

## Structural Effects of the GXXXG Motif on the Oligomer Formation of Transmembrane Domain of Syndecan-4

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Syndecan-4 (heparan sulfate proteoglycan), biologically important in cell-to-cell interactions and tumor suppression, was studied through mutation of the GXXXG motif of its transmembrane domain (Syd4-TM), a motif which governs dimerization. The expression and purification of the mutant (mSyd4-TM) were optimized here to assess the function of the GXXXG motif in the dimerization of Syd4-TM. mSyd4-TM was obtained in M9 minimal media and its oligomerization was identified by SDS PAGE, Circular Dichroism (CD) spectroscopy, mass spectrometry and NMR spectroscopy. The mutant, unlike Syd4-TM, did not form dimers and was observed as monomers. The GXXXG motif of Syd4-TM was shown to be an important structural determinant of its dimerization.

**Key Words :** Syndecan-4, Transmembrane, GXXXG, Oligomerization, NMR spectroscopy

### Introduction

Syndecan-4, distributed across cells' surfaces, regulates various human physiological functions, such as cell-to-cell interaction, cell adhesion and wound healing.<sup>1-4</sup> It is over-expressed by tumorigenesis in cell membranes.<sup>5-7</sup> The over-expression has been shown to be stimulated by tumor suppressing molecules, such as tumor necrosis factor- $\alpha$ , and to revert anti-adhesion molecules that enhance tumor cells' metastasis.<sup>8-10</sup>

Syndecan-4 comprises three major sections: extracellular, transmembrane and cytoplasmic domains. Its adhesive function with other cells or ligands occurs in the extracellular domain *via* glycosaminoglycan attachment. These extracellular functions are regulated by the its cytoplasmic domain that binds with PI-4,5-bisphosphate and activated protein kinase C- $\alpha$ . The signals from the cytoplasmic domain are mediated by structural changes in syndecan-4's transmembrane domain (Syd4-TM).<sup>4</sup> Therefore its transmembrane domain's structure greatly affects the function of syndecan-4.

The oligomerization of syndecan-4 is an important structural property in cell membranes.<sup>11-13</sup> Oligomerization is predominately affected by the core parts of each of syndecan-4's regions. In the transmembrane region, the conserved GXXXG sequence of the syndecan family is most important and mutation of this sequence in Syd4-TM can prevent oligomerization.<sup>14</sup> The effects of the GXXXG sequence have been previously reported but only by examining Syd4-TM with the other regions of syndecan-4 or fusion proteins since the expression and purification of pure Syd4-TM is difficult.<sup>14-17</sup> Pure Syd4-TM has been expressed and purified; it was shown to form dimers.<sup>18</sup>

This work reports the assessment of the importance of the GXXXG sequence to the oligomerization of Syd4-TM. The expression and purification of mutant Syd4-TM protein

were optimized. The mutated LG8LG12 Syd4-TM(mSyd4-TM: GXXXG  $\rightarrow$  LXXXL) was obtained at 4 mg/L. Its structure was investigated by SDS-PAGE, CD spectroscopy, mass spectrometry and NMR spectroscopy. Large quantities of mSyd4-TM were sought for the determination of its original structure by solid-state NMR spectroscopy in environments that mimic membranes, such as bicelles or lipid bilayers.

### Materials and Method

**Cloning.** Complementary 96-base oligonucleotide encoding sequences were chemically synthesized by IDT (Integrated DNA Technology, USA). Mutant Syd4-TM comprised 25 residues (VLAAL IVLGV VLILF AVFLI LLLVY) followed by 6 moderately polar residues consisting of two glycines and four lysines (GGKKKK) to improve solubility. The GXXXG sequence of Syd4-TM was selected for modification to prohibit the region's dimerization. Two glycine residues (G8 and G12) of Syd4-TM were mutated to leucine; the other residues remained unaltered. The sense and anti-sense primers were annealed at 95 °C and gradually cooled to room temperature over 30 min. N- and C-termini were generated after annealing, so that the DNA fragments could be flanked by AlwN I (Figure 1(b)). The annealed DNA samples were identified by 2% agarose gel electrophoresis and purified from the gel. They were then ligated into AlwN I digested pET31b(+) vector (Novagen, USA) expression plasmid. The insertion of the mSyd4-TM gene was confirmed by transforming the ligated mixture into *Novablue* competent cells (Novagen, USA). The plasmid DNA was purified and digested with XbaI and XhoI (New England Biolabs, USA) restriction enzymes. The restricted vectors including the fusion protein were isolated and identified from 1.5% agarose gel. Maximum expression efficiency of the fusion protein was achieved by transforming the recombinant plasmid

with single insert into three *Escherichia coli* (*E. coli*) strains as a expression host cells classified by BLR(DE3)pLysS, (Novagen, USA), C41(DE3) and C43(DE3) (Avidis, France). They were plated on LB agar media with carbenicillin (Amresco, USA) antibiotics and incubated overnight at 37 °C.

A well-incubated single colony was selected and transferred to 5 mL LB with carbenicillin and incubated overnight at 37 °C in an incubating shaker. 50 µL cell culture was transferred to 5 mL LB with carbenicillin and grown at 37 °C in the shaker. When the cells' OD at 600 nm reached 0.5, protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Noble Sciences, Inc., USA). The expression extent of the fusion protein in the IPTG-induced cultures was confirmed by 12% SDS-PAGE. Given the SDS-PAGE results, *E. coli* C41(DE3) cells transformed with pET31b(+) vector containing a single insert of the mSyd4-TM construct were selected for large-scale expression and stored.

**Expression of the KSI-mSyd4-TM Protein.** 500 µL C41(DE3) cells including the mSyd-TM fusion protein gene was transferred to 50 mL LB medium supplemented with carbenicillin and grown for 14-16 h at 37 °C in the shaker. For larger scale expression, 10 mL well grown culture was then transferred to 1 L M9 minimal media and incubated at 37 °C with shaking at 200 rpm. Structural studies were conducted by NMR spectroscopy, using uniformly <sup>15</sup>N labeled proteins over-expressed in M9 media containing 1 g/L <sup>15</sup>N-enriched ammonium sulfate (Cambridge Isotope Lab, USA).

When OD<sub>600</sub> reached about 0.5, IPTG was added at a final concentration of 1 mM to the growing culture to induce protein expression. After 16 h of induction, cells were harvested by centrifugation (60000 g, 4 °C, 30 min) and stored at -80 °C for 3 h for further purification.

**Purification of the KSI-mSyd4-TM Fusion Protein.** Frozen cells from the 1 L culture were resuspended in 100 mL lysis buffer containing 20 mM Tris(Amresco, USA), 500 mM NaCl (Sigma, USA) and 15% glycerol (Amresco, USA). 0.5 mg/mL Lysozyme (Sigma, USA) was added to catalyze the lysis. The cells were disrupted using a VC 505 ultrasonic processor with a tapered microtip (Sonics, USA). Heating of the cell mixture was prevented by holding the cell mixture in an ice bath. After ultrasonication, the lysate was centrifuged at 13,200 rpm for 30 min at 4 °C. The inclusion body pellet was collected, dissolved for column chromatography by stirring in 100 mL Ni-NTA binding buffer containing 20 mM Tris, 500 mM NaCl, 5 mM imidazole (Alfa Aesar, USA), and 6 M guanidine hydrochloride (Sigma, USA) at pH 8.0 overnight at room temperature. The dissolved inclusion body was centrifuged at 13,200 rpm for 30 min at 4 °C for the removal of residual impurities. Before loading the protein, the column was first packed with Ni-NTA agarose resin (Qiagen, USA), charged with 50 mM NiSO<sub>4</sub> and equilibrated in binding buffer. The pure supernatant was loaded onto the Ni-NTA column (Bio-Rad, USA). The column was washed with washing buffer, containing 20 mM Tris, 500

mM NaCl, 16 mM imidazole and 6 M guanidine hydrochloride at pH 8.0 to remove any unbound proteins. The bound proteins were then eluted with elution buffer containing 20 mM Tris, 500 mM NaCl, 500 mM imidazole and 6 M guanidine hydrochloride at pH 8.0. The eluted fusion proteins were immediately transferred to 10,000 molecular weight cutoff cellulose membrane tubing (Spectra/Por, USA) for dialysis. The membrane pores were too small to allow the diffusion of the fusion proteins with molecular weights above 10 kDa. If equilibrium was reached after 4 to 6 h, the outside solution (dialysate) was replaced with ddH<sub>2</sub>O four times to remove the guanidine and salts. With the elimination of the salts, the fusion protein was refolded and thus precipitated in the tubing. The precipitates were collected and lyophilized.

**Chemical Cleavage.** The lyophilized fusion protein was chemically cleaved by cyanogen bromide (CNBr) under acidic conditions. The protein was thoroughly dissolved in 70% formic acid (Fluka, USA). Fresh solid CNBr (Sigma, USA) was then added at 100 mg/mL to hydrolyze peptide bonds at the C-terminus of the methionine residues in the fusion protein. The mixture was reacted for at least 5 h with stirring in the dark at room temperature. The cleaved products were transferred to 1,000 molecular weight cutoff membrane tubing and dialyzed against distilled ddH<sub>2</sub>O at room temperature to remove impurities such as formic acid and CNBr. Similar to the preceding process, the dialysate was replaced with ddH<sub>2</sub>O four times. The dialyzed solution was then purged with nitrogen gas and lyophilized. 12% bis-tris PAGE was conducted to verify whether cleavage was successful and the target peptide was isolated.

**Purification of mSyd4-TM Peptide.** The released mSyd4-TM peptides with fusion protein were purified by semi-preparative reversed-phase HPLC on a Delta 600 HPLC system (Waters, USA). A Delta Pak C18 column (7.8 mm × 300 mm, Waters, USA) was used at a flow rate of 3 mL/min. Eluent A contained 95% water/2% acetonitrile (ACN, J. T. Baker, USA)/3% isopropanol (IPA, J. T. Baker, USA) and 0.1% trifluoroacetic acid (TFA, Sigma-aldrich, USA) and eluent B contained 28% ACN/47% IPA/20% trifluoroethanol (TFE Acros, Belgium)/5% water and 0.1% TFA. Both eluents were filtered through a 0.45 µm membrane filter and degassed prior to HPLC. Distilled and deionized water was used to prepare the buffers. The peptide sample was dissolved in 20% hexafluoroisopropanol (Sigma-aldrich, USA)/80% methylene chloride (J. T. Baker, USA). The insoluble part was removed by centrifugation (13,200 rpm, 4 °C, 30 min). The soluble protein solution was filtered through a 0.45 µm membrane filter, and injected into a six-port Rheodyne valve and a 10 mL sample loop. The column was preliminarily equilibrated with eluent A before the protein mixture was fractionated with a gradient of 0-100% eluent B over 100 min, depending on its solubility. Chromatograms were recorded by the absorbance at 220 nm and 280 nm using a photodiode array (PDA) detector. The fractions containing the peptides were collected manually and lyophilized. The purity and molecular weight of the purified

mSyd4-TM were confirmed by 12% tris-tricine PAGE and mass spectrometry.

**Mass Spectrometry.** After the pure peptides were isolated, mSyd4-TM was analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Samples were prepared by dissolving 80  $\mu\text{g}$  dried peptide powder in 1 mL mass buffer (30% ACN/70%  $\text{H}_2\text{O}$ /0.1% TFA). 12  $\mu\text{L}$   $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) matrix (AB SCIEX, USA) and 13  $\mu\text{L}$  peptide solution dissolved in buffer were premixed in a micro tube. A final concentration of mSyd4-TM was 12 pmol in 1  $\mu\text{L}$  protein-matrix mixture. 1  $\mu\text{L}$  mixture was spotted on a plate and dried until crystallized. The sample plate was introduced to a 4800 plus MALDI TOF/TOF mass spectrometer (AB SCIEX, USA). Measurement was in reflector mode for improved resolution; tandem mass (MS/MS) spectrometry was also performed. A diode-pumped Nd:YAG 355 nm UV laser was pulsed at 200 Hz.

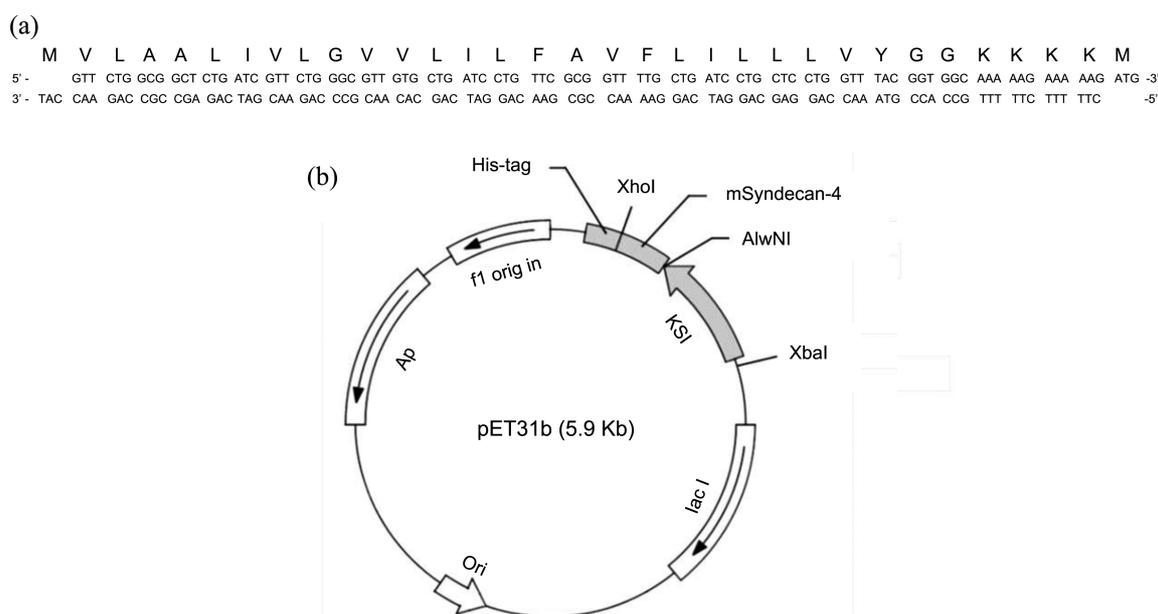
**Circular Dichroism.** Samples' circular dichroism (CD) were measured to assess the mSyd4-TM peptide's secondary structure before investigation of its tertiary structure by NMR spectroscopy. CD experiments were carried out using a Jasco J715 spectropolarimeter (Jasco, Japan) with 1 mm path length quartz cuvettes. Spectra were recorded between 190 and 250 nm with a data pitch of 0.2 nm, a bandwidth of 1 nm, a scanning speed with 50 nm per minute and a response time of 0.25 s. 1 mg peptide was first dissolved and dispersed in 700  $\mu\text{L}$  sterilized water. 100  $\mu\text{L}$  peptide solution was then dissolved in 500 mM dodecylphosphocholine (DPC) to a final volume of 400  $\mu\text{L}$  and final pH of 4. The concentration dependence of DPC was tested by using samples with final different concentrations of DPC: 0 mM, 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM. CD spectra were obtained at 25  $^\circ\text{C}$  and were the averages of five indivi-

dual spectra. Buffer without peptide was measured; its spectrum was subtracted as a baseline from the final spectra. CD signals,  $\psi/\text{mdeg}$ , were converted to mean residue molar ellipticity  $[\theta]/\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ .

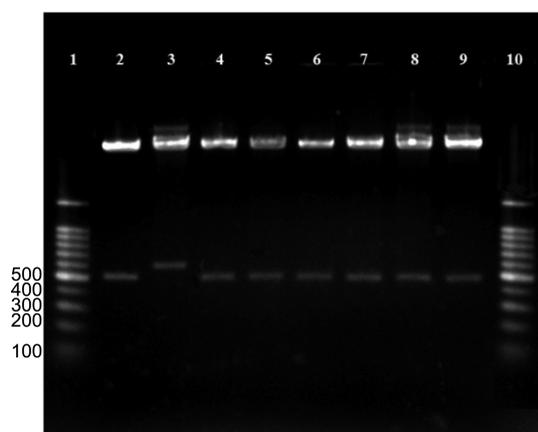
**NMR Spectroscopy.** NMR spectroscopy was the final assessment. Uniformly  $^{15}\text{N}$ -labeled mSyd4-TM powder samples were dissolved at a final concentration of 0.5 mM in 100 mM perdeuterated DPC- $d_{38}$  micelle containing 10 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM  $\text{NaN}_3$ , 90%  $\text{H}_2\text{O}$  and 10%  $\text{D}_2\text{O}$  at pH 4.0. Spectra were recorded on a Bruker AVANCE 800 MHz spectrometer (Bruker Biospin, Germany) using a triple-resonance indirect detection probe with xyz-gradient unit. Two-dimensional  $^1\text{H}$ - $^{15}\text{N}$  heteronuclear single quantum coherence (HSQC) spectra were recorded at 313 K using a 1 s recycle delay in phase-sensitive mode using the echo-antiecho method in the indirect dimension. Water resonance was suppressed by using a water flip-back pulse and the decoupling of  $^{15}\text{N}$  was achieved by GARP4 modulation. Data were acquired over eight scans with 1024 points in  $^1\text{H}$  and 256 increments in  $^{15}\text{N}$ . Data were processed using TOPSPIN 2.1 (Bruker Biospin, Germany).

## Results and Discussion

**Cloning.** The amino acid sequence of the recombinant mSyd4-TM and the corresponding complementary oligonucleotide sequences are shown in Figure 1(a). Figure 1(b) represents the construction of the KSI-(mSyd4-TM) expression vector. The annealed DNA comprising 96 base pairs was identified by 2% agarose gel electrophoresis and purified from the gel. The DNA samples were cloned into pET31b(+) vector used as an expression plasmid. In this vector, mSyd4-TM coding sequence was placed downstream of a 125 amino acids ketosteroid isomerase (KSI) gene as a



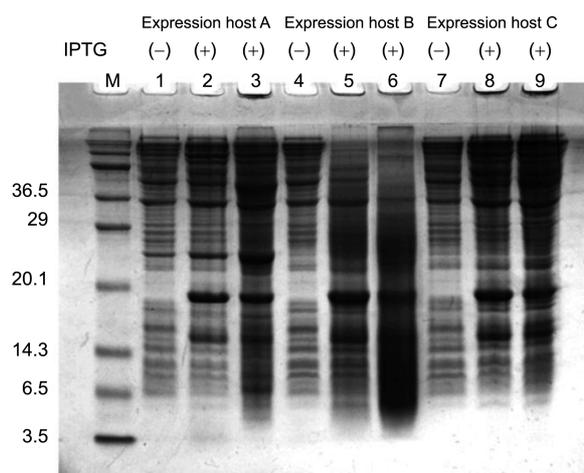
**Figure 1.** (a) Amino acid sequence of recombinant mutant Syndecan4-TM and the corresponding nucleotide coding sequence of the synthetic mutant Syndecan-4 gene. (b) The KSI-mSyd4-TM expression vector.



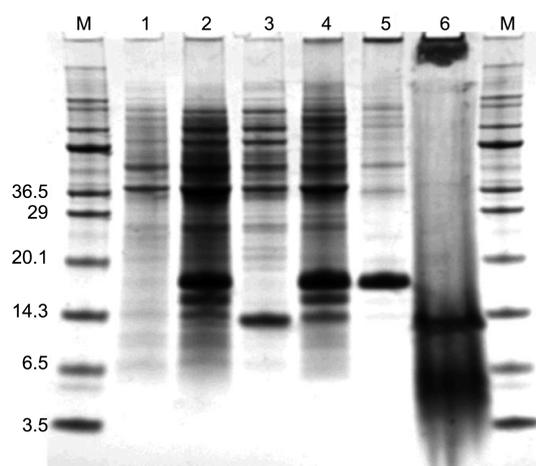
**Figure 2.** 1.5% agarose gel of the constructed expression vector extracted from *Novablue* cells. The extracted plasmids were digested with XbaI and XhoI restriction enzymes. The properly digested samples contained two segments, the vector pET31b(+) DNA (approximately 4800 bps) and the coding sequence (approximately 400–700 bps) of the KSI fusion protein. Lanes 1 and 10, DNA ladder; lanes 2, 4, 5, 6, 7, 8 and 9, single insert; lane 3, double inserts.

fusion partner and upstream of a His<sub>6</sub>-tag sequence for Ni-NTA affinity column purification. Use of the unique *AlwNI* cloning site in the vector map can allow the unidirectional insertion of the coding sequence adjacent to a methionine residue.<sup>19</sup> The *AlwNI*-ligated mixture was transformed into the cloning host, *Novablue*. To confirm the insertion of the mSyd4-TM gene into the vector system, the transformed plasmid was purified and digested with restriction enzymes XbaI and XhoI and assessed by 1.5% agarose gel electrophoresis (Figure 2). Approximately 450 bps represents the XbaI and XhoI restriction fragments of the vector pET31b(+) DNA. Therefore, the band above 500 bps shows the KSI fusion protein with single insertion of mSyd4-TM (Figure 2, lanes 2, 4–9) coding bases; the band between 600 and 700 bps in lane 3 shows the double insertion of mSyd4-TM coding bases. The sequence accuracy of each plasmid was determined by automated DNA sequencing. The mSyd4-TM expression vector was transformed into three *E. coli* strains to compare the expression and induction efficiency of the host cells (Figure 3). The C41(DE3) host cell showed the highest expression level, consequently it was chosen for large-scale expression. IPTG concentration and induction time were optimized prior to large-scale expression in M9 minimal media.

**Expression.** Cells were grown in M9 minimal media because the fusion protein in the cells could be uniformly or selectively labeled with <sup>15</sup>N nuclei for NMR structural studies. Selectively <sup>15</sup>N labeled amino acids or <sup>15</sup>N labeled ammonium sulfate was used. The fusion proteins were induced with 1 mM IPTG at an OD<sub>600</sub> of 0.5, followed by 16 h incubation in the shaking incubator at 37 °C. The expression of the fusion proteins was verified by 12% bis-tris PAGE. A band appeared around a molecular weight of 17.3 kDa, consistent with the expected molecular weight of the KSI-(Syd4-TM)-His<sub>6</sub>-tag protein (Figure 4, lane 2).

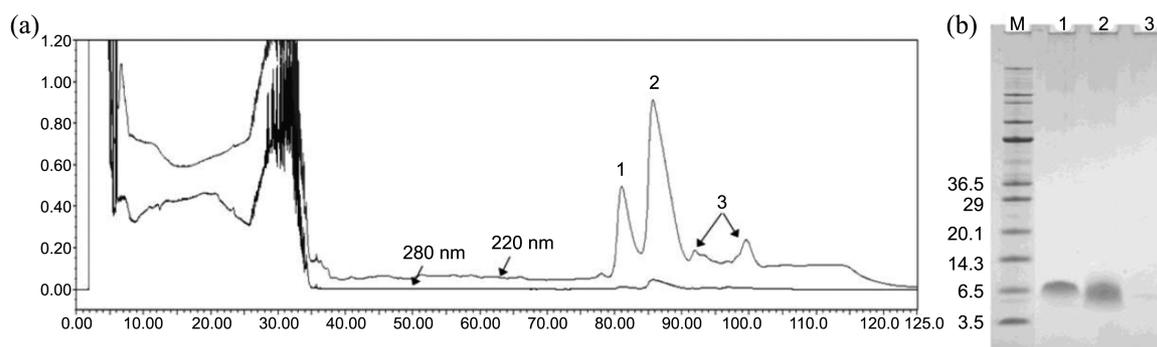


**Figure 3.** Selection of constructs with the highest expression and induction efficiencies of fusion proteins containing single inserts from expression host cells using 12% bis-tris gels. Three expression hosts were tested. Fusion proteins' expression levels were compared before IPTG induction, and (–) 4 h and overnight after IPTG induction, (+).



**Figure 4.** 12% bis-tris PAGE of mSyd4-TM after expression and purification. Lane M, molecular weight marker; lane 1, total cell before IPTG induction; lane 2, total cell after IPTG induction with a KSI-fused protein band; lane 3, supernatant after lysis with lysozyme; lane 4, pellet containing the fusion protein after lysis; lane 5, eluate of the KSI-fused protein from Ni-NTA affinity column chromatography; lane 6, protein mixtures containing target peptide, KSI, and other impurities after chemical cleavage.

**Purification.** To isolate the fusion protein, cells were resuspended in lysis buffer supplemented with lysozyme. After lysis by ultrasonication, the cell lysate, including the supernatant and the pellet, was examined to find the recombinant fusion protein. The supernatant after cell lysis showed a band of lysozyme around 14 kDa (Figure 4, lane 3) and the pellet with a band of the fusion protein (lane 4). The fusion protein was efficiently dissolved in the binding buffer containing 6 M guanidine hydrochloride. The KSI-(mSyd4-TM)-His<sub>6</sub>-tag protein was then purified by Ni-NTA affinity chromatography under denatured conditions. The fusion protein was eluted with elution buffer containing a



**Figure 5.** (a) Semi-preparative reversed-phase HPLC chromatogram of mSyd4-TM. The chromatogram was detected at 220 and 280 nm using a PDA detector. (b) 12% tris-tricine PAGE analysis of HPLC fractions.

high concentration of 500 mM imidazole by detaching histidine bound to the nickel. The eluted solution was dialyzed against ddH<sub>2</sub>O at room temperature and precipitated. After sufficient dialysis, the white precipitates were collected and lyophilized. The sample's purity after Ni-NTA column chromatography was confirmed (Figure 4, lane 5). In general, 100 mg KSI-(mSyd4-TM)-His<sub>6</sub>-tag protein was obtained per liter culture in M9 minimal media.

The lyophilized white protein powder was dissolved in 70% formic acid. Cyanogens bromide (CNBr) was to cleave the mSyd4-TM peptide from the KSI-(mSyd4-TM)-His<sub>6</sub>-tag fusion protein. After chemical cleavage of the fusion protein, it was dialyzed against ddH<sub>2</sub>O using the 1,000 molecular weight cutoff dialysis tubing to remove the formic acid and CNBr before lyophilization. The cleaved products (Figure 4, lane 6) showed a band at 14 kDa, corresponding to KSI fusion partner protein and the lower smeared band between 4–6 kDa is consistent with mSyd4-TM (Figure 4, lane 6). The lower smeared band was due to typical characteristics of membrane-bound proteins and so appeared above its own MW position. The band position of the membrane protein was distinguished from the soluble protein by the maximum SDS-binding level in SDS-PAGE.<sup>20–22</sup>

20 mg dried protein powder was dissolved first in 20% HFIP and then in 80% MC with sonication. Most of the KSI agglomerate appeared brown. After centrifugation and filtration, 10 mL solution was loaded onto a C18 column using a semi-preparative HPLC system (Waters, USA). The chromatogram was monitored at both 220 and 280 nm (Figure 5(a)), and the fractions containing the protein were collected manually and lyophilized. The purified protein fractions from HPLC were verified by 12% tris-tricine PAGE (Figure 5(b)). The major fraction of mSyd4-TM (3.4 kDa) was eluted from about 84–90 min (Figure 5). The band of lane 2 in Figure (b) shows smearing and so was placed above its own position as before.

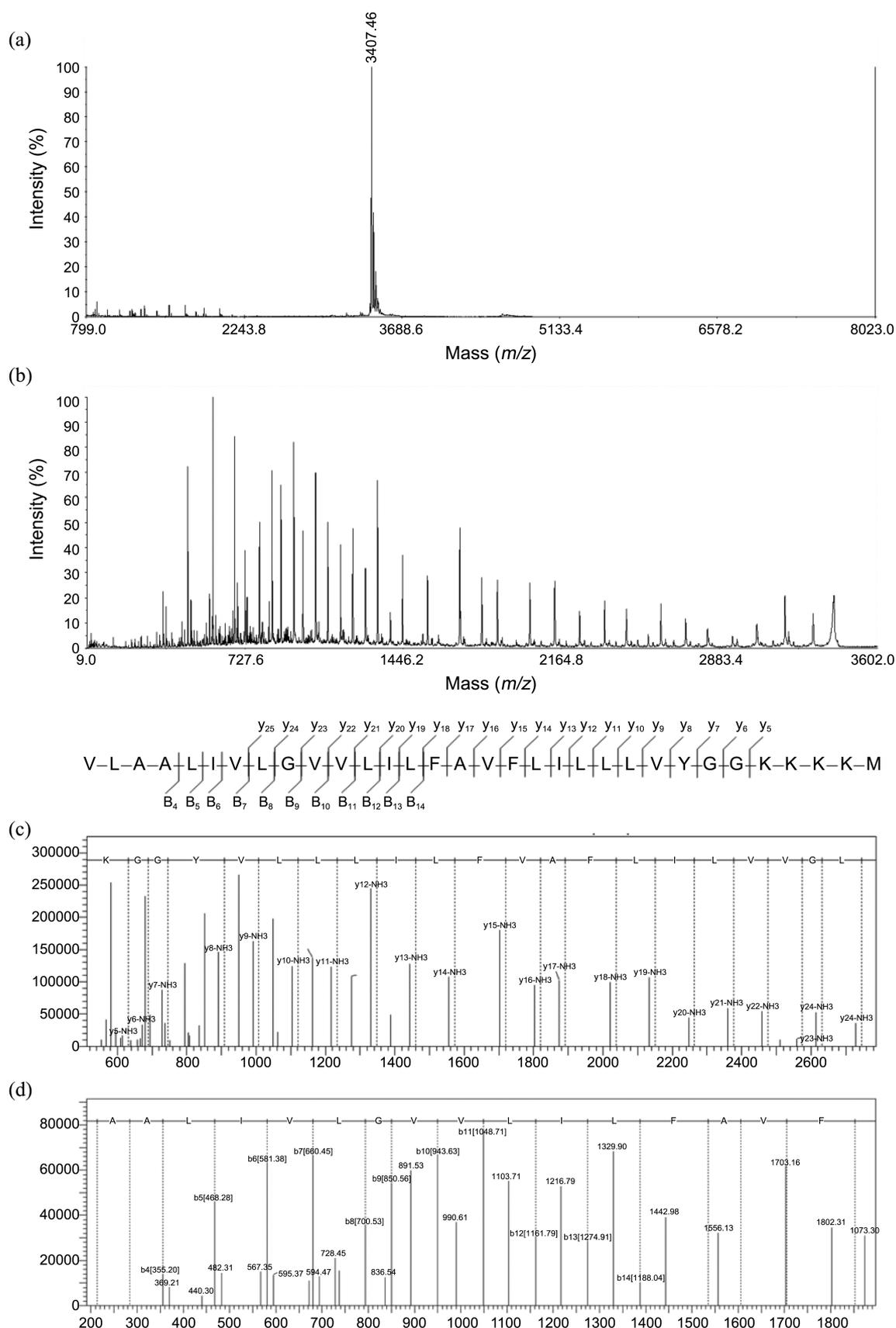
The purified peptide was further identified using MALDI-TOF mass spectrometry in reflector mode for enhanced resolution. The mass spectrum of fraction 2 after HPLC purification was represented by one peak at  $m/z$  3407.46 (Figure 6(a)), which is consistent with the exact theoretical mass of mSyd4-TM including the C-terminal homoserine

lactone (3406.22 Da). The mass spectrum indicates the high purity of the protein. The sequence of mSyd4-TM was additionally sought by tandem mass spectrometry (MS/MS, Figure 6(b)). The mass of the precursor ion was  $m/z$  3407.46—the major peak observed in reflector mode. MS/MS peptide sequencing of mSyd4-TM was performed using *de novo* sequencing software (ABSciex, USA, Figure 6(c) and 6(d)). The sequence appeared consistent with the y-NH<sub>3</sub> ion fragments of 21 residues (-LGVVLLILFAVFLILLVYGGK-) and the b-ion fragments of 11 residues (-LIVLGVVLLIL-) of mSyd4-TM. The mass spectrum of fraction 1 after HPLC purification showed two peaks at  $m/z$  3407.31 and  $m/z$  4605.83, the former corresponds to the mass of mSyd4 TM (Figure 7(a)). The latter was identified and so tandem mass spectroscopy for the precursor ion of  $m/z$  4605.83 was conducted (Figure 7(b)). *De novo* sequencing (Figure 7(c) and 7(d)) showed that the sequence was partially consistent with the y-ion fragments of 6 residues (-LLEHHH-) of His<sub>6</sub> tag and the b-ion fragments of 9 residues (-AALIVLGVV-) of mSyd4-TM. Therefore the peak at  $m/z$  4605.83 was attributed to mSyd4-TM with the LLE-His<sub>6</sub> tag.

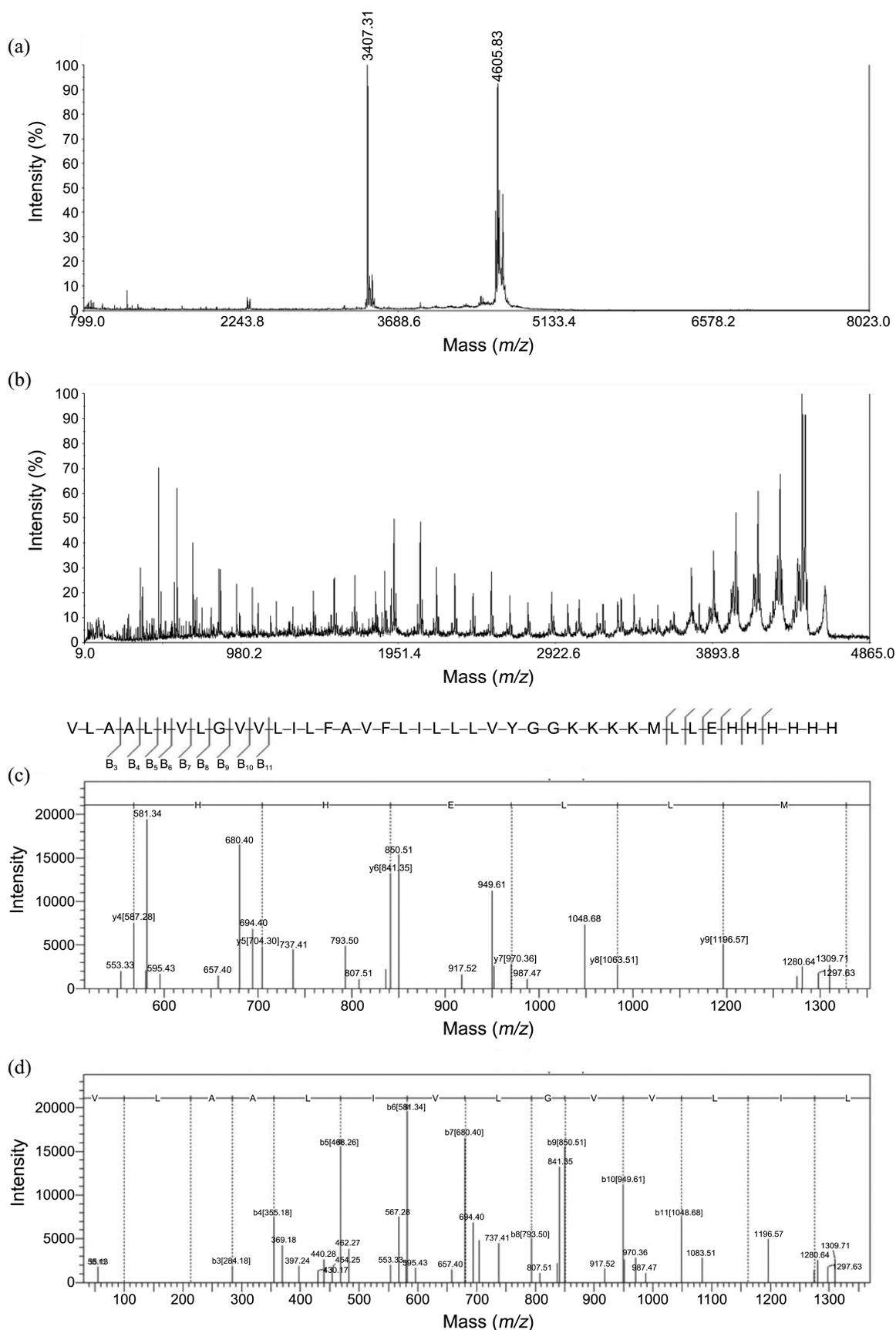
The final yield of mSyd4-TM in fraction 2 after HPLC purification was about 4 mg per 1 L M9 minimal medium.

**Structural Analysis.** The rough secondary structure of mSyd4-TM in micelles was examined by CD spectroscopy at 190–260 nm. The spectrum shows that the purified mSyd4-TM adopts an  $\alpha$ -helical structure, as evidenced by the double minima absorptions at 208 and 222 nm. This helical secondary structure was stable and remained unchanged in DPC concentrations of 20 to 100 mM. In water (*i.e.* 0 mM DPC), however, the peptide assumed a random coil structure (Figure 8).

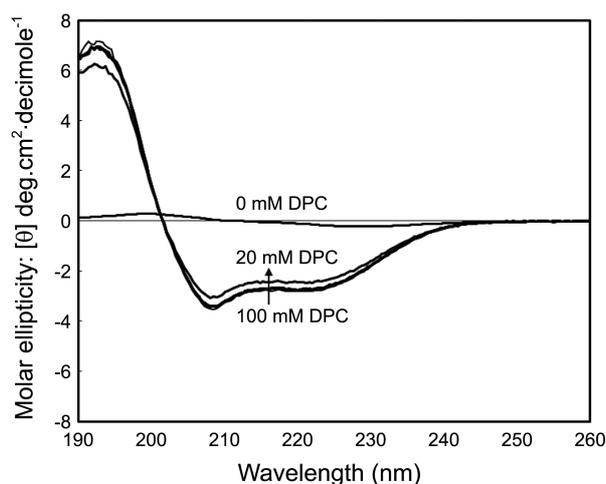
The structure of mSyd4-TM in DPC micelles, simulating a membrane environment, was analyzed by solution NMR spectroscopy. <sup>1</sup>H-<sup>15</sup>N HSQC spectroscopy was used to identify the protein's structure, as its finger print. The 31 cross-peaks in the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of mSyd4-TM corresponded to its number of amino acids (Figure 9(a)). The HSQC spectrum of wt-Syd4-TM showed an amount of peaks corresponding to double its number of amino acids (Figure 9(b)).<sup>16</sup> Glycine residues were present from 8 to 9 ppm for <sup>1</sup>H and from 105 to 110 ppm for <sup>15</sup>N, respectively. In this



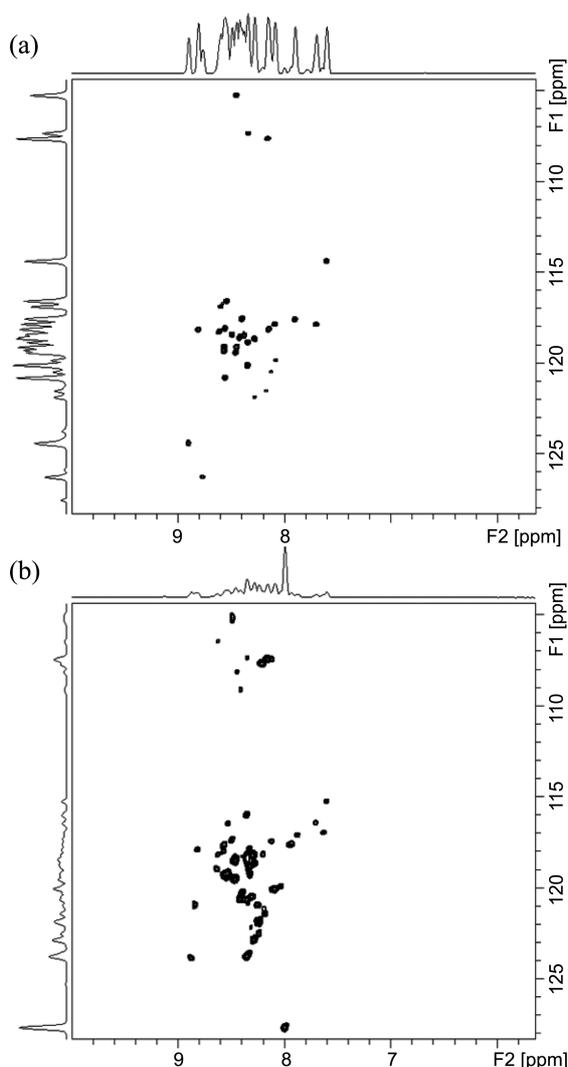
**Figure 6.** (a) MALDI-TOF mass spectrum of fraction 2 in the HPLC chromatogram in reflector mode. The monoisotopic peak of mSyd4-TM represents  $m/z$  3407.46, which is consistent with the theoretical mass of mSyd4-TM including C-terminal homoserine lactone (3406.22 Da). (b) Tandem mass (MS/MS) spectrum of mSyd4-TM. The mass of the precursor ion, 3407.46, was used as a base peak. MS/MS peptide sequencing spectra of mSyd4-TM's (c) y-H<sub>2</sub>O series ions and (d) b-series ions using *de novo* sequencing software.



**Figure 7.** (a) MALDI-TOF mass spectrum of fraction 1 HPLC chromatogram in reflector mode. The monoisotopic peak of mSyd4-TM represents  $m/z$  3407.31, the result of fraction 2 in Figure 6. (b) Tandem mass (MS/MS) spectrum of the peak at  $m/z$  4605.83. MS/MS peptide sequencing spectra for (c)  $y$ -series ions and (d)  $b$ -series ion using *de novo* sequencing.



**Figure 8.** Circular dichroism (CD) spectrum of purified mSyd4-TM dissolved in buffers containing 0-100 mM DPC.



**Figure 9.**  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra of (a) uniformly  $^{15}\text{N}$ -labeled mutant Syndecan4-TM and (b) uniformly  $^{15}\text{N}$ -labeled wild-type Syndecan4-TM recorded at 0.5 mM in perdeuterated DPC- $d_{38}$  containing optimized buffer at pH 4.0 in 800 MHz Avance solution NMR (Bruker Biospin, Germany).

region, wt-Syd4-TM contained five Gly residues and so showed ten peaks in its HSQC spectrum. mSyd4-TM contained three Gly residues by mutation of the GXXXG motif, and showed three peaks in its HSQC spectrum. These results show that the GXXXG motif of syndecan4-TM was important to its formation of dimers.

## Conclusion

The oligomerization of syndecan-4 was tested through the expression, purification and isolation of a large amount of mSyd4-TM without modification or other fusion protein. Its purity was identified by MALDI-TOS MS-MS and CD spectroscopy. Sufficient quantities of pure protein were produced to allow further structural studies using solution and solid-state NMR spectroscopy.

The structure of mSyd4-TM was assessed by SDS-PAGE and solution NMR spectroscopy, which unambiguously distinguished it from wt-Syd4-TM. The band position of mSyd4-TM in SDS-PAGE corresponded with mass of monomeric mSyd4-TM (3.4 kDa). The number of cross-peaks in its  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum was equal to its number of amino acids, demonstrating that mSyd4-TM was present as monomers in SDS or DPC micellar environments. Therefore, the GXXXG motif of Syd4-TM was shown to be a determining factor of its dimerization.

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## References

- Shaw, T. J.; Martin, P. *J. Cell Sci.* **2007**, *122*, 3215-3223.
- Bass, M. D.; Williamson, R. C.; Nunan, R. D.; Humphries, J. D.; Byron, A.; Morgan, M. R.; Martin, P.; Humphries, M. *J. Dev. Cell* **2011**, *21*(4), 681-693.
- Morgan, M. R.; Humphries, M. J.; Bass, M. D. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 957-969.
- Häcker, U.; Nybakken, K.; Perrimon, N. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 530-541.
- Roskams, T.; Rosenbaum, J.; De Vos, R.; David, G.; Desmet, V. *Hepatology* **1996**, *24*(3), 524-532.
- Roskams, T.; De Vos, R.; David, G.; Van Damme, B.; Desmet, V. *J. Pathol.* **1998**, *185*(3), 290-297.
- Beauvais, D. M.; Rapraeger, A. C. *Reprod. Biol. Endocrinol.* **2004**, *2*, 3-14.
- Charni, F.; Friand, V.; Haddad, O.; Hlawaty, H.; Martin, L.; Vassy, R.; Oudar, O.; Gattegno, L.; Charneau, N.; Sutton, A. *Biochim. Biophys. Acta* **2009**, *1790*(10), 1314-1326.
- Fischer, D.; Brown-Lüdi, M.; Schulthess, T.; Chiquet-Ehrismann, R. *J. Cell Sci.* **1997**, *110*, 1531-1522.
- Huang, W.; Chiquet-Ehrismann, R.; Moyano, J. V.; Garcia-Pardo, A.; Orend, G. *Cancer Res.* **2001**, *61*, 8586-8594.
- Shin, J.; Song, Y.; McFarland, D. C. *Mol. Cell Biochem.* **2012**, *363*, 437-444.
- Shin, J.; Lee, W.; Lee, D.; Koo, B.; Han, I.; Lim, Y.; Woods, A.;

- Couchman, J. R.; Oh, E. *Biochemistry* **2001**, *40*, 8471-8478.
13. Dews, I. C.; MacKenzie, K. R. *PNAS* **2007**, *104*, 20782-20787.
14. Xian, X.; Gopal, S.; Couchman, J. R. *Cell Tissue Res.* **2010**, *339*, 31-46.
15. Manon-Jensen, T.; Itoh, Y.; Couchman, J. R. *FEBS J.* **2010**, *277*, 3876-3880.
16. Dews, I. C.; Mackenzie, K. R. *PNAS* **2007**, *104*, 20782-20787.
17. Choi, S.; Lee, E.; Kwon, S.; Park, H.; Yi, J.; Kim, S.; Han, I.; Yun, Y.; Oh, E. *J. Biol. Chem.* **2005**, *280*, 42573-42579.
18. Park, T.; Lee, M.; Kim, J.; Kim, Y. *Process Biochem.* **2011**, *46*(5), 1166-1171.
19. Čipáková, I.; Gašperik, J.; Hostinová, E. *Protein Express Purif.* **2006**, *45*, 269-274.
20. Walkenhorst, W. F.; Merzlyakov, M.; Hristova, K.; Wimley, W. C. *Biochimica et Biophysica Acta* **2009**, *1788*, 1321-1331.
21. Grefrath, S. P.; Reynolds, J. A. *Proc. Nat. Acad. Sci. USA* **1974**, *71*, 3913-3916.
22. Rath, A.; Glibowicka, M.; Nadeau, V.; Chen, G.; Deber, C. M. *PNAS* **2008**, *106*, 1760-1765.
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