Nectin-like molecule 1 is a glycoprotein with a single $N$-glycosylation site at N290KS which influences its adhesion activity

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**A R T I C L E  I N F O**

Article history:
Received 27 November 2007
Received in revised form 24 February 2008
Accepted 11 March 2008
Available online 30 March 2008

Keywords:
Cell–cell adhesion
Nectin-like molecule 1 (Necl1)
N-linked glycoprotein

**A B S T R A C T**

Nectin-like molecule 1 (NECL1)/CADM3/IGSF4B/TSL1/SynCAM, from now on referred to as NECL1, is a neural tissue-specific immunoglobulin-like cell–cell adhesion molecule which has Ca$^{2+}$-independent homo- or heterophilic cell–cell adhesion activity and plays an important role in the formation of synapses, axon bundles and myelinated axons. Here we first detected the expression of NECL1 in human fetal and adult brains, and mouse brains at different developmental stages. The results indicated that two bands with molecular weights of about 62 kDa and 48 kDa were found in human fetal brain, while only one band with a molecular weight of about 48 kDa was found in human adult brain; two bands with molecular weights of about 62 kDa and 48 kDa whose expression level gradually increased were also found from mouse E16 to P14, while only one band with a molecular weight of about 48 kDa was found from P14. Bioinformatics analysis showed there were two putative $N$-glycosylation sites within human NECL1 at positions N25LS and N290KS and within mouse Necl1 at positions N23LS and N288KS, respectively. There was no O-glycosylation site in either human NECL1 or mouse Necl1. Based on the results of N-Glycosidase F treatment with human fetal brain tissue and lysates from transient transfection with human wild-type or glycosylation site mutant NECL1 in 293ET cells, we demonstrated that human NECL1 is an N-linked glycoprotein with a single glycosylation site at position N290KS. Cell aggregation assay further showed there was an increased adhesion activity after the glycosylation site mutation of NECL1 molecule.

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1. Introduction

In multicellular organisms, cells communicate with each other by their adhesive interactions which are essential for different cellular functions such as morphogenesis, proliferation, differentiation and migration [1]. This adhesion is mediated by cell adhesion molecules which have been classified into four types: immunoglobulin superfamily, selectin, integrin, and cadherin families. Nectins and Nectin-like molecules all belong to the immunoglobulin superfamily of cell–cell adhesion molecules and play roles in the above processes [2]. Up to now there are four members in the Nectin family (Nectin-1, 2, 3, 4) and five members in the Nectin-like molecule family (NECL1, 2, 3, 4, 5) based on binding ability to Afadin [2,3]. Among them, almost each member can recognize itself or others in homo- or heterophilic manners. For instance, Nectin-3 can interact with NECL1, NECL2, NECL5, Nectin-1, Nectin-2 and Nectin-3 itself [4]. NECL1 is exclusively and highly expressed in the nervous system and plays a role in the formation of synapses, axon bundles, myelinated axons and cerebellar morphogenesis [5–8]. It has been found that NECL1 can interact with NECL1, NECL2, NECL4, Nectin-1 and Nectin-3 but not with NECL5 or Nectin-2 [6,9–11]. The 1,197 bp length ORF of human NECL1 and 1,191 bp length ORF of mouse Necl1 produce the protein NECL1 with a theoretical molecular weight of 44 kDa. However, it was recently reported that in the rat brain, a major band approximately 48 kDa and a minor band approximately 62 kDa of Necl1 were detected [8,12]. This may be due to its post-translational modifications. However, little is known about post-translational modifications of NECL1 up to now. NECL2, which has significant homology with NECL1 in the overall coding sequences [5], has been reported to be an N-linked glycoprotein [13,14]. For this reason we speculate that the various molecular weight forms of NECL1 could also be the result of glycosylation. We report that two putative $N$-glycosylation sites were predicted within both human NECL1 and mouse Necl1. The expression pattern of NECL1 in human fetal and adult brain tissues and in mouse brain tissues of different developmental stages were characterized and it is shown that human NECL1 is an N-linked glycoprotein with one $N$-glycosylation site at N290KS which could play a role in cell adhesion.
2. Materials and methods

2.1. Polyclonal antibody preparation

Rabbit polyclonal antibody against human NECL1 was prepared as described before [8]. Briefly, the cDNA fragment encoding 47 amino acids of the cytoplasmic region of human NECL1 was cloned into pET-30a-DHFR vector. The fusion protein was expressed in E.coli (DE3) bacteria and purified using a nickel column. New Zealand white rabbits were immunized with the fusion protein to produce the antibody. Additionally, the antiserum was applied to GST-NECL2C immobilized column for absorption of non-specific antibodies. Monotype Antibody Purification Kit (Millipore) was further used to purify the pure IgG and the specificity of the antibodies has been detected.

2.2. Tissue and cell lysates preparation and Western blot analysis

Human fetal brain tissue was kindly provided by Beijing Jishuitan Hospital and adult brain tissues were provided by Beijing Tiantan Hospital. Human fetal and adult brain tissues, and different developmental stage mouse brain tissues were homogenized in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 10 mM NaF, 1 mM NaVO₃, 1% glycerol, 1% NP-40, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 2 μg/ml PMSF) on ice followed by shaking at 4 °C for 30 min. The lysates were centrifuged at 12,000 rpm for 20 min at 4 °C, then supernatant was collected. To prepare the whole cell extracts, cell pellets were suspended in the above lysis buffer and processed as the tissue lysates preparation. Protein concentration was determined using the Bradford method, lysates were aliquoted and stored at −80 °C until use. The Western blot analysis was performed as described [15] with rabbit pAb against NECL1 at a dilution of 1:200 and mouse anti-V5 mAb (Invitrogen) at a dilution of 1:3000.

2.3. Bioinformatics analysis

Bioinformatics prediction of N- and O-glycosylation sites on human NECL1 and mouse Necl1 were performed with the following database:

http://www.cbs.dtu.dk/services/NetOGlyc;
http://www.cbs.dtu.dk/services/NetNGlyc.

2.4. Lysates treated with N-Glycosidase F

Lysates were treated with N-Glycosidase F (Roche, Cat.No.1365185) according to the manufacturer’s instructions followed by Western blot with anti-NECL1 pAb (dilution: 1:200) or anti-V5 mAb (Invitrogen, dilution: 1:3000).

2.5. Plasmid construction

NECL1 ORF was amplified from the human fetal brain cDNA library with primers (see Table 1) and cloned into pcDNA3.1(+), pcDNA3.1/V5-HisA vectors (Invitrogen) to generate pcDNA-NECL1 and pcDNA3.1/V5-NECL1 (named as NECL1-WT), respectively. For NECL1 mutant constructs of pcDNA3.1/V5-NECL1-N25A, pcDNA3.1/V5-NECL1-N290A and pcDNA3.1/V5-NECL1-N25A+N290A, respectively, overlap PCR (to mutate N290 site) and QuickChange Kit (Stratagene, to mutate N25 site) were employed (see Table 1).

2.6. Cell culture, transfection and establishment of stable cell line

Human embryonic kidney 293ET cell line (kindly provided by Dr. Chengyu Jiang, Peking Union Medical College) was maintained in Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco), Cos7 cell line and L cell line (purchased from ATCC) were maintained in Dulbecco’s Medium (DMEM, Gibco). COS7 cell line and L cell line (purchased from ATCC) were maintained in Iscove’s Modi

2.7. Immunocytochemistry

Immunocytochemistry was performed as described [8]. Briefly, cells were washed with PBS twice and fixed with 4% paraformaldehyde for 10 min at RT, then permeabilized with 0.5% Triton X-100 for 10 min at RT and blocked with 3% bovine serum albumin (BSA) for 30 min at 37 °C. The cells were immobilized using mouse anti-V5 mAb (Invitrogen, dilution: 1:200) and then followed by a FITC conjugated goat anti-mouse antibody. After overlaying coverslips, the slides were imaged using a Leica confocal microscope.

2.8. Cell aggregation assay

Cell aggregation assay was performed as described [4]. Briefly, cells were washed with PBS, incubated with 0.2% trypsin and 1 mM EDTA at 37 °C for 3 min, and dispersed by gentle pipetting. The cells were then suspended in Hanks’ balanced salt solution (about 1 × 10⁶ cells/ml), placed in 12-well plates precoated with bovine serum albumin, and rotated on a gyratory shaker at 37 °C for about 15 min. Aggregation was stopped with the addition of 2% glutaraldehyde. The extent of aggregation of cells was photographed using a microscope. In order to prove

Table 1

<table>
<thead>
<tr>
<th>Applications of primers</th>
<th>Sequences</th>
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<tr>
<td>Amplitify NECL1 ORF</td>
<td>CCGAGTCCTTTATGATGACAGGGCCAGCG</td>
</tr>
<tr>
<td>Mutate 25 site</td>
<td>CGCTGAGATGGATATCACCGTGGG</td>
</tr>
<tr>
<td>Mutate 290 site</td>
<td>CTTCTGGAAGAGGGCAGAGG</td>
</tr>
</tbody>
</table>

The bold and italic letters represent the recognition sites of restriction enzymes HindIII and XhoI respectively; letters underlined represent the sequence after mutation.

Fig. 1. Verification of the specificity of anti-NECL1 antibody. (a) Homology alignment of the cytoplasmic regions of human NECL1 and NECL2, which share two conserved motifs: FERM binding motif and PDZ binding motif. (b) Antibody was produced, purified and detected as described. Antiserum before (indicated by + below) and after purification (indicated by + below) were used in Western blot with purified GST-NECL2 fusion protein and cell extracts from 293ET transfected with pcDNA-NECL1 as antigens. A specific band was detected about 48 kDa by antibody after GST-NECL2 affinity purification. CB: Coomassie Blue stain. (c) Human adult brain tissue extract and cell extracts from COS7 and 293ET transfected with control vector and pcDNA-NECL1 were loaded to SDS-PAGE and detected by anti-NECL1 antibody.

3. Results

3.1. Verification of the specificity of anti-NECL1 polyclonal antibody

DNAMAN software was used to compare the cytoplasmic regions of human NECL1 and NECL2 (Fig. 1a). The identity of them was up to 75%, and two conserved motifs were concluded in their cytoplasmic regions: FERM binding motif and PDZ binding motif. In order to prove
the specificity of anti-NECL1 pAb, the antibody was purified (Fig. 1b). Antiserum before and after purification were used in Western blot with purified GST-NECL2C fusion protein and cell extracts from 293ET transfected with pcDNA-NECL1 as antigens. We can see that after purification, the anti-NECL1 antibody only recognized the NECL1 protein and a specific band was detected about 48 kDa. The Western blot result of Fig. 1c indicated that the molecular weight of endogenous NECL1 in human adult brain tissue and exogenous NECL1 overexpressed in COS7 and 293ET cells were identical.

3.2. Determination of the molecular weight of NECL1 in human fetal brain and adult brain tissues and in mouse brain tissues at different developmental stages

Western blot was employed to detect the expression of NECL1 protein in human fetal and adult brain tissues (Fig. 2a) and in mouse brain tissues at different developmental stages (Fig. 2b) using specific anti-NECL1 antibody. One major band approximately 48 kDa was detected in both human fetal and adult brain tissues, while a minor band about 62 kDa was detected only in human fetal but not in adult brain tissues. Comparatively, a major band approximately 48 kDa was detected during mouse E16 up to P14 with gradually increased expression level, while a faint band about 62 kDa was detected during mouse E16 to P14 with also increased expression level, peaked at P14 and disappeared after P14. Both bands (48 kDa, 62 kDa) were larger than the theoretical molecular weight (44 kDa) possibly due to the post-translational modification(s), most likely glycosylation.

3.3. Two potential N-glycosylation sites are predicted in both human NECL1 protein and mouse Necl1 protein

Bioinformatics prediction as described in Material and methods showed that there are two potential N-glycosylation sites in the extracellular region at positions N25LS and N290KS of human NECL1 and at the positions N23LS and N288KS of mouse Necl1. No putative O-glycosylation site is predicted within NECL1 in either species. Because the identity of NECL1 between human and mouse is up to 94.9%, and the amino acid sequences of the cytoplasmic region of human and mouse NECL1 are identical, their potential N-glycosylation sites coincide perfectly (Fig. 3a). The prediction results and the structural schematic diagram of human NECL1 are presented in Fig. 3b, c and d.

3.4. NECL1 is indeed N-glycosylated but with only one N-glycosylation site

To address whether NECL1 is an N-linked glycoprotein, protein extracts from human fetal brain and mouse P14 brain were treated with N-Glycosidase F and detected with anti-NECL1 antibody. The results in Fig. 4a and b showed both two bands (48 kDa, 62 kDa) shifted to about 44 kDa which is perfectly matched with the theoretical molecular weight of NECL1 after treated with N-Glycosidase F. Since both two bands (48 kDa, 62 kDa) disappeared after N-Glycosidase F treatment and appeared as only one band of about 44 kDa, we claimed that both two bands (48 kDa, 62 kDa) are glycosylated by two possibilities, one is the glycosylation that occurs at two sites and the other one is glycosylation at only one site in varying degrees. It could be interesting to determine if these two predicted glycosylation sites are all responsible for the glycosylation of NECL1 or not. Since the identity of NECL1 between human and mouse is up to 94.9%, human wild-type NECL1 and N25A or N290A mutant NECL1 with V5 tag were overexpressed in 293ET cells. First we verified the distribution of wild-type NECL1 and its mutants by immunocytochemistry with anti-V5 antibody. As shown in Fig. 5c, the membrane localization of all of the NECL1 proteins (wild-type and mutants) meant that the glycosylation sites didn’t affect the trafficking of NECL1, as well as the folding of the protein. Next treatment of the lysates with N-Glycosidase F followed by Western blot using anti-V5 antibody (Fig. 5d) showed that overexpression of wild-type NECL1 was partially glycosylated (molecular weight, about 52 kDa) but after treatment with N-Glycosidase F, the band shifted to 48 kDa which equals to the theoretical molecular weight (44 kDa) of NECL1 plus approximately 4 kDa added by V5 tag. The N25A mutant showed the same molecular weight pattern with wild-type. However, for the N290A or N25A+N290A mutant, there was only one band with a molecular weight of 48 kDa. These results implied that it is N290A but not Asn290 responsible for the glycosylation of NECL1.

3.5. N-glycosylation of NECL1 affects cell–cell adhesion activity

It has been reported that the glycosylation of many adhesion molecules could affect the adhesion activity, such as NCAM [16]. So herein we asked whether the glycosylation of NECL1 also plays some roles in adhesion activity. Empty vector, NECL1-WT, NECL1-N25A and NECL1-N290A were stably overexpressed in L cells. First we examined the expression and distribution of these NECL1s on L cells. As shown in Fig. 6a and b, the wild-type and N25A mutant NECL1s were glycosylated in L cells while N290A mutant NECL1 was unglycosylated based on the comparison with Fig. 5d. Also, all NECL1 proteins were located on the membrane of L cells as those in 293ET cells. Cell aggregation could be obviously observed within the NECL1-WT, NECL1-N25A and NECL1-N290A stably transfected L cells compared to the empty vector transfected L cells (Fig. 6c). Moreover, the cell aggregates caused by aggregations within NECL1-N290A stably transfected L cells were significantly greater than those found with NECL1-WT and NECL1-N25A stably transfected L cells. The cell aggregates observed in NECL1-N25A stably transfected L cells were not significantly different from those within NECL1-WT stably transfected L cells (Fig. 6d). This result suggested the possibility that glycosylation of NECL1 can influence adhesion activity to a certain extent.

4. Discussion

In this study, we first reported human NECL1 is a glycoprotein with one N-glycosylation site at position N290. The two bands of NECL1 with molecular weights of about 48 kDa and 62 kDa observed in
human and mouse brain tissues were demonstrated to be due to different contents of glycosylation. The glycosylation site was detected and further confirmed by overexpression of NECL1 and its mutant forms in 293ET cell line, which indicated that Asn290 residue is essential for NECL1 glycosylation, and the glycosylation could influence the adhesion activity in a certain extent. Kakunaga et al. reported that, in rat for NECL1, there is a band with a molecular weight of about 62 kDa observed during postnatal days 6–14, after which time it disappeared [6]. Interestingly, we observed that one major band of approximately 48 kDa was detected in both human fetal and adult brain tissues, while a minor band of about 62 kDa was only detected in human fetal but not in adult brain tissues. In mouse,
one band of approximately 48 kDa was detected from mouse E16 with gradually increased expression level; another faint band of about 62 kDa was detected during mouse E16 to P14. Its expression level roughly increased from E16 to P14 with an expression peak at P14, then the band disappeared. Two bands (48 kDa, 62 kDa) both shifted to 44 kDa after treatment with \( \text{N-Glycosidase F} \), so we conclude that these two bands of 62 kDa and 48 kDa are glycosylated. Since there are two predicted putative glycosylation sites within mouse and human NECL1, it could be argued that these two molecular weights of NECL1 have been caused by glycosylation at these two sites. Overexpression of NECL1 and its glycosylation site mutants indicated that NECL1 only has one glycosylation site at Asn290. Thus, two bands could be further explained by glycosylation at Asn290 in varying degrees.

It must be mentioned that the molecular weight pattern among the three different species (human, mouse and rat) is similar during development, although it is difficult to get the human brain tissues

![Image](image_url)
from different developmental stages. It has been reported that many glycoproteins contain several forms with different contents of glycosylation, and one of the well-studied is NCAM (neural cell adhesion molecule). Seki et al. showed that NCAM is highly polysialylated (NCAM-H) during the late embryonic and early postnatal stages, but less polysialylated (NCAM-L) during the early embryonic and adult stages [17]. So the results from Kakunaga’s data as well as ours suggest that glycosylation of NECL1 maybe also play some role in the development of brain among different species.

Until now, the different glycosylated forms of NCAM have been shown to be dominantly regulated by two polysialyltransferases, PST (ST8SiaIV) and STX (ST8Sia II). PST and STX both would form polysialic acid in NCAM, however, polysialic acid synthesized by PST is larger (∼60 sialic acids) than that synthesized by STX (∼40 sialic acids) [18,19]. It was also reported PST cooperating with STX could add a longer polysialic acid to NCAM than PST alone. As the molecular weight of a sialic acid is about 310 Da, the N-glycans added by PST and STX should be ∼18 kDa and ∼12 kDa, individually. Considering that there are 6 N-glycosylation sites lying in NCAM and 3 different isoforms of NCAM, the Western blot result of NCAM always indicates a smear band around 200 kDa [19]. Although the precise mechanism of how NECL1 is glycosylated at the single site during brain development and the type of glycan needs to be further investigated, the model of NCAM polysialylation should give us some useful clues for the glycosylation of NECL1.

The result of cell aggregation assay showed that there is an increased adhesion activity after the glycosylation site was mutated. It has been proved that the steric properties of polysialic acid (PSA) can hinder the apposition of cell membranes regulated both by NCAM and by other molecules [16]. Sialic acid is a negative charge enriched and hydrophilic molecule, which can form a big hydrate radius [20]. The existing of PSA in NCAM would increase the distance between NCAMs and reduce the binding force between them, which caused the distance between other adhesion molecules being increased to inhibit their binding. Therefore the adhesion between cells would be disrupted by NCAM-H. This change could be necessary for neurite
outgrowth and axon expansion during the late embryonic and early postnatal stages. The homo-interaction between NECL1 may be also regulated by glycosylation similar to the NCAM mechanism. However, how the glycosylation affects the adhesion activity of human NECL1 remains to be further addressed. It is also a mystery why overexpression of NECL1 in the cells (293ET, L cells) leads to low glycosylation.

It has also been shown that NECL1 is a Ca2+-independent homo- or heterophilic cell–cell adhesion molecule which can interact with NECL1, NECL2, NECL4, Nectin-1 and Nectin-3 by forming cis-dimers first, followed by forming trans-dimers but not with NECL5 and Nectin-2 [6,9]. The crystal structure of the first Ig domain of NECL1 indicates that Phe82 is a key residue for the trans-adhesion activity of NECL1 [21]. It will be also interesting to ask whether the glycosylation affects the adhesion activity of human NECL1 in the cells (293ET, L cells) leads to low glycosylation.

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Acknowledgements

We thank Dr. Chengyu Jiang (Peking Union Medical College) for 293ET cell line and Mrs. Sheila Lennarz for critical reading of the manuscript. This work was supported by the National Sciences Foundation of China (Grant Nos: 30571039, 30721063); 973 (Nos: 2005CB522507, 2004CB518604, 2006CB504100); “863” (No: 2006AA02Z137); Program for New Century Excellent Talents in University (No: NCET-07-0505).

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