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Formation of Recombinant Triple-Helical $[\alpha1(IV)]_2\alpha2(IV)$ Collagen Molecules in CHO Cells

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Collagen IV molecules represent a major structural component of basement membranes providing a network of support for the supramolecular structure. Like other collagens, collagen IV forms a triple-helical molecule composed of three α chains. Six different α chains exist for collagen IV, although the most common isoform consists of two α1(IV) and one α2(IV) chain. To understand the molecular mechanism of triple-helical formation of collagen IV, we expressed recombinant α1(IV) and α2(IV) mouse collagen chains in Chinese hamster ovary (CHO) cells. An expression vector containing the full length cDNA for the mouse α1(IV) chain was stably transfected into CHO cells and a cell line, A222, which expressed recombinant α1(IV) chains was selected. These A222 cells were then infected with a retroviral expression vector containing the mouse α2(IV) chain and a cell line, A222-A2, stably expressing both recombinant α1(IV) and α2(IV) chains was obtained. Immunoprecipitation of A222 cell lysates revealed a high level of α1(IV) chain monomer, which was unable to form a homotrimer. Analysis of A222-A2 cell lysates revealed the presence of both monomeric α2(IV) and α1(IV) chains as well as a higher molecular weight collagen IV species. Second dimensional SDS-PAGE analysis demonstrated that the high molecular weight species was a heterotrimer consisting of two α1(IV) and one α2(IV) chain. This heterotrimer collagen IV species was pepsin-resistant indicating the formation of a stable triple-helical structure. Pulse-chase experiments showed that the monomer α1(IV) chain was secreted, but at a much slower rate than the heterotrimer. Together these results demonstrate that the α1(IV) chain is not capable of forming homotrimers and suggest that the coexpression with the α2(IV) chain is necessary to form a triple-helical structure.

Type IV collagen is the major collagenous component in basement membrane (1,2). It consists of both a globular NC1 domain at the C-terminus and a triple-helical collagenous domain with frequent interruptions (3). Each collagen IV molecule associates at both the NC1 domain and at the N-terminus to form a mesh-like network which provides the scaffolding of the basement membrane (4). Presently, six genetically distinct human α chains for collagen IV have been identified (5-14). Pairs of human genes for these chains, COL4A1/COL4A2, COL4A3/COL4A4, and COL4A5/COL4A, show a similar gene organization in which each pair of the genes is located closely in a head-to-head orientation on chromosomes 13, 2, and X, respectively (4). Type IV collagen has been linked to acquired and genetic diseases such as Goodpasture syndrome, Alport syndrome and diffuse leiomyomatosis and the molecular defects have been identified in COL4A3, COL4A4, COL4A5, and COL4A6, respectively (15). Little is known about what controls the chain composition of type IV collagen molecules, although the existence of six different chains could potentially produce a number of isoforms. The most ubiquitous form of collagen IV is a heterotrimer consisting of two α1(IV) and one α2(IV) chains (16,17). Collagen IV molecules consisting of two α3(IV) and one α4(IV) chain were identified in glomerular basement membrane (12). There are also reports for the existence of homotrimers of $[\alpha1(IV)]_3$ and $[\alpha3(IV)]_3$ chains (7,18).

In order to understand the mechanism of chain selection and assembly, we have constructed expression vectors containing full length cDNA for α1(IV) and α2(IV) chains and expressed the recombinant chains in CHO cells. We have found that α1(IV) and α2(IV) chains cannot form homotrimers and that trimer formation requires the presence of both chains.

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MATERIALS AND METHODS

Construction of plasmids. Full-length mouse cDNAs for α1(IV) and α2 (IV) were constructed from a series of overlapping clones isolated from a λgt11 cDNA library using RNA from differentiated F9 teratocarcinoma cells (19,20,21). The full length 6.4 kb α1(IV)-cDNA was cloned into the expression vector pMT-2 containing the adenovirus major late promoter and dehydrofolate reductase (DHFR) gene (22). The resultant construct, pMT2-α1(IV), was used for transfection to Chinese hamster ovary (CHO) cells (ATCC; CRL9087), which are deficient in DHFR. The full length 5.4 kb α2(IV)-cDNA was cloned into the retrovirus expression vector pLXSN derived from murine molony leukemia virus (23). The resultant construct, pLα2(IV)SN, was used for the transfection to ectropic packaging cell line φ2.

Cell culture. CHO cells deficient in DHFR were cultured in F12 media (Gibco LifeTechnologies) with 10% fetal bovine serum (FBS). The plasmid pMT2-α1(IV) DNA was transfected to these cells by the calcium precipitation method (24). After 2 weeks in α-MEM containing 10% dialyzed FBS, twenty four colonies were picked, and each clone was propagated in stepwise increasing concentrations of 0.01, 0.05, 0.1, 0.5, 2.0 μM methotrexate (MTX, Sigma). Two clones were resistant to 2.0 μM MTX and the expression levels of mRNA were checked by dot blot hybridization using an α1(IV) cDNA fragment. One of these cell clones, A222, showed a high level of expression and was used in future experiments.

Retrovirus packaging cell lines, φ2 and PA317, were cultured in DMEM containing 10 % FBS. The plasmid pLα2(IV)SN DNA was transfected to φ2 ectropic packaging cells. After two days, the medium which contained transiently produced viruses was collected, filtrated through a 0.45 μm membrane, and used for infection to PA317 amphotropic retrovirus packaging cells. Infected PA317 cells were cultured in DMEM supplemented 10 % FBS and 0.7 mg/ml of G418 (Gibco Life Technologies). After two weeks, twelve colonies were picked, and each clone was checked for the viral titer using NIH3T3 cells and for mRNA expression levels with an α2(IV) cDNA fragment. The high viral titer clone 16-4 was used to infect the A222 cells by incubation for 3h with media containing the virus and 8 μg/ml polybrane. After infection, these cells were cultured in MEM containing 10 % FBS, 0.5 μM MTX and 0.5 mg/ml G418. Cells resistant to both 0.5 μM MTX and 0.5 mg/ml G418 were cloned by limited dilution to produce a cell line called A222-A2.

Immunoprecipitations. Rabbit anti-mouse type IV collagen antibodies were prepared using type IV collagen purified from the EHS tumor (25,26,33), and were absorbed by Sepharose 4B conjugated with laminin purified from the EHS tumor. The synthesis and secretion of recombinant type IV collagen were monitored by labeling cells in methionine- and cysteine-free medium containing 75 μCi/ml of [^{35}S]-Translabel (ICN Pharmaceuticals Inc.) for 4h followed by immunoprecipitation of both the conditioned media and cell extracts using rabbit anti-mouse type IV collagen antibodies. Cells were extracted in Dulbecco's phosphate buffered saline containing 2% Triton X-100, 4 mM N-ethylmaleimid (NEM), 1 mM phenylmethansulfonylfluoride (PMSF), 5 mM EDTA, 20 μg/ml aprotinin, 5 mg/ml leupeptin and 1 mg/ml soybean trypsin inhibitor. The cell extracts and media were incubated with anti-mouse type IV collagen antibodies and protein A-Sepharose CL-4B (Sigma) for 1h at 4° C. Immunoprecipitates were separated by electrophoresis on 4.5% SDS-PAGE and analyzed by fluorography after treatment with Autofluor (National Diagnostics). Rat type I collagen was used as a molecular weight standard. For two-dimensional gel electrophoresis, immunoprecipitates from the medium were first run under non-reducing conditions. The gel slice was then incubated in the sample buffer containing 5 % 2β-mercaptoethanol for 1h and boiled for 2 min, and placed in vertical position on the top of a second dimension gel.

For the collagenase digestion, immunoprecipitates were suspended to 50 mM Tris HCl (pH 7.5) containing 50 mM $CaCl_2$ and 0.17 mg/ml bacterial collagenase (type III, Sigma). After incubation for 1h at 37° C, proteins were precipitated with 70 % ethanol and analyzed on 4.5 % SDS-PAGE. Immunoprecipitates from the culture media of S^{35}-labeled A222 and A222-A2 cells were digested with 0.1 mg/ml pepsin (Sigma) in 0.5 M acetic acid (pH 2.0) containing 1 mM PMSF, 4 mM NEM, 5 mM EDTA and 5 μg rat type I collagen as carrier protein. Digestion was stopped by adding 1 mM pepstatin and proteins were precipitated by 10 % trichroloacetic acid and analyzed on 4.5 % SDS-PAGE followed by fluorography.

For pulse chase experiments, cells were metabolically labeled with both [^{35}S]-methionine and [^{35}S]-cysteine for 4h. After washing the dishes, the cells were chased with excess unlabeled methionine and cysteine for up to 20 h. The media and cell extracts at each time point were analyzed by immunoprecipitation using anti-type IV collagen antibodies as mentioned above.

RESULTS

Full length cDNA for the mouse α1(IV) chain was cloned into the expression vector pMT-2 under the control of the adenovirus late promoter and transfected to DHFR-deficient CHO cells followed by selection with stepwise increasing concentrations of methotrexate (MTX) for gene amplification. One of cell lines, A222, was resistant to 2 μM MTX and showed a high level of expression of α1(IV) but no α2(IV) chains. A retrovirus expression vector for recombinant α2(IV) was then used to infect the A222 cells and a cell line, A222-A2 which expressed both α1(IV) and α2(IV) was established and used for further experiments.

Expression of the recombinant chains was analyzed on SDS-PAGE by anti-collagen IV antibody immunoprecipitation of metabolically labeled proteins from CHO cell lines, MT7, A222 and A222-A2. Analysis of A222 immunoprecipitates of the cell extracts showed the expected size of the α1(IV) chain monomer (185 kDa) (33) under reducing conditions (Fig. 1, G), while control MT7 CHO cells did not synthesize the α1(IV) or α2(IV) chains (Fig. 1, A, D). The same 185 kDa species was also found in the condition medium of A222 cells (Fig. 1, B). This species appeared to be monomer since no high molecular weight material was observed (Fig. 1, B, E). These results indicate that A222 cells produce monomer recombinant α1(IV) which is secreted into the media. MT7 cells infected with recombinant α2(IV) virus also produced monomer 170 kDa α2(IV) chains but no trimer was observed (data not shown). Analysis of the A222-A2 cells, however, revealed the presence of both recombinant α1(IV) and α2(IV) chain monomers with MW 170 kDa which was seen just below the position of monomer α1(IV) chain from the cell extract in nonreducing and reducing conditions (Fig. 1, C, F, H). In addition to these monomer chains, A222-A2 cells produced a band migrating under nonreducing conditions higher than the 300 kDa marker (Fig. 1, C, F). This protein species disappeared under reducing conditions and the 170 kDa band increased in intensity (Fig. 1, H). This protein species was found only in A222-A2 cells and not in the A222 cells. Collagenase treatment of the immunoprecipitates

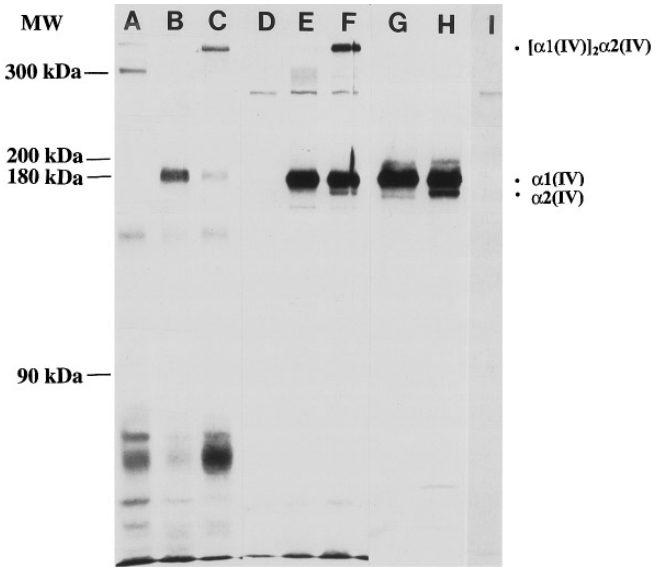

FIG. 1. Synthesis and secretion of recombinant α1(IV) and α2(IV) in CHO cells. Immunoprecipitates from the medium (lanes A-C) and cell extracts (lanes D-I) were analyzed on 4.5% SDS-PAGE under reducing (G, H) and non-reducing (A-F, I) conditions. Lanes A, D, MT-7 cells, lanes B, E, and G, A222 cells; lanes C, F, and H, A222-A2 cells. Lane I contains cell lysate from A222-A2 cell extracts digested with bacterial collagenase.

from the cell extract of A222-A2 cells completely digested the monomer α1(IV) and α2(IV) chains and the high molecular weight species indicating that they are indeed collagen IV chains (Fig. 1, I) and further confirmed that the high molecular weight protein is a complex of α1(IV) and α2(IV) chains. A faint protein band migrating around 250 kDa from the cell extract of all three cell lines (Fig. 1, D, E, F) appeared to be a non-collagenous protein since it was resistant to collagenase.

Two-dimensional gel electrophoresis under reducing conditions demonstrated that the collagen IV trimer species from the condition medium of A222-A2 cells consisted of the α1(IV) and α2(IV) chains in a ratio of 2 to 1 (Fig. 2). The recombinant collagen IV species from A222-A2 cells was subjected to pepsin digestion to examine if it had a triple-helical structure. This collagen IV molecule remained as a high molecular band under nonreducing conditions (Fig. 3, A2). In reducing conditions, it was separated into two components, migrating slightly faster than monomer α1(IV) and α2(IV) chains due to cleavage of the C-terminal globular domain (Fig. 3, B2). Pepsin treatment completely digested monomer α1(IV) chain from A222 cells (Fig. 3, A1, B1). Together these results suggest that the A222-A2 cells contain recombinant $[\alpha1(IV)]_2\alpha2(IV)$ molecules with identical characteristics to that of native molecules.

Pulse-chase experiments demonstrated that very little $[\alpha1(IV)]_2\alpha2(IV)$ trimer was secreted into the medium from A222-A2 cells after 1 h chase, but significant amounts were found in the media after a 2 h chase (Fig. 4). In contrast, monomers were accumulated in the cells, secreted at a much slower rate into the medium and detectable only after an 8 h chase (Fig. 4). The protein band observed between the trimer and monomer in the cell extract was not collagen IV since it was digested with collagenase (data not shown).

DISCUSSION

The structure and tissue distribution of various collagen IV molecules have been studied extensively at the protein and gene levels. However, little is known about composition of type IV. Although the existence of six different α chains may generate many isoforms, only limited numbers of isoforms including $[\alpha1(IV)]_2\alpha2(IV)$ and $[\alpha3(IV)]_2\alpha4(IV)$ have been identified (12,16,17). Here we have used CHO cells transfected with an expression vector for the α1(IV) collagen chain to create a cell line A222 which produces high levels of this protein. The α1(IV) chain expressed by these cells, however, did not form a homotrimer, but remained as a single chain in a random coil configuration which was easily digested by pepsin. Our results using this molecular approach suggest three α1(IV) chains are unable to assemble into a stable trimer. Our results are in disagreement with a previous report of the existence of α1(IV) chain homotrimers in rat embryo-derived yolk sac cells (18). One possibility for the discrepancy may be due to a low level of α2 (IV) chain present in their studies using cyanogen bromide and pepsin digestion.

We found that coexpression of the α2(IV) chain is necessary for formation of a triple-helical structure with the α1(IV) chain. Secretion of the collagen IV tri-

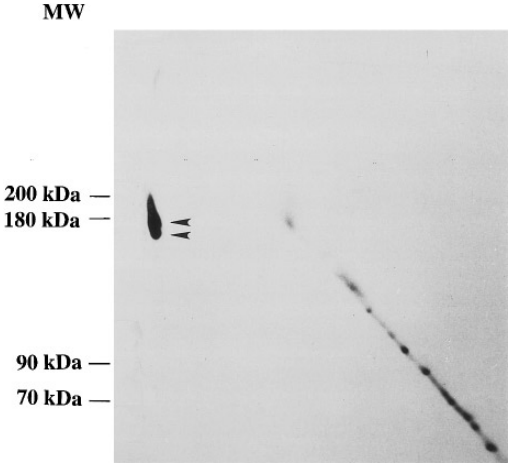

FIG. 2. Analysis of the high molecular weight band from A222-A2 cells by two dimensional gel electrophoresis. Immunoprecipitates from the A222-A2 media were first run under non-reducing conditions on 4.5% SDS-PAGE. A strip of the first gel was run under reducing conditions with 2β-mercaptoethanol on 4.5% SDS-PAGE. Two spots corresponding to the size of α1(IV) and α2(IV) chains are indicated (arrowheads).

mer is relatively slow (1 hr) compared to that of the fibrillar collagens which occurs in about 20 min (29). This slow process may be due to the slow folding of the many non-collagenous sequences that interrupt the triple-helical collagenous domains. Monomer α1(IV) chain was also secreted, although this occurred at a much slower rate. The secreted monomer α1(IV) chain appears to be non-disulfide bonded and remains as a random coil structure. The ability of the monomer α1(IV) chain to be secreted is in agreement with that observed in the culture medium of fibroblasts from a patient with osteogenesis imperfecta (30). These results are in sharp contrast to the assembly of the chains observed in collagen I molecules. It has been reported that cells lacking the transcription of the α1(I) gene via a retroviral insertion show undetectable levels of intracellular α2(I) chain, in spite of normal levels of expression of α2(I) mRNA (31). The reason for this is unclear , but may be due to the rapid intracellular degradation of the α2(I) chain. Furthermore, although a triple-helical assembly is required for secretion of type I collagen, the α1(IV) chain monomer lacking disulfide bonds is still secreted. This may reflect the difference in function of NC1 domain of the type IV collagen and the C-terminal propeptide of fibrillar collagens which are responsible for chain selection and assembly.

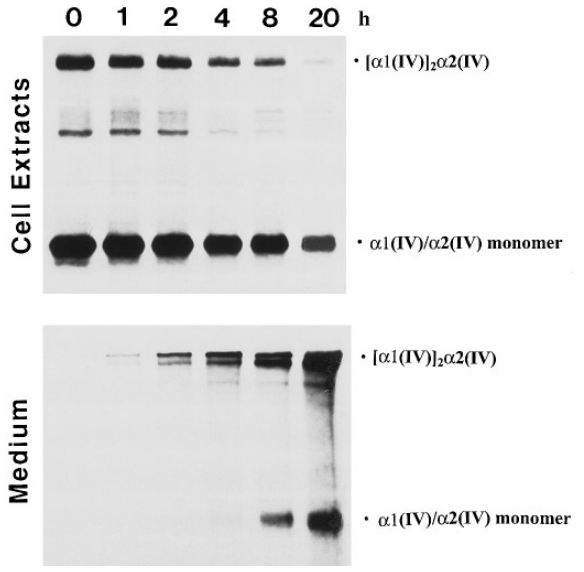

FIG. 4. The pulse-chase experiments of the recombinant α1(IV) and α2(IV) chains. A222-A2 cells were metabolically labeled and the cells were chased from 0 to 20 h. Anti-type IV collagen antibody immunoprecipitates from the media and cell extracts were analyzed by 4.5% SDS-PAGE and fluorography. The results show that trimeric protein was secreted in medium earlier than the monomer.

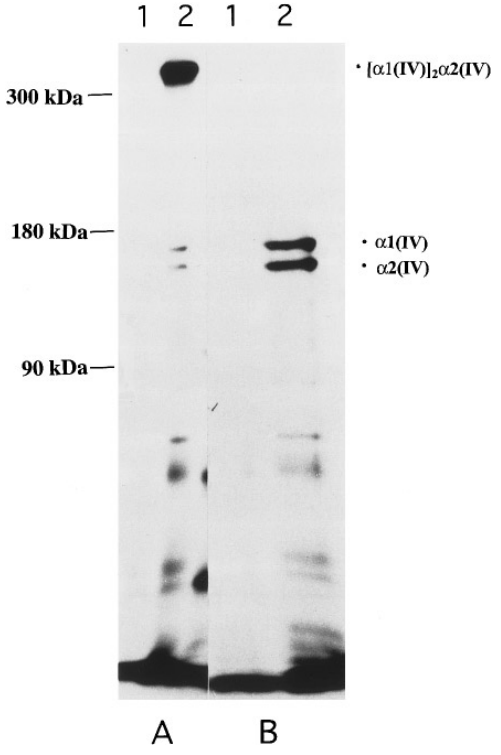

FIG. 3. Pepsin digestion of the recombinant collagen IV. Immunoprecipitates from the media of A222 and A222-A2 cells were digested with pepsin and analyzed by 4.5% SDS-PAGE and fluorography. Lane 1, A222 cells; lane 2, A222-A2 cells. A, non-reducing condition; B, reducing condition with 2β-mercaptoethanol. Pepsin-resistant proteins were found in A222-A2 cells.

In the case of collagen IV, the NC1 domain is not post-transcriptionally processed and functions extracellularly in linking two type IV collagen molecules in the macromolecular network. Recent evidence suggests that lateral aggregation of the triple helical domains brings the C-terminus in a close vicinity to facilitate the correct interaction of the two NC1 domains of type IV collagen (32). However, it is still unclear whether the NC1 domains of α(IV) chains dictates chain selection and assembly. The high level of expression of recombinant α1(IV) and α2(IV) chains in CHO cells combined with site specific mutagenesis and transfection with other chains should be useful to define the mechanisms of recognition and assembly of type IV collagen chains.

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