Cloning of $\alpha 2$ chain of type VI collagen and expression during mouse development

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We have previously described the molecular cloning of a cDNA probe which detects a 6 kb mRNA termed pOb24. pOb24 mRNA appeared to be a marker of the preadipose state both *in vitro* and *in vivo*. A pOb24 genomic fragment was isolated and used to screen cDNA libraries in order to isolate the full-length pOb24 cDNA and to identify the corresponding protein. The screening yielded a new cDNA clone which detected a 3.7 kb mRNA species in addition to the 6 kb mRNA species. Sequences at the 3' end of the 6 kb and 3.7 kb mRNAs indicate that both mRNAs are generated from the same gene through the use of two different polyadenylation sites. The protein encoded by the 3.7 kb mRNA appeared to be homologous to

the human $\alpha 2$ chain of type VI collagen (A2COL6). The expression of the A2COL6 gene was not confined to adipose tissue; mRNA species can be detected in ovaries, adrenal glands and lungs but not in liver and skeletal muscle. The expression appeared specific for initial phase(s) of cell differentiation since it is parallel to that of the MyoD1 gene during muscle embryogenesis *in vivo*. In the myogenic C2C12 cell line, the A2COL6 gene exhibited the same regulation as MyoD1 and myogenin genes. These results indicate that A2COL6 gene expression is a marker of the preadipose state, but may also be a marker of other differentiation programmes such as that of muscle.

INTRODUCTION

The understanding of the mechanisms underlying adipose cell differentiation requires the identification of specific proteins which are preferentially expressed during the preadipose state, defined as the differentiation step which precedes the emergence of lipogenic enzymes and lipid accumulation. Until recently, at the molecular level, lipoprotein lipase appeared to be the best candidate. Nevertheless, it is clear that lipoprotein lipase mRNA and activity accumulate during the process of terminal differentiation [1]. We have previously described the molecular cloning, by differential screening, of a cDNA probe which detects a 6 kb mRNA named pOb24. The expression of pOb24 mRNA is of interest since it was shown to be absent from growing undifferentiated Ob1771 preadipocytes as well as from growing and growth-arrested 3T3-C2 fibroblasts. The pOb24 mRNA content increased rapidly at the early stages of differentiation and subsequently decreased at later stages, i.e. at a time when mRNAs encoding glycerol-3-phosphate dehydrogenase (GPDH) and adipsin increased dramatically. The rapid emergence of pOb24 mRNA in growth-arrested Ob1771 cells was due to a dramatic increase in the transcriptional rate of the gene [2]. *In vivo*, it is noticeably absent from muscle and mainly present in adipose tissue of adult mice where it is confined to the stromal-vascular fraction which contains adipose precursor cells [2]. Taken together, these results indicate that pOb24 gene expression is a marker of the preadipose state both *in vitro* and *in vivo*. The fact that pOb24 mRNA appeared to be transiently expressed during adipose conversion was reminiscent of the status of MyoD and myogenin mRNAs during differentiation of

myoblasts into myotubes [3,4] and suggests that the pOb24 gene product(s) might play an important role in the process of adipose cell differentiation. In an effort to characterize the 6 kb species of pOb24 mRNA better, a new pOb24 cDNA probe has been characterized which has allowed us to detect both this species and a new 3.7 kb species. These two mRNAs are generated from the same gene by using different polyadenylation sites. The 3.7 kb mRNA species is very abundant compared with the 6 kb mRNA species and sequence studies indicate that it shows strong similarities to the human $\alpha 2$ chain of type VI collagen mRNA (named A2COL6) encoding an extracellular matrix protein.

Because of the abundance in adipose tissue of the 3.7 kb mRNA species (A2COL6) compared with the 6 kb mRNA species (pOb24), it was decided to re-examine their tissue distribution in mice before and after birth and to test whether their absence from muscles of developing mice excluded the possibility that the expression of the A2COL6 gene could eventually take place during embryonic life in tissues other than adipose tissue. The results showed that this is indeed the case for embryonic skeletal muscle and led us to investigate the status of A2COL6 gene expression in mouse C2C12 myoblast cells that differentiate in culture into myotubes.

MATERIALS AND METHODS

Construction and screening of genomic and cDNA libraries

A genomic library, containing inserts of approximately 20 kbp from *Mbo*I partial digests of L929 DNA, constructed in the λ EMB3 cloning vector, was screened by the *in situ* hybridization technique of Benton and Davis [5]. Two positive clones among

Abbreviations used: GPDH, glycerol-3-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; A2COL6, $\alpha 2$ chain of type VI collagen; WAT, white adipose tissue.

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The nucleotide sequence data reported in this paper have been submitted to the EMBL DataBank under the accession number X62332.

approx. 5×10^5 clones were identified after probing with the 0.35 kb *HinfI*–*BglII* fragment of pOb24 cDNA. These clones, which contained a 13 kbp insert, were later shown to be identical by restriction analysis. Phage DNA was prepared and then several genomic DNA fragments were subcloned into PUC18 or pSP72 plasmids.

Two cDNA libraries were constructed in λ ZAPII (Stratagene) and λ gt10 (Amersham) vectors: mRNAs for these libraries were prepared from growth-arrested Ob1771 cells 1 day and 4 days after confluence respectively. Single-stranded cDNA was synthesized using oligo(dT) as primer in the case of the λ gt10 cDNA library and random hexanucleotides as primers in the case of the λ ZAPII cDNA library. Double-stranded DNA was synthesized as previously described [2]. The cDNA was methylated, ligated with *EcoRI* linkers, cleaved with *EcoRI* and subsequently ligated to *EcoRI*-digested phosphatase-treated vector DNAs. It was then packaged *in vitro* according to the instructions of the manufacturer (Stratagene). The λ gt10 cDNA library, containing about 5×10^5 – 10^6 independent clones, was screened using a cloned genomic fragment. The screening led to the purification of ten cDNA clones. Two inserts of 3 kbp and 1.7 kbp were subcloned into PUC18 plasmid and designated C3PU18-11 and C3PU18-7 respectively. The C3PU18-7 insert was used to screen the λ ZAPII cDNA library. From 1.5×10^6 independent clones only one positive clone containing a larger insert was obtained and named pB12.

DNA sequencing and analysis

Several DNA fragments from the different clones were generated by digestion with various restriction enzymes and subcloned. Progressive unidirectional deletions of the inserted DNA were created using the *ExoIII*/mung bean nuclease kit (Stratagene Cloning Systems) in order to obtain overlapping sequences.

Denaturated recombinant plasmid DNAs were sequenced by the dideoxy chain-termination method and the use of modified T7 DNA polymerase. Some specific oligonucleotide primers were synthesized to obtain sequences in both strands. Sequence data were decoded and examined using BISANCE sequence analysis programs [6].

Animals and tissues

Four-week-old male and female BALB/cByJlco mice were obtained from Iffa Credo (Lyon, France). To obtain different adult mouse tissues and developmental stages of embryos, female mice (on different days after coitus, which is considered as day 0 of gestation) and male mice were killed by cervical dislocation. Several tissues of adult mice and embryos were immediately removed, put into liquid nitrogen and ground to a powder. The homogenates were subsequently treated with a thiocyanate-buffered solution and RNA was prepared as described below.

Cell line, culture conditions and fusion of myoblastic cells

C2C12 cells [7] were plated at 10^3 cells/cm² in 60 mm-diameter dishes and maintained as undifferentiated myoblasts in a nutrient-rich growth medium containing Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 5% (v/v) adult bovine serum, 200 units of penicillin/ml, 50 μ g of streptomycin/ml, 33 μ M biotin and 17 μ M pantothenate. This medium is termed standard medium. At confluence (day 0), which

usually occurred within 5 days of seeding, the medium was removed and replaced by Dulbecco's modified Eagle's medium containing only 5% (v/v) heat-inactivated horse serum to induce myoblast fusion and the formation of multinucleated fibres. This medium was defined as differentiation medium and was changed every 2 days. Two or three days after the onset of cell fusion, muscle fibres exhibited spontaneous contractions.

RNA analysis

RNA was prepared as described by Chomcynski and Sacchi [8]. Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography (Pharmacia PL Biochemicals) as described by Aviv and Leder [9]. Poly(A)⁺ RNA was electrophoresed on denaturing 1.2% agarose gels containing 1.1 M formaldehyde and transferred to Hybond-N membranes. Hybridizations were performed with approx. 10^6 c.p.m./ml of randomly primed ³²P-labelled DNA probes. After being washed, the membranes were exposed to Hyperfilm MP Amersham at –70 °C with intensifying screens. Results were quantified by densitometry using an LKB Ultrascan XL laser densitometer [2].

Probes

Plasmid pA21 was a cDNA clone complementary to the mRNA encoding mouse β -actin and α -actin [10]. Plasmid pRGAPDH-13 contained a full-length cDNA probe complementary to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA [11]. Plasmid pC8 was a cDNA clone complementary to mRNA encoding mouse GPDH [12]. Plasmid EMSV-Myo8 contained a full-length cDNA probe for mouse myogenin mRNA [13] and plasmid pEMC11s contained a full-length cDNA for MyoD1 mRNA [14].

Materials

Modified T7 DNA polymerase (Sequenase) was from United States Biomedical Corp. Specific oligonucleotide primers were synthesized by Eurogentec (Seraing, Belgium). [α -³²P]dCTP was purchased from Amersham International. Random priming labelling kits were from Boehringer Mannheim (Germany). Culture cell media and heat-inactivated horse serum were from Gibco BRL (Cergy Pontoise, France). Fetal calf serum was from I.B.F. Biotechnics (St. Quentin-Fallavier, France). Bovine serum and all other chemicals were purchased from Sigma Chimie (Villeneuve la Garenne, France).

RESULTS

Characterization of two pOb24 mRNAs with distinct 3' ends

pOb24, a cDNA previously isolated from an Ob1771 cDNA library, contains a 1.2 kbp insert which corresponds to the 3' end of a 6 kb mRNA that is preferentially expressed at the preadipose state during adipose cell differentiation. In order to isolate the full-length pOb24 cDNA, a pOb24 genomic fragment (see the Materials and methods section) was used to screen a λ gt10 cDNA library constructed with mRNAs from early marker-expressing Ob1771 cells (day 4 after confluence). The screening yielded two different kinds of cDNA clones. The first one, named C3PU18-7, was shown to contain an insert of 1.7 kbp in length. When used for Northern-blot analysis, this cDNA revealed the 6 kb mRNA only. The second one, named C3PU18-11, was found to contain a 3 kbp insert which revealed, by Northern-blot analysis, two species of 3.7 and 6 kb in early marker-expressing Ob1771 cells (Figure 1). Their absence from, or low levels of

Figure 1 Expression of 6 kb pOb24 and 3.7 kb mRNAs in preadipose and non-preadipose cells using different cDNA probes

Poly(A)⁺ RNA (3 µg) from confluent 3T3-C2 fibroblasts and Ob1771 preadipose cells were electrophoresed and separately hybridized with C3PU18-11 and C3PU18-7 cDNAs.

Figure 2 Restriction map of cDNAs and sequencing strategy

A schematic diagram of the clones isolated from λ gt10 and λ ZAPII cDNA libraries is represented. The pOb24 cDNA previously cloned [2] is also reported. Each clone was sequenced in both strands, and inserts are aligned to show overlapping areas. The black box indicates a region encoding for a collagen-like domain. Sa, *Sac*I; S, *Sam*I; X, *Xba*I; B, *Bgl*II.

expression in, 3T3-C2 fibroblasts indicated that both mRNAs were expressed in a differentiation-dependent manner. Since no overlapping sequence appeared to be present between the 1.7 and 3 kb cDNA species, obtaining a larger DNA complementary to the pOb24 mRNA, which would overlap with the 3.7 kb mRNA, proved to be necessary to understand their structural relationships. For this purpose, the C3PU18-7 cDNA insert was used to screen a λ ZAPII cDNA library from early marker-expressing cells (1 day after confluence). A cDNA clone, named pB12, which overlapped with the C3PU18-11 cDNA, was isolated. Both strands of the cDNA inserts within the different clones were sequenced employing strategies summarized in Figure 2. The 3' non-coding region of the 3.7 kb mRNA contains an AAUAAA consensus polyadenylation signal located 13 nucleotides upstream from the poly(A) tail. The 3' non-coding region of the 6 kb pOb24 mRNA extends downstream by 1850 nucleotides

and possesses an AAUAA non-consensus polyadenylation site located seven nucleotides upstream from the poly(A)⁺ tail (Figure 3). This polyadenylation sequence was found in two clones obtained from independent libraries. It is interesting to note that the AAUAA polyadenylation signal present in pOb24 mRNA has been previously shown to be tenfold less efficient than with the usual AAUAAA sequence [15]. This feature may contribute to the low amount of the 6 kb mRNA compared with the 3.7 kb mRNA but other transcriptional and/or post-transcriptional events may be involved. Taken together, the above results demonstrate that alternative polyadenylation sites are involved in the formation of the 6 kb and 3.7 kb mRNAs at the 3' end but do not exclude additional differences at the 5' end.

The protein encoded by the 3.7 kb mRNA is apparently homologous to the human α 2 chain of type VI collagen

In order to identify protein(s) encoded by the 3.7 kb mRNA, the C3PU18-11 cDNA was sequenced. Figure 4 shows the nucleotide and predicted amino acid sequences. The first characteristic of the amino acid sequence was the presence of glycine at every third position, which is analogous to a collagen-like domain. This domain extends from nucleotide number 1 (defining the collagen domain) to nucleotide number 1005 and corresponds to 335 amino acid residues. The comparison of this sequence (Figure 5) with sequences reported in GenBank databases reveals 95% similarity with the collagenous domain of the human α 2 chain of type VI collagen (A2COL6), the sequence of which has recently become available [16], and 75% similarity with the collagen domain of the chicken A2COL6 [17] (not shown). Similarities with human and chicken A2COL6 C-terminal domain are 88% and 75% respectively (not shown), and no extensive similarity was found in the 3' untranslated region. A2COL6 was previously cloned in chicken and human and reported mRNA sizes were 4.2 and 3.5 kb respectively. The 3' untranslated sequence of the chicken, mouse and human A2COL6 is 970 nucleotides [18], 450 nucleotides (this paper) and 295 nucleotides [19] in length respectively and reflects directly the differences in size observed for A2COL6 mRNAs in these species. Together, these results indicate clearly that the product of the 3.7 kb mRNA is the murine homologue of the human and chicken A2COL6.

Expression of A2COL6 and pOb24 mRNAs in different mouse tissues

Previous studies have shown that the 6 kb pOb24 mRNA is confined to brown adipose tissue and to various depots of white adipose tissue of 4-week-old mice and very old mice [20]. In contrast, this mRNA species could not be detected in liver or skeletal muscle [2]. Expression of the A2COL6 gene was studied further in adult mice. The results of Figure 6 show that the two mRNA species were expressed at different levels: depending on the tissue, the 3.7 kb mRNA was about 7–16 times more abundant than the 6 kb mRNA. Both the 3.7 kb and 6 kb mRNA were preferentially expressed in periepididymal and periovarian adipose tissues. However, it appeared that the A2COL6 gene expression was not confined to adipose tissues. Different tissues, such as lung, adrenal and mainly ovary, showed a high content of A2COL6 and pOb24 mRNAs. Another group of tissues (testis, tongue, skin, kidney, heart, intestine and spleen) showed a low content of A2COL6 mRNA whereas the 6 kb pOb24 mRNA could not be detected. Whether this absence is merely due to the sensitivity of the technique and/or to a differentiated tissue-specific expression remains uncertain. As reported previously for pOb24 mRNA, the A2COL6 mRNA

The nucleotide sequence was determined from C3PU18-11 cDNA and numbered (upper numbers) by taking 1 as the start of the collagen domain indicated by the black box in Figure 1. The untranslated region is indicated in lower case. The protein sequence is also numbered (lower numbers).

Figure 6 Distribution of A2COL6 and pOb24 mRNAs in various adult mouse tissues

RNAs from various tissues were prepared as described in the Materials and methods section. Poly(A)⁺ RNA (3 µg) was analysed by Northern-blot analysis. The blot was successively hybridized with labelled A2COL6/pOb24 and GAPDH cDNA probes. The sizes of the mRNA bands were determined from standards of ribosomal RNA. GAPDH mRNA signals were used as an internal control. Identical results were obtained in two separate experiments. Epid., epididymal.

Figure 7 Expression of A2COL6/pOb24 and MyoD1 mRNAs in total mouse embryo and embryonic skeletal muscle

RNA of total mouse embryos (a) and RNA of embryonic skeletal muscles (b) were prepared and analysed as described in the Materials and methods section for the presence of mRNA A2COL6/pOb24, MyoD1 and GAPDH. E, Embryonic day; P, postnatal day.

accumulation appeared late during embryogenesis (day 16) and reached its maximum level after birth. The presence of A2COL6 and pOb24 mRNAs in embryonic skeletal muscle and the parallel evolution of their expression with the myoblast marker MyoD1 mRNA during muscle development suggest that the A2COL6 gene is not only a marker of the preadipose state, but it may also be a marker of the muscle differentiation programme. To test this hypothesis, expression of the A2COL6, MyoD1 and myogenin

Figure 8 Expression of mRNA for (a) A2COL6/pOb24, (b) myogenin, (c) MyoD1 and (d) actin during differentiation of C2C12 cells

At confluence, standard medium was removed and replaced by the differentiation medium (see the Materials and methods section), defined as time zero. Poly(A)⁺ RNA (3 µg) was prepared at the indicated times and analysed as described in the Materials and methods section. The values were normalized to GAPDH signals which were used as internal controls and expressed by taking the maximum signal obtained for each probe as 100%. The results are representative of three independent experiments performed on three different series of cells. Exp, exponentially growing cells. ●, β-Actin mRNA; ○, α-actin mRNA.

genes was studied *in vitro* during the myogenic differentiation of C2C12 cells [19].

Expression of the A2COL6 gene during myogenic differentiation

As illustrated in Figure 8, A2COL6 and pOb24 mRNAs show the same kinetics of accumulation as that of master genes of muscle cell differentiation such as MyoD1 and myogenin. All were absent from exponentially growing undifferentiated cells. They became expressed in growth-arrested confluent cells and accumulated during the first few hours after the induction of myotube formation (maximum at 4 h). However, the expression of these genes was transient since the content of these various mRNAs decreased rapidly and remained low during terminal differentiation. At the same time, β-actin mRNA, a myoblast marker [22], decreased gradually and α-actin mRNA, a myotube marker [23,24], increased during cell fusion and attained its maximum level of expression 72 h after the induction of myotube formation when cells began to contract. These results show that A2COL6 gene expression is concomitant with that of two major myogenic regulatory factors.

Table 1 Emergence of mRNA for A2COL6/pOb24, MyoD1 and myogenin in growth-arrested C2C12 cells

Growth arrest was initiated by the addition of 2 μ g of aphidicolin/ml, 5 mM hydroxyurea or 5 mM thymidine to C2C12 cells actively growing in standard medium. At 24 h after drug addition, 3 μ g samples of poly(A)⁺ RNA were prepared and analysed for the presence of mRNA for A2COL6/pOb24, MyoD1, myogenin, β -actin and α -actin. The values were expressed by taking the maximum signal obtained for each probe as 100%. The results are means $\pm$ range of two independent experiments performed on two different sets of cells. Only the 3.7 kb mRNA species of A2COL6/pOb24 is shown, but identical results were obtained with the 6 kb species. The α -actin signal was below the threshold of detection.

mRNA	Relative expression (%)				
	Exponentially growing cells				
	Confluent cells	No addition	+ Aphidicolin	+ Hydroxyurea	+ Thymidine
A2COL6	100 $\pm$ 4.6	8 $\pm$ 1.2	50 $\pm$ 1.7	45 $\pm$ 4.1	86 $\pm$ 3.3
MyoD1	100 $\pm$ 3.2	2 $\pm$ 1.1	94 $\pm$ 2.5	40 $\pm$ 3.0	68 $\pm$ 3.5
Myogenin	95 $\pm$ 2.4	4 $\pm$ 1.6	83 $\pm$ 3.6	91 $\pm$ 3.6	100 $\pm$ 4.1
β -Actin	73 $\pm$ 2.8	100 $\pm$ 3.8	72 $\pm$ 1.9	55 $\pm$ 2.9	87 $\pm$ 2.2

Expression of the A2COL6 gene as a function of the cell cycle

It is now well established that growth arrest at G₁-phase of the cell cycle is required for commitment of cell precursor cells giving rise to myoblasts [25] or preadipocytes [26]. The effect of growth arrest on the content of mRNA for A2COL6/pOb24, MyoD1, myogenin and actin was investigated in C2C12 cells. Growth arrest was induced merely by cell contact at confluence or after a 24 h exposure of growing dispersed cells to 2 μ g/ml aphidicolin, 5 mM hydroxyurea or 5 mM thymidine. As shown in Table 1, the three markers (A2COL6/pOb24, MyoD1 and myogenin) were not detectable in exponentially growing cells. Growth arrest led to the accumulation of A2COL6/pOb24 mRNA in C2C12 cells as already described for growth-arrested Ob1771 cells [1,2]. In addition, MyoD1 and myogenin mRNA accumulated. The emergence of these various markers appears to be specific since, under each condition, the β -actin mRNA level was moderately affected whereas α -actin mRNA did not emerge.

DISCUSSION

Type VI collagen is a heterotrimer consisting of α 1(VI), α 2(VI) and α 3(VI) chains which are synthesized as polypeptides with *M_r* values of 140 000, 140 000 and 260 000 respectively [27–29]. In this work, we have identified the α 2(VI) chain (A2COL6) as a marker of preadipose and myoblast states. The protein represents a hybrid molecule consisting of a short triple-helix collagenous domain which is flanked by two large globular domains which contribute more than 80% of the molecular mass. Sequence

analysis of the collagenous domain revealed five Arg-Gly-Asp (RGD) tripeptide sequences which may be used as cell-binding sites via integrins [30,31]. Four of these motifs are in identical positions in chicken, mouse and human (Figure 9); this observation strongly suggests that these conserved sites are implicated in the cell attachment of type VI collagen. In agreement with this assumption, Aumailley et al. [32] have clearly demonstrated that cells are able to bind to A2COL6 via RGD sequences in a specific manner and established that the unique structural and binding properties of type VI collagen distinguish it from other collagenous proteins. Type VI collagen interacts with type I and type II collagen and with the proteoglycan decorin [33]. These various observations suggest that type VI collagen plays an essential role in regulating the structural organization of the extracellular matrix. Moreover, integrins provide a link between the extracellular matrix and the cytoskeleton and it is interesting to note that the interaction of integrins with extracellular matrix components, via RGD sequences, is critical in triggering the metabolic events leading to terminal stages of myogenic differentiation [34], and similar observations have been made recently for the late events of adipose cell differentiation (A. Ibrahimi and C. Dani, unpublished work). The results presented here indicate that the expression of the A2COL6 gene is not unique for adipose tissue since both A2COL6 and pOb24 mRNAs (3.7 kb and 6 kb) can be detected at different levels in other tissues of adult mice. Furthermore, the A2COL6 gene is expressed *in vivo* during the differentiation of skeletal muscle. The emergence of MyoD1, myogenin and A2COL6/pOb24 mRNA during the differentiation of the myogenic C2C12 cell line showed similar kinetics. In addition, the expression of the A2COL6, MyoD1 and myogenin genes was triggered similarly by growth arrest. The MyoD1 and myogenin gene products are phosphorylated nuclear transcription factors [35] able to bind specifically to a DNA sequence found in the promoter region of several muscle-specific genes. The A2COL6 gene must be a target for transcriptional factors. However, the latter are not MyoD1 or myogenin since MyoD1 and myogenin mRNAs remain undetectable in growth-arrested Ob1771 cells (A. Ibrahimi and C. Dani, unpublished work). We assume that these unidentified factors are specific for some initial step(s) of cell differentiation in various types of mesenchymal cells.

It is suggested that studies of integrins expressed in preadipocytes and myoblasts, which are able to bind type VI collagen during differentiation, might be of interest, as well as studies on the signalling pathways triggered by these specific interactions.

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	109–115	169–175	232–238	241–247	282–288
Chicken	<u>GERGDEG</u>	<u>GRRGDPG</u>	<u>GSRGDPG</u>	<u>GPRGDAG</u>	<u>GGRGELG</u>
Mouse	<u>GERGDQG</u>	<u>GRRGDPG</u>	<u>GSRGDPG</u>	<u>GPRGDSG</u>	<u>GGRGDFG</u>
Human	<u>GERGDQG</u>	<u>GRRGDPG</u>	<u>GSRGDPG</u>	<u>GPRGDSG</u>	<u>GGRGDFG</u>

Figure 9 Comparison of the RGD tripeptide sequences in chicken, mouse and human A2COL6

The chicken sequence was reported by Trueb et al. [17], the human sequence by Saitta et al. [16], and the mouse sequence from this paper. Numbers indicate their position in the collagen domain. The RGD tripeptides are underlined.

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