Molecular Cloning and Characterization of Chick CD9

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Summary: CD9 is a tetra-membrane-spanning glycoprotein involved in cell adhesion, migration, growth signaling and tumor cell metastasis. In this study a CD9 cDNA clone has been isolated from chick cDNA library. The chick CD9 clone contains an open reading frame of 224 amino acids. The deduced amino acid sequence from chick CD9 shows 63.3% identity with the human counterpart. Human and monkey CD9 upregulate the diphtheria toxin (DT) binding activity of human heparin-binding EGF-like growth factor, while chick CD9 lacks the ability to upregulate the DT binding activity. Comparison of the amino acid sequence of chick CD9 with that of human CD9 gives a clue to understanding the upregulation mechanism.

Key words HB-EGF, diphtheria toxin, CD9, DNA sequence, tetra-membrane-spanning glycoprotein, TM4SF

INTRODUCTION

CD9, a tetra-membrane-spanning glycoprotein of 24-27 kDa, was initially reported to be expressed in preB cells and platelets [1,2] but further studies have revealed that it is expressed in a wide variety of hematopoietic and nonhematopoietic cells [3-6]. Increasing evidence suggests that this protein is involved in cell signaling [7,8], cell growth [9], cell motility [10,11], cell adhesion [12-14], tumor cell metastasis [15-17] and development and maintenance of the neural system [18-21].

One characteristic feature of CD9 is the high potential of this protein to form complexes with other membrane proteins [11,22-24]. Earlier studies demonstrated that CD9, originally reported as DRAP27 [5], associates with diphtheria toxin receptor (DTR) and that DRAP27/CD9 upregulates the diphtheria toxin (DT) binding activity of DTR [6,22]. DTR is also known to be the membrane-anchored form of heparin-binding EGF-like growth factor (proHB-EGF) [25]. Not only the DT binding activity, but also the juxtacrine mitogenic activity of proHB-EGF is upregulated by CD9 [9]. CD9 also forms complexes with integrin $\alpha 3 \beta 1$ [23], $\alpha 6 \beta 1$ [24,26] and the neural adhesion molecule L1 [11]. In

monkey Vero cells, complexes comprising CD9, proHB-EGF and integrin $\alpha 3 \beta 1$ localize at cell-cell adhesion sites, suggesting the involvement of those complexes in intercellular signaling.

CD9 belongs to a member of the tetra-membrane-spanning protein superfamily (TM4SF), which includes CD37, CD53, CD63, CD81 and CD82 [27]. This family of proteins is characterized by four trans-membrane domains and conserved cysteine residues at the putative extracellular domain. Although the general function of this family has not been shown, this family of proteins has been described in a wide variety of animal cells including *C. elegans* and *Drosophila* [27]. Because of this family's members tendencies to form complexes with a variety of membrane proteins, they are thus thought to be 'adapters for membrane proteins,' or 'molecular facilitators,' that mediate the formation of large molecular complexes and allow them to function more efficiently [27,28].

This paper describes molecular cloning of CD9 cDNA from a chick embryo spinal cord cDNA library and compare the amino acid sequence with its mammalian counterparts. This report is the first description of a vertebrate CD9 other than from mammals. Using the isolated chick CD9 cDNA, the

upregulation activity of chick CD9 for the DT binding of proHB-EGF was examined.

MATERIALS AND METHODS

Cloning of chick CD9 cDNA

Fragments encoding the open reading frame of mouse CD9 cDNA was labeled with ^{32}P and used to screen a chick CD9 lambda ZAP embryonal spinal cord cDNA library (Clonetec). Hybridization was carried out under the following conditions: pre-hybridization at 60 °C for 2 hrs in church buffer (7% SDS, 560 μM $\text{Na}_4\text{P}_2\text{O}_7$, 85.5 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 2 mM EDTA) followed by hybridization under the same conditions for 16 hrs in the presence of the ^{32}P -labeled probe. The filters were washed three times with 2 $\times$ sodium citrate (SSC) containing 0.1% SDS at 55 °C for 15 min. Positive clones were isolated, subcloned into pBluescript II SK(−). Nucleotide sequences were determined by the dideoxy chain termination method using DNA Sequencing Kit with AmpliTaq DNA Polymerase (Perkin Elmer) and a DNA Sequencing System, Model 373S (Perkin Elmer).

Plasmid constructions

Plasmids encoding monkey CD9 (pRcT1843) were used as described previously [6]. pRc/Flag was constructed from pRc/CMV (Invitrogen Corp): The synthetic oligonucleotides encoding Flag sequence (5'-AAAAAATCTAGAGACTACAAGGACGACGATGACAAGTGAAGGGCCCTTTTT-3', and 5'-AAAAAAGGGCCCTTCACTTGTCATCGTCGTCCTTGTAGTCTCTAGATTTTT-3') were annealed, digested with *Xba*I and *Apa*I and then inserted into *Xba*I/*Apa*I sites of pRc/CMV. To express Flag-tagged monkey CD9 protein, the cDNA encoding monkey CD9 (pRcT1843) was amplified by polymerase chain reaction with 5'-GTAATACGACTCACTATAGGGC-3' and 5'-AAAAAATCTAGAATAGACCATCTCGCGGT-3' as primers. The amplified fragments were digested with *Hind*III and *Xba*I and inserted into the corresponding sites of pRc/Flag. Chick CD9 cDNA was subcloned into *Xba*I and *Apa*I sites of pRc/CMV, and the resulting plasmid named pRc/chCD9. To express Flag-tagged chick CD9 protein, DNA fragments were amplified using pRc/chCD9 as a template with 5'-GTAATACGACTCACTATAGGGC-3' and 5'-ATTAATTAGCGGCCGCCGGACCATTTCTCTGTTTCT-3' as primers. The amplified fragments were digested with

*Hind*III and *Not*I and inserted into the corresponding sites of pRc/Flag.

SDS-PAGE and immunoblot

Cells were lysed by 1% Triton X-100 in lysis buffer (0.15 M NaCl, 2 mM EDTA, 1 mM PMSF, 1 mM NaVO_4 , 50 mM Tris, pH7.5), then lysates were centrifuged for 20 min at 15,000 g. The supernatants were then boiled for 5 min in SDS-PAGE sample buffer without reducing agents, subjected to SDS-PAGE (15% gel) and electrotransferred to an Immobilon membrane. The membrane was blocked with 3% skim milk at room temperature for 60 min, then incubated at room temperature with the first antibody at a concentration of 2 $\mu\text{g}/\text{ml}$ in Tris-HCl buffered saline (TBS (20 mM Tris-HCl, 100 mM NaCl, pH 7.5)) containing 1% skim milk and 0.2 M NaCl. After washing with TTBS (TBS containing 0.05% Tween 20), the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Chemicon) at a concentration of 2 $\mu\text{g}/\text{ml}$, washed with TTBS, and analyzed with an ECL-Western blotting kit (Amersham International).

Cell culture and transfection

Mouse LH cells, which are a stable transformant of mouse L cells expressing human proHB-EGF, were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin G and 100 $\mu\text{g}/\text{ml}$ of streptomycin. Transfection of plasmids into LH cells was done as described previously [29]. Transfected cells were cultured for 48 hrs and then used for further studies.

DT binding assay

Purified DT was labeled with Na^{125}I using enzymebeads as reported previously [30]. The labeled DT had a specific activity of about 1.5×10^4 cpm/ng. LH cells were plated on 12-well plates at 5×10^4 cells/well and incubated for 12 hrs at 37 °C. The cells were incubated with ^{125}I -labeled DT (100 ng/ml) for 8 hrs at 4 °C in the binding medium (MEM containing nonessential amino acids, 5% FCS and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.2). Then cells were washed three times with PBS (phosphate-buffered saline) containing 1% FCS, lysed with 0.1N-NaOH and cell-associated radioactivity was measured by a γ -counter. Nonspecific binding of ^{125}I -labeled DT was assessed in the presence of a 100-fold excess of unlabeled DT. Specific binding was determined by subtracting the

nonspecific binding from the total binding obtained with ^{125}I -DT alone. The nonspecific binding was less than 10% of the total binding.

RESULTS

Molecular cloning of chick CD9 cDNA

A fragment of mouse CD9 cDNA was used to screen a cDNA library made from chick embryo spinal cord. By 2 cycle hybridization screening as described in MATERIALS and METHODS, one of the positive clones was isolated. Figure 1 shows the DNA sequence of the cloned cDNA and the pre-

dicted amino acid sequence. The chick CD9 cDNA comprises 865 nucleotides containing 20 nucleotides 5'-untranslated region, an open reading frame of 672 nucleotides and 173 nucleotides 3'-untranslated region with a poly adenylation signal TATAAA. The open reading frame encodes a protein of 224 residues in length, and the sequence around the first ATG (GGAATGC) agrees with the consensus sequence for the initiation of translation. The predicted sequence has four hydrophobic domains which would be deduced as transmembrane domains. One potential N-glycosylation site is located at residue 51 and the conserved CCG motif found in TM4SF proteins at positions 148-150. A comparison of the

-20	CGTTGCTATCCGTCAGCGGA	0
1	ATGCCTGTCAAAGGAGGCACTAAGTGCATCAAATACTTGCTCTTTGGCTTCAACTTCGTA	60
1	M P V K G G T K C I K Y L L F G F N F V	20
61	TTCTGGCTTGCAGGAACAGCAGTTCTTGCAATTGGACTGTGGCTTCGATTGATGTGCAG	120
21	F W L A G T A V L A I G L W L R F D V Q	40
121	ACCAAAAGCATCTTTGAACTGGAATCCAACAACATGACATTCTATACAGGAGTTTACATC	180
41	T K S I F E L E S N N M T F Y T G V Y I	60
181	CTAATTGGAGCTGGTGCACATCATGATGCTGGTGGCTTCTGGGATGCTGTGGTGCATTG	240
61	L I G A G A L M M L V G F L G C C G A L	80
241	CAAGAATCACAGTGATGCTTGGACTGTTCTTCGCTTCCTCTTTGTGATTTTGGCCCTT	300
81	Q E S Q C M L G L F F V F L F V I F A L	100
301	GAAATTGCTACTGCCATCTGGGGATTGCAAATAAGAAAGATTGTCGAAGAGTTACAG	360
101	E I A T A I W G F A N K E K I V E E L Q	120
361	GACTTCTACAGAGAAACCTATGAAAAGAGATCTCAACCAGCTGCCAGAGAAATCTTGAA	420
121	D F Y R E T Y E K R S Q P A A R E I L K	140
421	GCATTCCATTTTCTGCTGAACTGCTGTGGTATTACTGGAGGTCTTGAGCAGCAGTTGATG	480
141	A F H F A L N C C G I T G G L E Q Q L M	160
481	GGAATCCTGTCCAAAGAAGACCTTGCCTGAGTCATTTACTACATGCCATGCCCTAAAGCC	540
161	E S C P K K T L P E S F T T M P C P K A	180
541	ATTGATGATGCTTTAATTCAAACCTGAATGTCATTGGAGCAGTTGGCCTTGGTATTGCT	600
181	I D D V F N S K L N V I G A V G L G I A	200
601	GTGATAATGATTTTGGCATGATATTCAGTATGGTCTTTGCTGTGCTATCCGCAGAAAC	660
201	V I M I F G M I F S M V L C C A I R R N	220
661	AGAGAAATGGTCTAAGAGCTGTAAATCCAAGGTGTCTGCACTATAAAATCTGTCACTAAC	720
221	R E M V *	224
721	TCTAGTGTCTCAAAGCAGTTCTAAAAGAAAATTGTATGTTACCTTTTAAATGCTTTATT	780
781	TGGTACTGGTGTAGATTGCTTTTATGCTATAGGTTAAAATGATAAAGTTATATCTGAC	840
841	TCGAG	845

Fig. 1. Nucleotides sequence of chick CD9 cDNA and the predicted amino acid sequence. The putative transmembrane domains and the conserved CCG motif in TM4SF proteins are underlined and box-shaded, respectively. The potential N-glycosylation site is indicated by a double underline. The nucleotide sequence of chick CD9 cDNA has been deposited in the DDBJ, EMBL and GenBank under Accession No. AB032767.

Chick CD9	1	MPVKGGTKCIKYL	LF	GFNF	IF	WLAGI	AV	LAIGL	WLR	FD	SQ	TKS	I	F	E	Q	T	N	N	N	S	S	F	Y	T	G	V	58																			
Human CD9	1	MPVKGGTKCIKYL	LF	GFNF	IF	WLAGI	AV	LAIGL	WLR	FD	SQ	TKS	I	F	E	Q	T	N	N	N	S	S	F	Y	T	G	V	60																			
Monkey CD9	1	MPVKGGTKCIKYL	LF	GFNF	IF	WLAGI	AV	LAIGL	WLR	FD	SQ	TKS	I	F	E	Q	T	N	N	N	S	S	F	Y	T	G	V	60																			
Mouse CD9	1	MPVKGGTKCIKYL	LF	GFNF	IF	WLAGI	AV	LAIGL	WLR	FD	SQ	TKS	I	F	E	Q	T	N	N	N	S	S	F	Y	T	G	V	58																			
Rat CD9	1	MPVKGGTKCIKYL	LF	GFNF	IF	WLAGI	AV	LAIGL	WLR	FD	SQ	TKS	I	F	E	Q	T	N	N	N	S	S	F	Y	T	G	V	58																			
Feline CD9	1	MPVKGGTKCIKYL	LF	GFNF	IF	WLAGI	AV	LAIGL	WLR	FD	SQ	TKS	I	F	E	Q	T	N	N	N	S	S	F	Y	T	G	V	58																			
Bovine CD9	1	MPVKGGTKCIKYL	LF	GFNF	IF	WLAGI	AV	LAIGL	WLR	FD	SQ	TKS	I	F	E	Q	T	N	N	N	S	S	F	Y	T	G	V	58																			
TM 1																																															
Chick CD9	59	YILIGAGALMML	VG	FL	GCC	GA	LV	QES	Q	C	M	L	G	L	F	F	V	I	F	A	I	E	A	A	I	W	G	Y	118																		
Human CD9	61	YILIGAGALMML	VG	FL	GCC	GA	LV	QES	Q	C	M	L	G	L	F	F	V	I	F	A	I	E	A	A	I	W	G	Y	120																		
Monkey CD9	61	YILIGAGALMML	VG	FL	GCC	GA	LV	QES	Q	C	M	L	G	L	F	F	V	I	F	A	I	E	A	A	I	W	G	Y	120																		
Mouse CD9	59	YILIGAGALMML	VG	FL	GCC	GA	LV	QES	Q	C	M	L	G	L	F	F	V	I	F	A	I	E	A	A	I	W	G	Y	118																		
Rat CD9	59	YILIGAGALMML	VG	FL	GCC	GA	LV	QES	Q	C	M	L	G	L	F	F	V	I	F	A	I	E	A	A	I	W	G	Y	118																		
Feline CD9	59	YILIGAGALMML	VG	FL	GCC	GA	LV	QES	Q	C	M	L	G	L	F	F	V	I	F	A	I	E	A	A	I	W	G	Y	118																		
Bovine CD9	59	YILIGAGALMML	VG	FL	GCC	GA	LV	QES	Q	C	M	L	G	L	F	F	V	I	F	A	I	E	A	A	I	W	G	Y	118																		
TM 2																																															
Chick CD9	119	LQEFYRETYEK	-RS	-QPAARE	IL	KAF	HF	FA	IN	CCG	IT	GG	LE	Q	LM	ES	CP	KK	T	LES	FT	IM	E					176																			
Human CD9	121	VQEFYKDTYN	KL	KT	K	D	E	P	O	R	E	T	L	K	A	I	H	Y	A	L	CCG	L	A	GG	VE	Q	F	I	S	D	I	C	P	K	K	D	V	L	H	T	F	T	V	K	S		180
Monkey CD9	121	VQEFYKDTYN	KL	KT	K	D	E	P	O	R	E	T	L	K	A	I	H	Y	A	L	CCG	L	A	GG	VE	Q	F	I	S	D	I	C	P	K	K	D	V	L	H	T	F	T	V	K	S		180
Mouse CD9	119	LQEFYKDTY	KL	KS	K	D	E	P	O	R	E	T	L	K	A	I	H	M	A	L	CCG	I	A	GL	EQ	F	I	S	D	I	C	P	K	K	O	L	LES	F	O	V	K	E		178			
Rat CD9	119	LQEFYKDTY	KL	KN	K	D	E	P	O	R	E	T	L	K	A	I	H	M	A	L	CCG	I	A	GG	VE	Q	F	I	S	D	I	C	P	K	K	O	V	L	ES	F	O	V	K	S		178	
Feline CD9	119	VQEFYKDTYN	KL	KS	K	D	E	P	O	R	E	T	L	K	A	I	H	Y	A	L	CCG	L	A	GG	VE	Q	F	I	S	D	I	C	P	K	K	O	L	LES	F	O	V	K	E		178		
Bovine CD9	119	VQEFYKDTYN	KL	KN	K	D	E	P	O	R	E	T	L	K	A	I	H	Y	A	L	CCG	L	A	GG	VE	Q	F	I	S	D	I	C	P	K	K	O	L	LES	F	O	V	K	E		178		
TM 3																																															
Chick CD9	177	CHKAIDVFN	SK	LN	V	I	G	A	V	G	I	A	V	M	I	F	G	M	I	F	S	M	L	CC	A	I	R	R	N	R	E	M	V										224				
Human CD9	181	CHDAIKEVFN	SK	N	F	H	I	I	G	A	V	G	I	A	V	M	I	F	G	M	I	F	S	M	L	CC	A	I	R	R	N	R	E	M	V									228			
Monkey CD9	181	CHDAIKEVFN	SK	N	F	H	I	I	G	A	V	G	I	A	V	M	I	F	G	M	I	F	S	M	L	CC	A	I	R	R	N	R	E	M	V									228			
Mouse CD9	179	CHFAISEVFN	SK	N	F	H	I	I	G	A	V	G	I	A	V	M	I	F	G	M	I	F	S	M	L	CC	A	I	R	R	N	R	E	M	V									226			
Rat CD9	179	CHDAIDEVFN	SK	N	F	H	I	I	G	A	V	G	I	A	V	M	I	F	G	M	I	F	S	M	L	CC	A	I	R	R	N	R	E	M	V									226			
Feline CD9	179	CHFAIKEVFN	SK	N	F	H	I	I	G	A	V	G	I	A	V	M	I	F	G	M	I	F	S	M	L	CC	A	I	R	R	N	R	E	M	V									226			
Bovine CD9	179	CHFAIDEVFN	SK	N	F	H	I	I	G	A	V	G	I	A	V	M	I	F	G	M	I	F	S	M	L	CC	A	I	R	R	N	R	E	M	V									226			
TM 4																																															

Fig. 2. Alignment of the amino acid sequences of CD9 proteins from various species. The conserved amino acids are enclosed in the box. Gaps are indicated as -. An underline indicates the putative transmembrane domains. The most divergent region is the second extracellular domain.

amino acid sequence of the chick CD9 with other mammalian CD9 is shown in Fig. 2. The chick CD9 shows 63.3% identity with mammalian CD9. As shown in mammalian CD9, the transmembrane domains are highly conserved among chick and mammalian CD9, while the putative second extracellular domain shows relatively large inter-species differences.

Upregulation of the DT binding activity of DTR/proHB-EGF by CD9

CD9 associates with proHB-EGF/DTR. Human or monkey CD9 upregulates DT binding activity of human proHB-EGF/DTR when CD9 is co-expressed with this protein, though CD9 itself does not have the DT binding property [9,22]. Although this upregulation of the DT binding activity by CD9 has been shown to be due to protein-protein interaction, the precise mechanism of upregulation remains to be elucidated. In order to know whether chick CD9

upregulates the DT binding activity of human proHB-EGF/DTR, DT binding assay was performed using cells expressing chick CD9 as follows.

The isolated chick CD9 cDNA clone was subcloned into the eukaryotic expression vector pRc/CMV, the resulting plasmid is referred to pRc/chCD9. For generating CD9 derivatives with known epitope tags, plasmids encoding chick or monkey CD9 with the Flag tag in their C-terminal portions, designated as pRc/chCD9-F and pRcT 1843-F, respectively, were also constructed. Mouse LH cells, which are stable transformants of mouse L cells expressing human DTR/proHB-EGF, were transfected with plasmids encoding chick CD9 (pRc/chCD9), monkey CD9 (pRcT1843) and their Flag-tagged derivatives (pRc/chCD9-F and pRcT 1843-F). The cell lysates were subjected to western blotting analysis (Fig. 3). The anti-human CD9 mAb (TP82) did not cross-react with chick CD9 (data not shown), but anti-Flag antibody showed that Flag-

Fig. 3. Western Blotting analysis of chick CD9. LH cells were transfected with cDNA encoding monkey CD9, Flag-tagged monkey CD9 (monkey CD9-F) or Flag-tagged chick CD9 (chick CD9-F). Forty-eight hours after transfection cell lysates were analyzed by Western blotting using anti-Flag antibody M2 and anti-human CD9 mAb BU16.

Fig. 4. Effect of CD9 expression on DT binding activity of human proHB-EGF. LH cells were transfected with cDNA encoding monkey CD9, chick CD9 or their Flag-tagged derivatives (monkey CD9-F and chick CD9-F). Forty-eight hours after transfection specific binding of ¹²⁵I-labeled DT to the transfected cells was determined as described in Materials and Methods. Data represent the mean $\pm$ S.D. of the results obtained from triplicate samples.

tagged chick CD9 was expressed in LH cells as Flag-tagged monkey CD9 was expressed.

Fig. 4 shows the DT binding activity of LH cells transfected with the CD9 constructs. DT binding of LH cells was upregulated 10 fold by the expression of monkey CD9, while it was not upregulated by chick CD9. Flag-tagged monkey CD9 also upregulated the DT binding activity, the same as the native monkey CD9, while Flag-tagged chick CD9 did not upregulate the DT binding activity, though Flag-tagged chick CD9 was expressed in LH cells, the same as Flag-tagged monkey CD9. These results indicate that chick CD9 is not competent to upregulate DT binding activity of human DTR/proHB-EGF.

DISCUSSION

Increasing evidence suggests that CD9 and TM4SF proteins are involved in a wide variety of cellular processes during embryonal development and the maintenance of mature individuals [31,32]. In this study chick CD9 cDNA was isolated, and it is the first description of vertebrate CD9 other than in mammals. Because chick is one of the most characterized animals in developmental biology, isolation of a chick counterpart of CD9 would be a useful for the further studies of CD9 functions in development.

The highly conserved amino acid sequence of the

transmembrane domains of chick CD9, in line with those of other mammalian CD9, implies the importance of those domains in the fundamental role of this protein. Possibly, the regulated formation of complexes of CD9 with various other membrane proteins as a 'molecular facilitator' could be the central role of CD9 and TM4SF proteins [28]. The transmembrane domains might, thus, be important for complex formation.

CD9 is reported to be involved in cell signaling [7,8], cell growth [9], cell motility [10,11], cell adhesion [12-14], tumor cell metastasis [15-17] and development and maintenance of neural system [18-21]. However, despite such possibilities of fundamental roles, evidence to demonstrate the function of CD9 at the molecular level is limited. One of the functions of CD9 clearly demonstrated is the upregulation activity of CD9 for proHB-EGF. CD9 greatly upregulates DT binding activity and the juxtacrine mitogenic activity of proHB-EGF [9,22]. Although this upregulation of proHB-EGF activities by CD9 is achieved protein-protein interaction, the precise mechanism of upregulation remains to be elucidated. Present studies indicated that chick CD9 is not able to upregulate DT binding activity, probably due to amino acids alterations between chick CD9 and monkey CD9. Further studies will give a clue to understanding the essential regions of CD9 for upregulation, and consequently the upregulation mechanism.

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