identification has been impeded by the inability of mAb 4C7 to react with laminin in immunoblots or to cross species, as well as the lack of a well-characterized cell culture model in which human laminin $\alpha5$ is produced. The present results unequivocally prove that mAb 4C7 recognizes the human laminin $\alpha5$ subunit. Similar findings were reported recently by Tiger et al. [29]. Published results must be reclassified in view of this assignment. BeWo cells can be used in the future as a source of human laminin 10 for structure–function studies.

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