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Cloning and characterization of chicken $\alpha 5$ integrin: Endogenous and experimental expression in early chicken embryos

Yukinori Endo^{1,*}, Hiroko Ishiwata-Endo¹, and Kenneth M. Yamada^{1,*}

¹Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892-4370.

Abstract

Key roles for fibronectin and its integrin receptors have been postulated in the multiple cell-matrix interactions essential for chick embryo morphogenesis. However, mechanistic studies of these processes have been hampered by the current absence of sequence data and chicken cDNA clones for the major fibronectin receptor subunit, integrin $\alpha 5$ (ITGA5). We report here the sequence, endogenous expression pattern, and transfection of full-length chicken integrin $\alpha 5$. During early chicken embryonic development, $\alpha 5$ is highly expressed in cranial neural folds and migrating neural crest cells, suggesting potential roles in neural crest formation and migration. In fact, over-expression of this integrin in early neural tube selectively induces BMP5, a growth factor recently implicated in neural crest formation. Availability of these $\alpha 5$ integrin tools should facilitate studies of its functions in early embryonic development.

Keywords

$\alpha 5$ integrin; fibronectin; extracellular matrix; chicken embryo; neural crest

1. Introduction

Classical studies of cell-matrix interactions using embryonic chicken embryo systems have provided extensive insight into the mechanisms mediating development of a number of vertebrate tissues. For example, *in vitro* explants and *in vivo* experimental analyses of chicken embryos have continued to provide important knowledge about extracellular matrix (ECM) regulation of the formation and subsequent ECM-based migration of neural crest stem and progenitor cells (Le Douarin and Kalcheim, 1999; Brauer and Markwald, 1987; Newgreen, 1984; Baremaum et al., 2000; Endo et al., 2012).

Over the past three decades, numerous investigations of chick neural crest biology have identified crucial roles for a variety of ECM glycoproteins and receptors, such as fibronectin, laminin, collagen, and a variety of integrins (Tucker et al., 1988; Perris et al., 1989; Lallier and Bronner-Fraser, 1991; Perris et al., 1991; Rovasio et al., 1983; Rogers et al., 1990; Henderson and Copp 1997; McKeown, et al., 2013). For example, initial functional studies of fibronectin and the $\beta 1$ integrin (which forms a heterodimer with

*Corresponding author: Kenneth.Yamada@nih.gov or yukinorishiko@yahoo.co.jp, +1-301-496-9124 (Phone).

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multiple integrin alpha subunits) revealed critical roles in cell migration *in vivo* (Tucker et al., 1988; Boucaut et al., 1984; Duband et al., 1991; Perris et al., 1989; Bronner-Fraser, 1985 and 1986). *In vitro* studies using chick neural crest explants have suggested considerable potential complexity in requirements for various integrins for neural crest cell adhesion and migration, including $\alpha 3\beta 1$, $\alpha 4\beta 1$, and three different αV -containing receptors (Desban et al., 2006; Testaz and Duband., 2001; Testaz et al., 1999; Desban and Duband, 1997; Delannet et al., 1994; Duband et al., 1986). This complexity, at least *in vitro*, is not surprising because multiple sites on fibronectin can be used for avian neural crest cell interactions in tissue culture (Dufour et al., 1988). A notable omission in these studies, however, was the absence of tests for roles of the major fibronectin receptor $\alpha 5\beta 1$ because of a lack of specific anti- $\alpha 5$ integrin antibodies and other tools for experimentally altering $\alpha 5$ integrin expression.

The interactions of neural crest cells with laminin have also been analyzed. Besides a role for the laminin molecule itself by interacting with heparan sulfate proteoglycan in neural crest migration (Bronner-Fraser and Lallier, 1988), *in vitro* roles for the $\alpha 1\beta 1$ and $\alpha V\beta 3$ integrins have been identified for neural crest cell interactions with laminin in tissue culture (Desban and Duband, 1997; Desban et al. 2006).

In studies using other species besides chicken, the fibronectin receptor $\alpha 5\beta 1$ integrin has been cloned and characterized experimentally in species ranging from *Xenopus* to human (Argaves et al., 1986, Whittaker and DeSimone, 1993; Holers et al., 1989). Even though highly effective anti-functional monoclonal antibodies can be used in mouse and human systems to probe $\alpha 5$ functions, they also do not yet exist for chicken. However, monoclonal antibodies useful for western immunoblotting have been generated against chicken $\alpha 5$ integrin protein, which reveal a pattern of progressively decreasing expression as development progresses through day 17 (Muschler and Horwitz, 1991; Hofer et al., 1990).

Studies in mouse knockout models have provided intriguing hints of roles for the $\alpha 5$ integrin in neural crest cell interactions *in vivo*. For example, ablation of mouse $\alpha 5$ integrin gene expression leads to apoptosis of some migrating cranial neural crest cells in the hyoid arch (Goh et al., 1997) as well as defects in proliferation and survival in migrating cardiac neural crest cells (Mittal et al., 2010). These initial findings in mouse models indicate the need for further in-depth analyses of the *in vivo* roles of this integrin in more experimentally tractable chick embryo systems.

A major problem for such future functional analyses of chicken integrin $\alpha 5$, however, has been that its full gene sequence remains unknown except for a short 243 bp segment and a partially sequence of the N-terminal 24 amino acids (Guan et al., 2003; Hofer et al., 1990). Puzzlingly, the $\alpha 5$ gene sequence is not available in current chicken genomic data (GenBank Assembly ID: GCA 000002315.2), even though chicken genomic sequences have been available for years. Consequently, nucleic acid sequences are not currently available for use for sensitive *in situ* hybridization expression studies, nor for experimental analyses using transfection and RNAi approaches. In order to facilitate such studies, we have determined the chicken $\alpha 5$ integrin nucleotide sequence; we then used it for *in situ* hybridization to examine its endogenous localization during early neural crest development, and to generate a full-length cDNA clone for transfection studies. Because extracellular matrix proteins are known to modulate specific growth factor expression (e.g., see Endo et al., 2012), we performed proof-of-principle transfection experiments to examine whether increased expression of this integrin receptor might, by itself, selectively modulate growth factor expression during early chicken embryo development.

2. Results and Discussion

We determined the full-length $\alpha 5$ sequence using a combination of PCR with degenerate primers and RACE (rapid amplification of cDNA ends). We first aligned previously published amino acid sequences for human, mouse, zebrafish, and *Xenopus* $\alpha 5$ integrins and designed two sets of degenerate primers at two highly conserved regions for isolation of chicken *ITGA5-1* (900 bp) and *ITGA5-2* (998 bp) (Figure 1A). We then isolated *ITGA5-3* and *-4* including the 5'-UTR and 3'-UTR, respectively, by performing RACE. *ITGA5-3* included the longest 5'-UTR (240 bp) we could obtain, which we validated by sequencing chicken genomic DNA (data not shown). Among the 3'-RACE products, we isolated clone *ITGA5-4* containing the 3'-UTR with poly A tail (1486 bp). Finally, we confirmed the full-length $\alpha 5$ integrin coding gene sequence (4888 bp, gene accession number: KC439457) by repeated DNA sequencing using several sets of nested primers (Figure 1B).

In an NCBI BLAST search, the predicted chicken $\alpha 5$ protein had highest similarity to human and mouse $\alpha 5$ integrins (61% and 60%, respectively) in comparisons with all α integrins; for comparison, human and mouse $\alpha 5$ integrins are 90% identical. The lower homology of the chicken $\alpha 5$ sequence with these mammalian $\alpha 5$ integrin subunits suggests that comparisons of specific sequences or domains can provide insight into key functional sites of the $\alpha 5$ integrin. There is higher conservation of the third and fourth $\alpha 5$ integrin beta-propeller domains, which may indicate functional significance (Figure 2A). More notably, the Ala¹⁰⁷-Tyr²²⁶ loop sequence previously reported by Mould et al. (2000) to be associated with human $\alpha 5$ integrin ligand specificity is highly homologous in chicken, with 77% amino acid identity in comparison of chicken to human sequences (Figure 2B). Interestingly, however, the human $\alpha 5$ sequence DFSWAA within this region thought to confer selectivity for binding of the peptide RGDGW and ability to recognize RRETAWA (Mould et al., 2000) is not fully conserved in the corresponding DLNSEA chicken sequence. In fact, because the mouse $\alpha 5$ amino acid sequence DFGSAA is also not completely identical, further analysis of this site by mutagenesis should be considered.

Because the cytoplasmic domains of integrins have important functions in mediating the formation of integrin cell adhesion complexes mediating cytoskeletal interactions and signaling, we aligned the chicken $\alpha 5$ cytoplasmic domain amino acid sequence with other species. We found that the chicken protein had 92% identity to zebrafish, and 84% to human and mouse. Interestingly, however, the carboxyl-terminal 6 amino acids of the cytoplasmic domain were not well-conserved compared to the remainder of the cytoplasmic domain and the similarly highly conserved propeller domains in the extracellular domain (50% versus ~90% identity, respectively, Figure 2C). The non-critical nature of these amino acids is consistent with a previous finding that deletion of the carboxyl terminus of the human $\alpha 5$ integrin did not significantly affect *in vitro* cell adhesion, cytoskeleton, or cell motility (Bauer et al., 1993). It may also explain why even fusion of a bulky GFP molecule to the carboxyl-terminal sequence retains biological function (e.g., see Laukaitis et al., 2001).

Since monoclonal antibodies against chicken $\alpha 5$ integrin have been produced (for this study, we used A21F7 from Muschler and Horwitz, 1991), we were able to confirm the identity of our putative $\alpha 5$ integrin sequence by cDNA overexpression in mammalian cells followed by western immunoblotting. We used lysates from stage HH9 whole embryos for immunoblot standards (Figure 3A), because $\alpha 5$ protein expression decreases during later development (Muschler and Horwitz, 1991). Expression of our full-length chicken $\alpha 5$ integrin construct in mouse B16-F10 cells revealed very heavy immunostaining of a band at 150 kDa, confirming that our isolated chicken cDNA encodes $\alpha 5$ integrin (Figure 3B).

We then tested whether our chicken $\alpha 5$ integrin cDNA could express a functional fibronectin receptor. In transfection rescue experiments using the integrin $\alpha 5$ -deficient CHO cell line CHO-B2, we found that the defective cell adhesion of these cells to fibronectin could be rescued directly by expression of our chicken $\alpha 5$ integrin, as well as by a human $\alpha 5$ integrin cDNA, but not by a human αIIb integrin cDNA (Figure 3C). These results confirm that our isolated chicken cDNA encodes a functional $\alpha 5$ integrin fibronectin receptor.

We next used our cDNA to evaluate gene expression in early chicken embryos using whole-mount *in situ* hybridization. Using an RNA probe from *ITGA5-1* (Figure 1A, see Experimental Procedures), we found that the $\alpha 5$ integrin was weakly and ubiquitously expressed, but was particularly highly expressed in the anterior neural ridge (ANR) and cranial neural fold at stage HH8, as well as in the neural crest cells migrating into the embryonic body at HH 9 (arrowheads in Figure 4A, C and D). These $\alpha 5$ integrin mRNA expression patterns suggest a potential role of this integrin not only in cranial neural crest cell migration, but also possibly in the neural fold or neural crest formation.

We have previously shown that fibronectin enhances BMP5 activity, which is involved in chicken cranial neural crest formation (Endo et al., 2012). Consequently, we evaluated whether the full-length $\alpha 5$ integrin cDNA would be functionally active *in vivo*, testing the hypothesis that increased expression of this integrin might by itself induce specific growth factor or transcription factor expression. We first confirmed that transfection by our chicken $\alpha 5$ integrin plasmid could increase $\alpha 5$ integrin protein in cranial regions of chicken embryos by western blotting, documenting a 1.5 fold increase in chicken $\alpha 5$ integrin protein (Figure 5A). We then compared gene expression of four BMPs and seven transcription factors after this moderate over-expression of $\alpha 5$ integrin; isolation of neural folds and qPCR quantification revealed specific up-regulation of only *BMP5* expression. Furthermore, we found that the increased *BMP5* gene expression was specific to $\alpha 5$ integrin, with no response to αIIb integrin over-expression (Figure 5C). This finding demonstrates that our full-length clone can be used to test hypotheses in living early chick embryos, and it suggests that regulation of levels of an integrin receptor can specifically affect expression of a morphogenetic growth factor.

In summary, we have generated the full length chicken $\alpha 5$ integrin fibronectin receptor cDNA for use in functional studies of this molecule. We have identified its specific expression in the cranial neural fold during early chicken embryonic development, and find that its over-expression results in highly selective expression of the *BMP5* gene.

3. Experimental procedures

3.1. Experimental animals and RNA isolation for cDNA synthesis

Fertile chicken eggs were obtained from Charles River Laboratories, CT and were incubated at 38°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH), 1951. Cranial neural fold regions of HH stage 8 embryos were dissected using tungsten needles (Fine Science Tools) in ice-cold Hanks' balanced salt solution (HBSS, Invitrogen) supplemented with 1 mM MgCl_2 , 1 mM CaCl_2 , and 1 mM HEPES at pH 7.5. Isolated chicken embryo tissues were homogenized in TRIzol (Invitrogen), snap frozen in dry ice/ethanol, and then stored at -80°C overnight.

3.2. Isolation of integrin $\alpha 5$ cDNA by RT-PCR and RACE

cDNA was synthesized using SuperScript II and III Reverse Transcriptase (Invitrogen) for RT-PCR and isolation of full-length integrin $\alpha 5$. For RACE (Bertling et al., 1993; Frohman, 1993), the SMART RACE cDNA Amplification Kit (Clontech) was used according to the

manufacturer's protocol. RT-PCR or RACE was performed using KOD DNA polymerase (Toyobo) using a GeneAmp PCR system 9700 (Applied Biosystems). The sets of degenerate primers used for RT-PCR were: *ITGA5-1* (sense; 5'-TTYAGTGCNGAYTTYAC-3', antisense; 5'-CACGACGGTGAGCGAAG-3', 900 bp), *ITGA5-2* (sense; 5'-TCAATGATGTGGCTGTGGG-3', antisense; 5'-CATKGGGTTNCCYAGG-3', 998 bp), and the sets of primers used for RACE were *ITGA5-3* (sense; 5'-CTAATACGACTCACTATAGGGC-3', antisense; 5'-CACCTGCCCTTGCCAGAAGTAACTR-3', 925 bp) and *ITGA5-4* (sense; 5'-CTGCGGTGAGGACAACATCTGTG-3', antisense; AAGCAGTGGTATCAACGCAGAGT-3', 2711 bp). The degenerate primers were designed based on human, mouse, zebrafish, and *Xenopus* DNA sequences: N: N wobble (A+C+G+T), Y: Y wobble (C+T), R: R wobble (A+G), K: K wobble (G+T). The cDNA fragments were cloned into the PCRII-TOPO vector (Invitrogen) and confirmed by DNA sequencing.

3.3. Integrin $\alpha 5$ and $\beta 1$ integrin antibodies

Chicken integrin $\alpha 5$ (A21F7) and $\beta 1$ (V2E9) antibodies were from the Developmental Studies Hybridoma Bank and Chemicon International, respectively. These antibodies were used for western blotting after 4-12% polyacrylamide gel (Invitrogen) electrophoresis under non-reducing conditions.

3.4. In situ hybridization

Chicken $\alpha 5$ integrin gene fragment *ITGA5-1* (900 bp, see Figure 1A) was used as the RNA probe template. After restriction enzyme (Roche) linearization of the cDNA plasmid, digoxigenin-labeled antisense and sense RNA probes were synthesized by SP6 or T7 polymerases (Roche). In situ hybridization in whole-mounts was performed as described previously (Endo et al., 2002) with minor modifications. Briefly, after fixation in 4% paraformaldehyde (PFA, Sigma) / PBS at 4°C overnight, embryos were dehydrated in ice-cold 100% methanol. After re-hydration in PBST (PBS with 0.1% TWEEN 20, Sigma), the embryos were incubated with 1 μ g/ml proteinase K (Roche) for 10 minutes at room temperature (RT) and re-fixed in 4% PFA, 0.1% glutaraldehyde (Sigma) / PBST for 25 minutes at RT. The samples were washed with PBST and then incubated with digoxigenin (Roche)-labeled RNA probes at 65°C for 18 hours after 2 hours pre-hybridization. Following washing with TTBST (TBS containing 0.1% TWEEN 20, 0.5% Triton X-100, Sigma), the samples were incubated with anti-digoxigenin-AP antibody (Roche) in 10% heat-inactivated (63°C for 30 minutes) goat serum (Jackson) / TTBST at 4°C overnight. After extensive washing with TTBST for 5 hours, the in situ color reactions were developed in a mixture of BCIP and NBT solutions (Sigma) in color reaction buffer (100 mM Tris-HCl at pH 9.5, 150 mM NaCl, 25 mM MgCl₂, 0.5% Triton X-100) at RT. Stained whole embryos or sections were imaged on Stemi SV11 or Axiovert 135 microscopes (Zeiss), respectively, and images were adjusted for brightness and contrast using Photoshop 7.0.

3.5. Construction of integrin $\alpha 5$ expression vector

Based on the isolated DNA sequence information, full length of integrin $\alpha 5$ gene including the original stop codon was isolated from cDNA (see above) using the following primer pair: sense; 5'-GGATCCTCCGAGGGGACTCCGAAGC-3', antisense; 5'-GGTGCTGCCCCGGTATGACG-3' using KOD DNA polymerase. The PCR product was at first cloned into the PCRII-TOPO vector, and then was subcloned into the pEF6 expression vector (Invitrogen) using same primer set. The sequence of the full length cDNA was confirmed by DNA sequencing.

3.6. Cell lines

Mouse melanoma cells (B16-F10, ATCC) were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Integrin $\alpha 5$ -deficient Chinese hamster ovary cells (CHO-B2, Schreiner et al., 1989) were kindly provided by Dr. Rick Horwitz and were cultured as described previously (Laukaitis et al., 2001). Lipofectamine 2000 (Invitrogen) was used for transfection of control EGFP (Clontech), chicken or human integrin $\alpha 5$, and human integrin αIIb expression plasmids into B16-F10 or CHO-B2 cells according to the manufacturer's protocol. After 48 hours, the cells were lysed in RIPA buffer and cell lysates used for western blotting; alternatively, the cells were used for the cell adhesion assay described below

3.7 Cell adhesion assay

Cell adhesion was assayed as described previously (Humphries, 2001; Hozumi et al., 2006). Briefly, Lab-Tek II glass chamber slides (Nalge Nunc International, #154941) were coated with 20 µg/ml fibronectin in Dulbecco's PBS without calcium or magnesium (DPBS) overnight at 4°C. After washing with DPBS, non-specific sites on the substrate were blocked with 1% BSA in DMEM at 37°C for 1 hour. CHO-B2 cells were cultured for 48 hours after transfection with control EGFP, chicken or human integrin $\alpha 5$, or human integrin αIIb plasmids. They were suspended using trypsin-EDTA and allowed to recover from the enzymatic treatment by incubation for 30 min in 10% FBS / DMEM at 37°C. The cells were then washed three times with serum-free 0.1% BSA / DMEM, and 1×10^5 cells in 0.1% BSA / DMEM were seeded in the fibronectin-coated glass chamber wells. After incubation for 1 hour at 37°C, the chambers were gently washed twice with DPBS, and adherent cells were fixed in 4% PFA / DPBS at room temperature for 20 min. Adherent cells were counted in randomly selected 2.8 mm² regions (n=5).

3.8 Transfection and quantitative PCR

Culture of HH4⁺ to HH5 embryos and electroporation were performed exactly as described previously (Endo et al., 2012; Endo, 2012). For checking transfection efficiency, 0.5 mM fluorescein isothiocyanate (FITC)-labeled control morpholino (Gene Tools) was mixed with EGFP as the control or with the chicken $\alpha 5$ integrin expression vector. Materials for electroporation were injected onto the entire cranial neural ectoderm region of the embryos placed in an electroporation chamber from Unique Medical Imada (Japan). After electroporation, the embryos were cultured at 38°C for 10-11 hr to HH9. Then, cranial neural plates were dissected, and total RNA was isolated as above. cDNA synthesis was performed using the SuperScript III or VILO cDNA synthesis kit (Invitrogen), and quantitative PCR (qPCR) was performed using Power SYBR green (Applied Biosystems) using the 7900HT Fast Real-Time PCR system (Applied Biosystems). The sets of primers used for qPCR were described previously (Endo et al., 2012).

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Highlights

- We report the full-length sequence of chicken integrin $\alpha 5$ (ITGA5).
- ITGA5 mRNA is expressed in cranial neural folds and migrating neural crest cells.
- Over-expression of ITGA5 in the neural tube selectively induces BMP5.
- This full-length cDNA and sequence provide new tools for in vivo research on ITGA5.

Figure 1. Isolation of chicken $\alpha 5$ integrin gene

(A) Schematic map for isolation of full length chicken $\alpha 5$ integrin gene (4888 bp). Gene fragment *ITGA5-1* and *-2* were isolated by RT-PCR, and *ITGA5-3* and *-4* were isolated by 5'- and 3'-RACE, respectively. *ITGA5-3* and *ITGA5-4* includes 5'UTR (240 bp) and 3'UTR (1486 bp), respectively. (B) Predicted amino acid sequences of chicken $\alpha 5$ integrin.

Figure 2. Sequence comparisons of chicken with human and mouse $\alpha 5$ integrin

(A) Comparison of mouse and chicken amino acid sequences at specific domains; the numbers indicate percent amino acid identity. (B) Alignment of an amino acid segment of human $\alpha 5$ integrin reported to be important for ligand-binding specificity with mouse and chicken sequences. (C) Alignment of the cytoplasmic domains of $\alpha 5$ integrin protein between different species.

Figure 3. Chicken α5 integrin functions as a fibronectin receptor

(A) Western blotting of α5β1 integrin. Lysate from whole embryos of stage HH9 was blotted under non-reducing condition for α5 integrin (1) and β1 integrin (2). (B) Chicken α5 integrin was overexpressed in mouse B16F10 cells, and then the cell lysate was blotted for anti-chicken α5 integrin antibody. (C) Cell adhesion assay. Chicken or human α5 integrin, human αIIb integrin, or control EGFP were overexpressed in α5 integrin-deficient Chinese hamster ovary cells (CHO-B2) for 48 hours. Their capacity to mediate cell adhesion to fibronectin was evaluated by quantifying numbers of cells adherent to randomly selected 2.8 mm² microscope fields (n=5).

Figure 4. $\alpha 5$ integrin mRNA is expressed in the cranial neural fold of chicken embryos

The mRNA expression was examined by whole-mount in situ hybridization (A and B as a sense control) and in some embryos the expression was examined on cryostat sections after whole-mount in situ hybridization (C and D). $\alpha 5$ integrin mRNA is highly expressed in the anterior neural ridge and neural crest at HH8 embryo (A and C, arrowheads) and in the migrating cranial neural crest cells (B, arrowheads).

Figure 5. Elevated expression of $\alpha 5$ integrin in the neural tube upregulates *BMP5* expression
 (A) Western blotting of $\alpha 5$ integrin (ITGA5) protein in cranial regions of chicken embryos transfected with chicken $\alpha 5$ integrin plasmid. Lysates from cranial regions of stage HH9 embryos overexpressing control EGFP or chicken $\alpha 5$ integrin were western blotted under non-reducing conditions for $\alpha 5$ integrin and GAPDH. (B) Gene expression of neural crest genes by qPCR. Cranial neural plate of HH9 control EGFP or $\alpha 5$ integrin over-expressed embryos (each $n=3$) were dissected, and total RNA was used for qPCR. (C) *BMP5* expression by qPCR after expression of different integrin subunits. Cranial neural plate of HH9 embryos over-expressing control EGFP, chicken $\alpha 5$ integrin, or human αIIb integrin (each $n=3$) were dissected, and total RNA was used for qPCR quantification.