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The NC1 domain of human collagen IV is necessary to initiate triple helix formation

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Abstract

Type IV collagen is a heterotrimeric molecule, which contains the N-terminal 7S, a central triple-helical domain, and the globular C-terminal NC1 domain. A zipper-like mechanism of triple helix formation, starting from the C-terminus, has been proposed for most collagens but for collagen type IV there has only been indirect evidence so far. In this study we expressed trimeric human collagen type IV to compare the effects of different structural variants on the formation of collagen IV molecules. Our data show that the NC1 but not 7S domain is essential for the chain association and initiation of triple helix formation. This strongly suggests an N-to-C terminal mechanism of triple helix formation. Additionally, we could show that the human α2(IV) chain can form chimeric α1.α1.α2(IV) heterotrimers with mouse subunits when expressed in PF-HR9 cells.

Keywords: Collagen IV; Triple helix

Type IV collagen represents a member of the large collagen family and is found in virtually all basement membranes [1]. Basement membranes (BMs) are thin sheet-like extracellular matrices separating tissues and cells, and contribute to multiple cellular functions. The tissue-specific functions of individual BMs are defined by characteristic supramolecular arrangements of members of protein families like laminins, nidogens, and collagen IV, as well as additional components [2,3].

Type IV collagen is a heterotrimeric molecule, which is in its most abundant form composed of two α1(IV) chains and one α2(IV) chain. Six different α-chains, α1(IV) to α6(IV), have been identified [4] which contain three domains, the N-terminal 7S domain, a large central triple-helical domain, and the globular C-terminal NC1 domain. The terminal domains are involved in tetrameric (7S) and dimeric interactions (NC1) and define a three-dimensional network of collagen type IV molecules [5]. The six subunit chains are able to form three heterotrimers, α1.α1.α2(IV), α3.α4.α5(IV), and α5.α5.α6(IV), which are involved in the formation of three distinct network systems [4] which are characteristic for specialized BMs in various tissues.

The characteristic element of all collagens is the triple helix formed by three subunit chains and its assembly is based on the repetition of the typical Gly-Xxx-Yyy repeats. In contrast to the fibrillar collagens, like I, II, and III, with uniform repetitive sequence patterns, the type IV collagen triple-helical domain contains more than 20 imperfections [5]. Type IV collagen is able to form a triple helix, despite these imperfections with a melting temperature comparable to collagen I, due to a high content of proline and hydroxyproline. These imperfections inhibit the formation of crystal-like fibrils, but local interactions of triple-helical domains contribute to the stability of the collagen IV network.

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A zipper-like mechanism of triple helix formation, starting from the C-terminus, has been proposed for collagen type I [6,7]. In contrast, an N-to-C terminal zipper-like mechanism was postulated for collagen XVII [8], suggesting that both directions are an option for the formation of collagens. Yet, there are only some data on type IV collagen, as melting experiments with isolated collagen IV showed a formation of the helix in a C-to-N direction [9]. In contrast to the in vitro situation, chaperone-like proteins in the endoplasmic reticulum, like prolyl *cis–trans* isomerase or hsp47, can vastly accelerate the triple helix formation in vivo [10,11].

In this paper we expressed type IV collagen constructs in a murine eukaryotic cell system and compared the effects of different structural variants on the formation of collagen IV molecules. We were able to express trimeric human collagen type IV. Our data show that the NC1 but not 7S domain is essential for the chain association and initiation of triple helix formation, which strongly suggests an N-to-C terminal mechanism of triple helix formation. Expression of human α2(IV) chain in the mouse cell line without the human α1(IV) chain still produced some triple-helical collagen indicating the formation of mouse–human chimera. Therefore, the human α1(IV) chain can be replaced by the equivalent murine α1(IV) chain.

Materials and methods

Generation of cDNA expression constructs. A full-length human α2(IV) cDNA clone (pCMVα2) was kindly provided by R. Nischt (University of Köln) and used for the construction of the expression vector pcDNAα2 in the pcDNA3 (Invitrogen, Karlsruhe, Germany) plasmid. Numbering correlates with entry NM_001846. Wild type construct *pcDNAα2H*: a RGSHis$_6$-tag (-AGA GGA AGC CAT CAC CAC CAT CAC CAC-) was introduced after position 5424, followed by a stop codon and a *Xba*I site by a PCR-based strategy (amplifying position 5204–5424) and a *Xba*I fragment (5226–5424) was recloned in pcDNAα2. N-terminal His-tagged *pcDNAα27sH*: a *Nhe*I site and RGSHis$_6$-tag added to 5′ of the fragment 397-659 by PCR and recloned into the plasmid pRC/Ac7mut fusing it with the signal peptide domain of BM40 similarly as described [12]. The modified 5′-region was isolated (*Hin*dIII, *Eco*RI) and used to replace the corresponding region in pcDNAα2. N-terminal His-tagged deletion *pcDNAα27sΔH*: amino acids 1–15 were deleted by amplifying position 397–476 and fusion to the human BM40 signal peptide as described before. Deletion of the 7S domain in *pcDNAα27sΔΔ*: pcDNAα2H was digested with *Nhe*I and *Eco*RV, blunted, and re-ligated introducing an in-frame deletion of the complete 7S-domain (position 397–832). Deletion of the NC1 domain in *pcDNAα2NCΔH*: position 4677–5424 were deleted by a PCR strategy introducing a C-terminal RGSHis$_6$-tag, a stop signal (TAG), and a *Xho*I site for further cloning. The PCR-product was subcloned and the *Apa*LI/*Xho*I fragment was inserted into pcDNAα2. Mutation of the integrin α1β1 binding site [13] in *pcDNAα27sRL*: For the mutation of Arg^{461}Leu in the putative integrin binding site two overlapping PCR fragments (1239–1659; 1644–1783) were generated introducing the mutation at position 1659–1661 (AGA to ACT), purified, and fused by PCR. After subcloning the *Bsu*36I–*Bsr*GI fragment replaced the complementary wild type sequence in pcDNAα27sH.

In all cases, primers were designed and annealing temperatures were calculated by using the program Oligo (Medprobe, Oslo, Norway). Deep vent polymerase was used according to the manufacturer's recommendations (New England Biolabs, Beverly, USA).

Cell culture and stable transfections. Mouse cell lines PF-HR9 and a derived cell line Do4 expressing the human α1(IV) chain kindly provided by K. Kühn (Martinsried) were cultured as described previously [14] and transfected by the calcium phosphate protocol [15]. Briefly, 5×10^5 cells were plated per 10 cm dish 20 h prior to transfection, and 10 μg plasmid DNA at pH 6.95 was used. After 24 h the medium was changed and selection by G418 (750 μg/ml) was started after 48 h. After 2 weeks G418-resistant colonies were isolated and expanded individually for further testing and evaluation.

Expression and purification of proteins. Stably transfected lines were cultured to 80–90% confluency, washed, and conditioned serum-free medium was collected after 2 days. Collected media containing RGSHis$_6$-tagged proteins were purified by affinity chromatography using Ni–NTA Sepharose (Qiagen, Hilden, Germany) as described. Columns (2 ml) were equilibrated with 2 M urea, 300 mM NaCl, and 50 mM sodium phosphate buffer, pH 7.4, media adjusted to 2 M urea, 300 mM NaCl, and 50 mM sodium phosphate were added, washed with equilibration buffer and with buffers containing 1, 5, and 10 mM imidazole. Bound proteins were eluted with equilibration buffer containing 50 mM imidazole. Fractions were concentrated for gel analysis by TCA-precipitation.

Immunoprecipitation. Serum-free culture media (1 ml) were concentrated by acetone precipitation, dissolved in 100 μl of 1% SDS in 10 mM Tris/HCl, pH 7.5, at 100 °C for 5 min and diluted with the same volume of 1% Triton X-100, 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM NEM, and 1 mM PMSF, and centrifuged for 15 min at 15000*g*. The supernatant was mixed with 1 μg of the respective antibody and incubated for 16 h at 4 °C. The precipitate was pelleted (15 min, 15000*g*), washed four times with dilution buffer, once with 150 mM NaCl, 0.05% SDS, and 10 mM Tris, pH 7.5, and then used for gel electrophoresis. Antibodies specific for the NC1 domains of human α1(IV) and α2(IV) were kindly provided by R. Timpl (Martinsried).

Immunoblotting. Conventional immunoblotting was performed by standard methods using semidry blotting to nitrocellulose membranes. Briefly, concentrated protein samples equivalent to 1 ml serum-free culture media were separated under non-reducing conditions or after reduction with 10 mM DTT for 5 min at 95 °C by SDS–PAGE on 5% gels. Prior to blotting, the gel was equilibrated for 30 min in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% methanol) containing 1% β-mercaptoethanol for better transfer of disulfide linked proteins. The gel was blotted at 0.8 mA/cm^2 on BA83 membranes (Schleicher and Schuell). Membranes were blocked by 5% milk powder in PBS, pH 7.4, and incubated with a monoclonal RGSHis$_6$-specific antibody (mouse, 1:10000, Qiagen, Germany) for 16 h at 4 °C, washed in PBS, pH 7.4, Tween 20 0.05%, and incubated for 2 h at 20 °C with an HRP-conjugated secondary antibody (rabbit anti mouse, 1:1000, rabbit polyclonal, Dako, Hamburg, Germany). Detection of HRP was done with ECL-Substrate according to standard protocols and visualized by exposure on X-ray film. In some cases, single protein bands were cut out after staining the gel in 50 mM $ZnCl_2$ 20 mM Tris, pH 6.5, divided equally, and one-half was reduced for 30 min at 37 °C in 25% glycerol, 200 mM Tris, pH 6.8, 5% SDS and 0.5 mg/ml bromophenol blue. Gel bands were placed on 5% Laemmli gels, the proteins were fractionated and used for immunoblotting as described above.

Results

Expression of α2(IV) chains in mouse PF-HR9 cells

The cDNA of human α2(IV) and its mutants was recombinantly expressed in the mouse cell line PF-HR9 [14] or in a cell line pretransfected with a construct expressing the human α1(IV) chain (Do4). Expressed

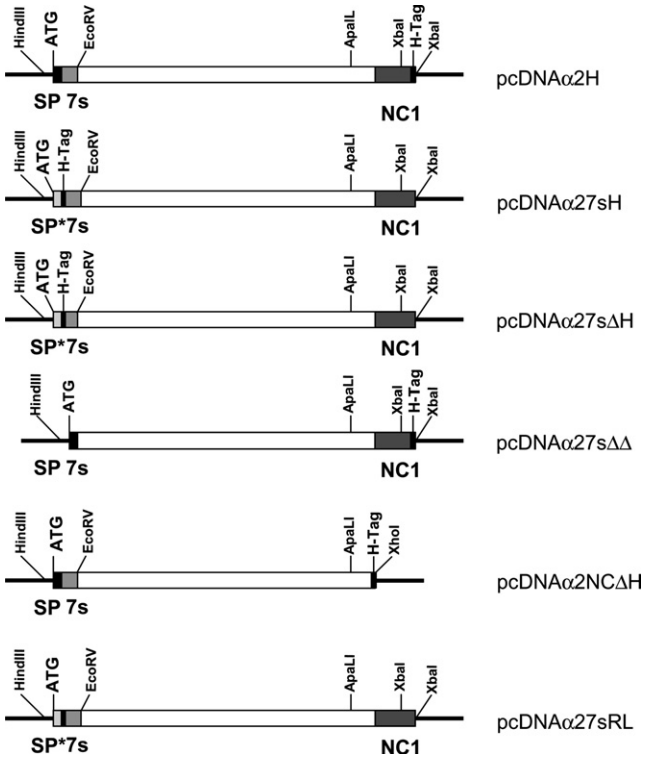

Fig. 1. Recombinant expression of variants of the human α2(IV) subunit chain. Schematic representation of expression constructs containing the wild type collagen IV signal peptide (SP; black) or the alternative BM40 signal peptide (SP*; gray). The 7S-domain (shaded) and the C-terminal NC1 domain are marked (shaded gray). Constructs were expressed in PF-HR9 or Do4 cells together with human wild type α1(IV).

mutants of the human α2(IV) chain and position of tag-sequences are shown in Fig. 1. Secreted collagen IV protein of transfected Do4 cells was detected by conventional immunoblot analysis using RGSHis$_6$-specific antibodies (Fig. 2). The wild type variants (α2H, α27sH) and a variant (α27sRL) comprising a mutation within the recognition site for the integrin α1β1 [13] displayed a similar 175 kDa product consistent with normal collagen IV due to the extensive hydroxylation and glycosylation of collagen IV [16]. The variants containing terminal deletions (α2NCΔH and α27sΔΔ) showed the expected reduction in molecular size, whereas the partial deletion of the 7S domain (α27sΔH) causes only a minimally reduced size. Two constructs (α2H and α27sΔΔ) were expressed additionally in PF-HR9 cells and showed similar recombinant proteins as compared to expression in Do4 cells.

Formation of heterotrimers after recombinant expression of human collagen IV

Triple-helical collagen IV was detected by immunoblotting of non-reduced samples, as triple-helical collagen IV is stabilized by disulfide bridges within the collagenous domains [13]. Therefore, bands of about 480 kDa indicate the presence of triple-helical collagen IV molecules, as seen with the wild type constructs

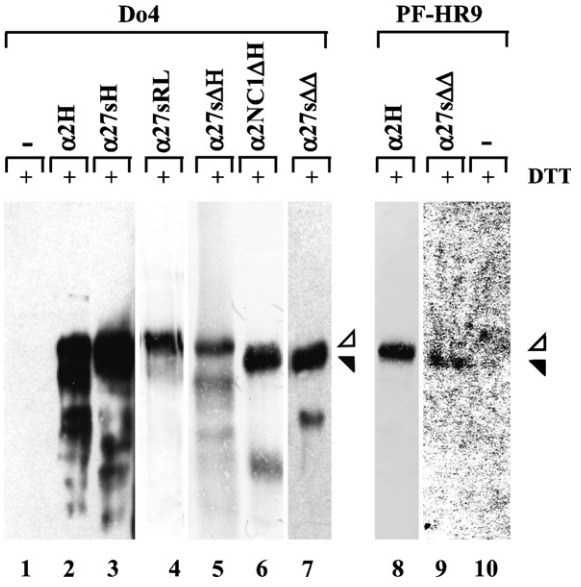

Fig. 2. Detection of secreted recombinant proteins. Serum-free media containing collagen IV variants expressed in the mouse cell lines Do4 or PF-HR9 were fractionated by SDS–PAGE under reducing conditions (10 mM DTT) and recombinant proteins were detected after immunoblotting with an anti-RGSHis$_6$-specific antibody. Untransfected control (−) and mutants are indicated. Positions of single chains (open arrowhead) and deletion variants (filled arrowhead) expressed in PF-HR9 and Do4 are indicated.

(α2H, α27sH), mutant α27sRL as well as the partial (α27sDH) or full deletion of the 7S domain (α27sΔΔ) (see Fig. 3). In contrast, no secretion of detectable amounts of the NC1 deletion variant (α2NCDH) was seen. Using non-reducing conditions, all constructs showed a strong signal indicating the expression and

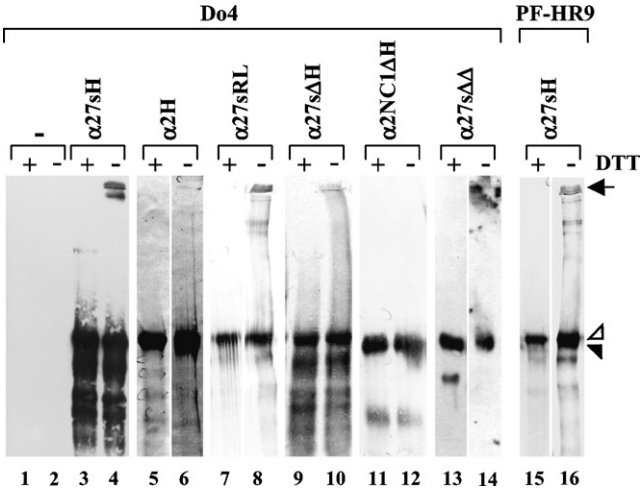

Fig. 3. Detection of trimeric recombinant collagen IV. Serum-free media were fractionated by SDS–PAGE under reducing (+) and non-reducing (−) conditions. Recombinant proteins were detected after immunoblotting with an anti-RGSHis$_6$-specific antibody. Untransfected control (−) and mutants are indicated. The position of trimeric collagen IV at 480 kDa (arrow) and the positions of the recombinant wild type chains (open arrowhead) and smaller deletions constructs (filled arrowhead) are indicated.

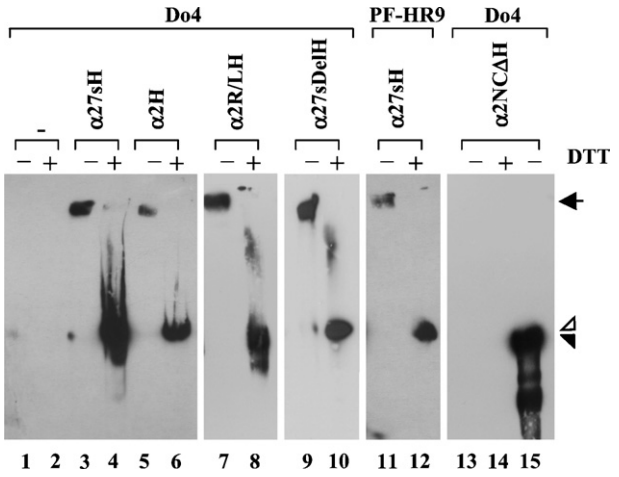

Fig. 4. Analysis of the 480 kDa band. Serum-free media were fractionated by SDS–PAGE under non-reducing conditions and the 480 kDa complex (arrow) was isolated and fractioned under reducing (+) and non-reducing (−) conditions. Recombinant proteins were detected after immunoblotting with an anti-RGSHis$_6$-specific antibody. Positions of the single chains (open arrowhead) and deletion variants (filled arrowhead) expressed in PF-HR9 and Do4 are indicated. In lane 15 serum-free α2NCΔH media were loaded as a positive control.

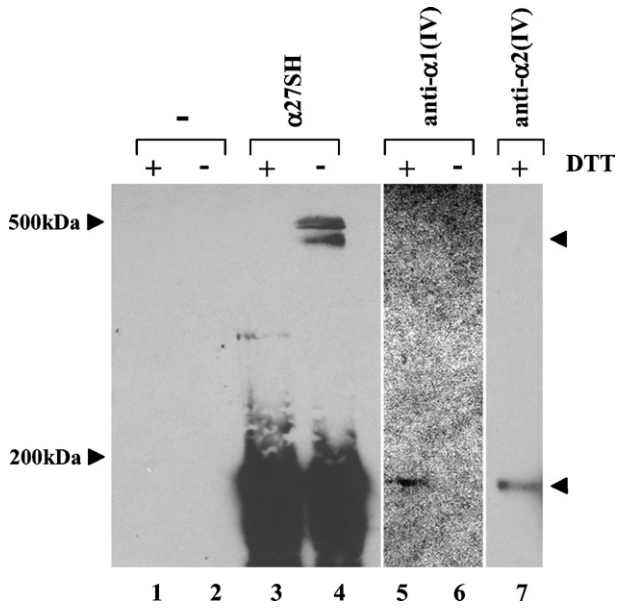

Fig. 5. Chain composition of recombinant His-tagged wild type collagen IV. Serum-free media of collagen IV wild type variant α27SH expressed in Do4 were immunoprecipitated with either anti-α1(IV) NC1 or anti-α2(IV) NC1 antibodies, fractionated by SDS–PAGE under reducing (+) and non-reducing (−) conditions. Recombinant proteins were detected after immunoblotting with an anti-RGSHis$_6$-specific antibody. In lanes 1–4 (see lanes 1–4, Fig. 3) serum-free media from untransfected control (−) and α27SH expressed in Do4 are shown as a control to indicate the position of the respective chains. Positions of the single chains (lower arrowhead) and 480 kDa complex (upper arrowhead) are indicated.

secretion of large amounts of single chains unable to get integrated into higher molecular bands.

To study specifically the α2(IV) variants integrated into the 480 kDa band, this product was isolated and analyzed after reduction and immunoblotting (see Fig. 4). In all cases, the 480 kDa bands contained the expressed α2(IV) variant. This analysis could not be performed for the variant lacking the complete 7S domain (α27sΔΔ), due to the weakness of the 480 kDa band. The increased intensity of immunoreactive bands after elution may be due to the more efficient transfer of single chains. In most cases constructs with C-terminal tag displayed weaker signals as compared to constructs with N-terminal tag most likely due to less efficient binding of the RGSHis$_6$-specific antibody to its epitope.

In contrast, the precipitation with the anti-α1(IV) NC1 antibody and consecutive detection with RGSHis$_6$-specific antibody was only able to detect collagen IV containing both α2(IV) and α1(IV) chains. Compared to the precipitation with the anti-α2(IV) NC1 antibody only a much weaker signal was observed, indicating that only part of the α2(IV) chain is integrated into triple-helical collagen IV. For the anti-α1(IV) NC1-specific precipitation a band was detected only under reducing conditions but not under non-reducing conditions as expected because of the poor transfer of large proteins (see Fig. 5).

Formation of human–mouse collagen IV chimera

The α27sH construct was additionally expressed in the mouse cell line PF-HR9. The secreted product was found to be included in the 480 kDa by immunoblotting (see Fig. 3) and could also be detected after reduction of this band. Therefore, the human α2(IV) chain associates with the mouse α1(IV) into a chimeric collagen IV molecule, as homotrimers of α2(IV) chains are not known to exist (see Fig. 4).

Discussion

A C-to-N association mechanism has been shown for fibrillar collagens [7] as well as FACIT collagens which share an interrupted triple helix with collagen IV [17]. Previous studies were indicative for a similar formation of collagen IV triple helices starting from the C-terminus, but either truncated collagen IV molecules were used under in vitro conditions [9] or the ability of NC1 domains for association was analyzed [4,18,19]. In contrast, an alternative N-to-C mechanism has been postulated for collagen type XVII [8]. In this paper we now present supporting evidences that collagen IV indeed also associates at the C-terminal NC1 domain and consecutively the triple helix is formed starting from its C-terminus.

The expression of recombinant α2(IV) chains in the cell line PF-HR9 enabled the efficient expression and processing of the variants, as this line produces large

amounts of extracellular matrix components and is therefore able to perform all steps of post-translational processing. Recombinant collagen IV chains appear to be extensively hydroxylated and glycosylated as they displayed a size of 185 and 175 kDa for the α1(IV) and the α2(IV), respectively, characteristic for correctly processed wild type collagen IV chains (data not shown). Only variants including the C-terminal NC1 domain are able to form trimers, but not the mutant lacking the NC1 domain. This further supports previous data that the NC1 domains are essential for the specific and stable interactions of subunit chains in the heterotrimer [9,18]. Trimer formation is indicated by the formation of the 480 kDa complex under non-reducing conditions, as inter-chain disulfide bridges are formed. Higher order complexes and networks cannot be analyzed in detail by these methods. In contrast, partial or full deletion of the N-terminal 7S-domain has no effect on the formation of trimeric collagen IV molecules. This further supports the model of a C-to-N zipper mechanism of the collagen IV molecules. Similar results were seen by using two different terminal tag-elements (RGSHis$_6$-tag and substance P-tag, data for substance P-tag not shown).

A point mutation located within the binding site for integrin α1β1 had no significant effect on the triple helix formation and showed that site-specific mutagenesis is possible for analyzing subdomains of the α2(IV) chain. Additionally, we could show that the human α2(IV) chain can form chimeric α1.α1.α2(IV) heterotrimers with mouse subunits when expressed in PF-HR9 cells. Coexpression of both human subunits results in human and chimeric recombinant molecules. Therefore, the species-specific sequence differences do not interfere with the collagen-specific aggregation.

In the present study, we investigated for the first time association of collagen IV chains in a cellular system. Our data show that the NC1 but not 7S domain is essential for chain association and initiation of triple helix formation. This proves that chain association is not possible without the NC1 domain, which strongly favors an N-to-C terminal mechanism of triple helix formation.

Acknowledgments

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