Ninjurin2, a Novel Homophilic Adhesion Molecule, Is Expressed in Mature Sensory and Enteric Neurons and Promotes Neurite Outgrowth

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A large number of cell adhesion molecules mediate cell-to-cell and cell-to-extracellular matrix interaction during development, differentiation and regeneration of the peripheral nervous system. Here, we report the identification of a novel cell surface adhesion molecule, ninjurin2 (for nerve injury induced protein 2). Ninjurin2 is a homolog of a homophilic cellular adhesion molecule, ninjurin1, that was previously isolated as a gene induced in Schwann cells after nerve injury. Ninjurin1 and 2 share conserved hydrophobic regions for their transmembrane domains; however, they do not contain comparable adhesion motifs nor do they interact with each other. In the peripheral nervous system, ninjurin2 is expressed constitutively in mature sensory and enteric neurons but not in glial cells or in autonomic ganglia. Ninjurin2 is upregulated in Schwann cells surrounding the distal segment of injured nerve with a time course similar to that of ninjurin1, neural CAM, and L1. Ninjurin2 promotes neurite outgrowth from primary cultured dorsal root ganglion neurons, presumably via homophilic cellular interactions. Ninjurin2 is also highly expressed in hematopoietic and lymphatic tissues. Finally, the ninjurin2 gene is located on human chromosome 12p13 in which several disorders of unknown etiology have been mapped, including inflammatory bowel disease and acrocallosal syndrome.

Key words: cell adhesion; postmitotic; peripheral nervous system; hematopoietic and lymphatic organs; chromosome 12p13; inflammatory bowel disease; acrocallosal syndrome

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MATERIALS AND METHODS

Cloning and sequence analysis. All sequencing analysis was performed on an Applied Biosystems (Foster City, CA) 373DNA sequencer using Taq DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems). Se-
quence editing, mapping, alignment, and contig generation were performed using the DNAstar software package. Expressed sequence tags (ESTs) were obtained from the Washington University–Mercè EST project and sequenced completely. Based on the identified human ninjurin2 cDNA sequence, primers for mouse ninjurin2 (5′-ATGCT-GGACGTCGCTCTTTATG-3′ and 5′-TATGAGACCAAGAT-GGTGCAGCATT-3′) were synthesized, and partial mouse ninjurin2 sequence was amplified by PCR. Rapid amplification of cDNA ends (RACE) PCR was performed using KlenTaq-LA (Barnes, 1994) and human and mouse brain cDNA libraries, per the manufacturer’s instructions (Clontech, Cambridge, UK). Products were cloned into the EcoR V site of pBluescript (Stratagene, La Jolla, CA), and full-length human and mouse cDNA sequences were determined. Putative transmembrane domains were predicted by PSORT algorithm (Nakai and Kanehisa, 1992).

For the ninjurin2 expression construct (pNINJ2), the coding region of the human ninjurin2 cDNA was amplified by KlenTaq-LA from the brain marathon RACE cDNA library, using primers 5′-GTCGAGATCT-CTCATGAAATCAGAAGAA-3′ and 5′-CTTAAAGCTTTAGGAGGATTCTTGGAGC-3′. The product was cloned into pCB6 expression vector (Breuer, 1994). Ninjurin1 expression construct (pN-1N1J) was described previously (Araki and Milbrandt, 1996). The human ninjurin2 genomic clone was obtained from a PAC genomic library (Roswell Park Cancer Center, Buffalo, NY).

Immunohistochemical analysis. The human RNA was probed with an RNA blotted onto nitrocellulose. The blot was incubated with the primary antibody. The signal was visualized using a chemiluminescent detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was then exposed to X-ray film. The resulting image was analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The results were quantified by densitometry using the ImageQuant software (Molecular Dynamics). The fold increase in signal intensity was calculated by dividing the intensity of the control sample by the intensity of the experimental sample.

RESULTS

Identification, sequence analysis, and genomic localization of ninjurin2

Ninjurin2 was identified by performing a basic local alignment search tool (BLAST version 2.0) (Altschul et al., 1990) search of the dbEST database using the full-length human ninjurin1 cDNA sequence as a query. An EST clone (GenBank accession number H91351) that showed partial similarity with ninjurin1 was obtained from the Washington University–Mercè EST project and sequenced. The 5′ end of the cDNA was obtained by RACE–PCR using a human brain Marathon RACE library as a template. The mouse ninjurin2 cDNA was subsequently obtained by PCR amplification using primers corresponding to the human ninjurin2 cDNA.

The nucleotide sequence of the human ninjurin2 cDNA predicts an open reading frame of 142 amino acids, which is 73% identical to mouse ninjurin2. Human ninjurin2 protein is 55% identical to human ninjurin1 but has no significant homology to any other known proteins in the database. Ninjurin2 has two hydrophobic regions, both of which can form transmembrane domains (PSORT algorithm; Nakai and Kanehisa, 1992). These transmembrane domains are the most highly conserved regions between ninjurin1 and 2. Ninjurin2 does not contain any N-linked glycosylation sites or a signal sequence. The residues 26–37 of ninjurin1, which contain the homophilic adhesive motif (Araki and Milbrandt, 1996), is not conserved in ninjurin2. Overall, these features suggest that ninjurin2 is a membrane protein like ninjurin1, but that it is functionally distinct from ninjurin1.

To analyze the genomic locus of ninjurin2, we identified and...
sequenced a human ninjurin2 genomic clone. The ninjurin2 gene has three introns, and the location of all three of these introns is precisely conserved between ninjurin1 and ninjurin2 (Fig. 1). A BLAST search against the high throughput genome sequence database revealed that human ninjurin2 is located on chromosome 12, region p13. A search of the On-line Mendelian Inheritance in Men (OMIM) database showed that some human diseases of unknown pathogenesis have been linked to this region, including acrocallosal syndrome (polydactyly and loss of corpus callosum) and inflammatory bowel disease.

Ninjurin2 is located on the plasma membrane and mediates homophilic adhesion

To characterize the ninjurin2 protein and examine its cellular localization, we generated polyclonal antiserum against a mixture of three synthetic peptides derived from the ninjurin2 N terminus (amino acids 1–30, 15–45, and 31–60, as shown in Fig. 4C). Anti-ninjurin2 antibodies were then purified by immunoaffinity chromatography. These antibodies recognized a ∼20 kDa protein expressed in CHO cells that were stably transfected with pNINJ2 but that was absent in either control CHO cells or CHO cells stably expressing ninjurin1 (Fig. 2).

The high sequence homology with ninjurin1 in the putative transmembrane domain and immunocytochemical analysis of CHO cells stably expressing ninjurin2 suggested that ninjurin2 is located on the cytoplasmic membrane (Fig. 3B). To examine this possibility, we performed immunostaining of live CHO cells stably expressing ninjurin2 using conditions in which antibodies do not penetrate the cell membrane and are not internalized. Anti-ninjurin2 antibodies showed intense staining on the cell surface (Fig. 3C), whereas negative control β-actin antibodies showed no staining under similar conditions (data not shown). This clearly indicated that ninjurin2 is located on the plasma membrane and that the N-terminal region of ninjurin2 (to which the antibodies were raised) is located extracellularly.

The cell surface localization of ninjurin2 and its homology with the adhesion molecule ninjurin1 suggested that ninjurin2 might also be an adhesion molecule. To test whether ninjurin2 mediates cellular adhesion, we performed standard cell aggregation assays using Jurkat cells (Shimizu et al., 1990). Jurkat cells were stably transfected with either pN1N2 or pCB6 (nonrecombinant vector). Expression of ninjurin2 in the Jurkat cells transfected with pN1N2 (N2 cells) but not in pCB6 was confirmed by immunoblot analysis (data not shown). Jurkat cells stably expressing ninjurin2 demonstrated increased aggregation compared with cells transfected with pN1N1 expression construct (lane 2), and CHO cells stably transfected with pN1N2 construct (lane 3) were electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with affinity-purified anti-ninjurin2 antibodies. Ninjurin2 was visualized by using enhanced chemiluminescence.

Figure 1. Sequence analysis of ninjurin2. A, Alignment of human ninjurin1 and ninjurin2. Identical residues are boxed, and putative transmembrane domains are underlined. Arrowheads denote intron–exon junctions. Note that these sites are conserved between the ninjurin1 and 2 genes. B, Alignment of human and mouse ninjurin2 amino acid sequences. Identical residues are boxed, and putative transmembrane domains are underlined.

Figure 2. Protein blot analysis of ninjurin2. Proteins from lysates prepared from native CHO cells (lane 1), CHO cells stably transfected with a pN1N1 expression construct (lane 2), and CHO cells stably transfected with pN1N2 construct (lane 3) were electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with affinity-purified anti-ninjurin2 antibodies. Ninjurin2 was visualized by using enhanced chemiluminescence.

pN1N2 (N2 cells) but not in pCB6 was confirmed by immunoblot analysis (data not shown). Jurkat cells stably expressing ninjurin2 demonstrated increased aggregation compared with cells transfected with pCB6 (Fig. 4A,B). Increased aggregation of ninjurin2-expressing Jurkat cells (N2 cells) was quantified in aggregation assays in 96-well culture plates. After 1 hr, the percentage of cells in aggregates was 83 ± 4% in N2 cells and 14 ± 4% in control cells. To identify which region of ninjurin2 is responsible for ninjurin2-mediated cell adhesion, we tested three partially overlapping peptides corresponding to the N-terminal extracellular region of ninjurin2 for their ability to inhibit ninjurin2-mediated cellular aggregation (Fig. 4C,D). Peptide (P) 1 and P2 inhibited aggregation in a dose-dependent manner, with
pressing ninjurin2 identified by RNA blots, other tissues showed expression of ninjurin2 with restricted distribution patterns. In the kidney, ninjurin2 was detected specifically in the glomeruli (Fig. 6A). In the adrenal gland, ninjurin2 expression was observed only in the glomerular layer of the cortex (Fig. 6B). In the CNS, neuronal expression of ninjurin2 was very low; however, ninjurin2 was detected in radial glial cells during development and in adulthood (data not shown).

**Ninjurin2 is expressed in mature postmitotic sensory and enteric neurons**

To determine whether ninjurin2 plays a role in the PNS, expression of ninjurin2 was examined in detail by immunohistochemistry. Expression of ninjurin2 was detected in most neurons of the sensory and enteric ganglia, but in contrast to ninjurin1 (Araki and Milbrandt, 1996), ninjurin2 was not expressed in the supportive glial cells. Ninjurin2 expression was intense in the adult rat trigeminal (Fig. 6C), DRG (Fig. 6D), and nodose ganglia (data not shown). The intensity of ninjurin2 immunoreactivity was similar in most neurons and did not correlate with neuronal size. In the enteric nervous system, ninjurin2 was detected in neurons of both the submucosal and myenteric plexuses (Fig. 6F). In contrast, ninjurin2 expression in the superior cervical ganglion (Fig. 6E) and parasympathetic ganglion in the salivary gland (data not shown) was very low.

To explore the developmental regulation of ninjurin2 expression in the PNS, immunohistochemical analysis was performed on mouse DRG at E14, E19, and postnatal day 2 (P2) and in the mouse enteric ganglia at E17, P1, and P3. In the DRG, ninjurin2 expression was very weak at E14 (Fig. 7A), became apparent at E19 (Fig. 7B), and by P2, the intensity of staining was comparable with the adult level (Fig. 7C). An examination of the enteric ganglia revealed that ninjurin2 is not expressed at E17 (Fig. 7D), but weak ninjurin2 immunoreactivity was observed in neurons of the myenteric ganglia at P1 (Fig. 7E) and was more intense at P3 (Fig. 7F). In contrast, ninjurin2 expression was undetected in the submucosal plexus.

Neuronal proliferation in the myenteric ganglia is almost complete by P3, whereas in the submucosal plexus, neurons are generated later in development (by P14) (Pham et al., 1991). This raised the possibility that ninjurin2 is expressed only in postmitotic enteric neurons. To test this hypothesis, BrdU was injected into 3-d-old mice. Animals were killed 1 hr later, and gut sections were examined immunohistochemically to assess ninjurin2 expression and BrdU incorporation. We found that ninjurin2-immunoreactive cells in the myenteric plexus lacked BrdU incorporation (Fig. 8C,D), whereas some neuron-specific enolase (NSE)-positive cells in the submucosal plexus were BrdU-positive (Fig. 8E,F). These results suggest that ninjurin2 expression occurs after enteric neurons become postmitotic.

**Ninjurin2 is upregulated after nerve injury in Schwann cells**

We were interested in characterizing the role of ninjurin2 after nerve injury, because the related ninjurin1 is highly induced in Schwann cells after nerve injury. To examine the expression of ninjurin2 after nerve injury, immunohistochemistry was performed to detect ninjurin2 in normal and injured nerves. In normal sciatic nerve, ninjurin2 expression was weak (Fig. 9A), but it was greatly upregulated 7 d after nerve transection in the distal segment of the injured nerve (Fig. 9B). The digitizing pattern of ninjurin2 immunoreactivity in the injured nerve (Fig. 9D)
closely resembled that observed for ninjurin1 (Fig. 9C), indicating that ninjurin2 is expressed by Schwann cells.

To further characterize the regulation of ninjurin2 expression after nerve injury, we examined ninjurin2 mRNA levels for up to 8 weeks after nerve injury (Fig. 9E). A 1.0 kb ninjurin2 mRNA was detected at low levels in normal nerve but was highly upregulated after nerve injury and reached peak levels 7–14 d after injury. This time course of expression is similar to what has been observed for other nonmyelinating Schwann cell marker molecules, including ninjurin1 and p75 (Taniuchi et al., 1988; Araki and Milbrandt, 1996). The message level remained high for up to 56 d when the nerve was completely transected, but when the injury was partial and nerve regeneration could occur, ninjurin2 mRNA expression returned to low levels after 28–56 d (data not shown).

**Ninjurin promotes neurite outgrowth from primary cultured DRG neurons**

Upregulation of ninjurin2 in Schwann cells after nerve injury suggested that it may promote nerve regeneration by homophilic adhesive interactions as has been observed with other adhesion molecules (Seilheimer and Schachner, 1988; Lemmon et al., 1989). To test this possibility, we examined neurite outgrowth in DRG neuron–CHO cell cocultures (Eichler and Rich, 1989). DRG neurons were dissected from E16 rat embryos, and dissociated neurons were seeded at low density onto confluent monolayer of either wild-type CHO cells or CHO cells stably expressing ninjurin2. The culture was fixed after 6 hr of culture, and neurites were visualized by immunostaining of neurofilament H. As described previously (Araki and Milbrandt, 1996), 50 neurons were randomly selected from each culture condition, and the length of the longest neurite per neuron, which did not have contacts with nearby neurons, was measured. As shown in Figure 9F, neurons plated on CHO cells expressing ninjurin2 extended significantly longer neurites than the ones plated on wild-type CHO cells. These results indicate that ninjurin2, like ninjurin1, promotes neurite outgrowth from primary cultured neurons.

**DISCUSSION**

We have identified a novel member of the ninjurin family of adhesion molecules. Members of this family share high homology in the putative transmembrane domains, lack signal peptide sequences, and have the N-terminal hydrophilic region located...
extracellularly. The extracellular regions, especially the domains involved in adhesive interactions, are diverse. Although interactions between family members are commonly observed with other adhesion molecules (Brummendorf and Rathjen, 1996), no heterophilic interactions were observed between ninjurin2 and ninjurin1. Consistent with these results, residues comprising the ninjurin1 adhesion motif are not conserved in the ninjurin2 adhesion motif located between residues 16 and 45.

The tissue distribution of ninjurin proteins indicates that they are involved in multiple functional systems in the body like most other known adhesion molecules. Ninjurin1 showed a wide distribution primarily among organs of epithelial origin, whereas
ninjurin2 showed more restricted distribution; ninjurin2 was highly expressed in lymphatic and hematopoietic organs. In addition, the majority of human ninjurin2 cDNA sequence in the dbEST database is derived from "germinal center B cells" libraries (data not shown). This suggests that ninjurin2 expression in the lymphatic cells is involved in B lymphocyte function, perhaps during maturation of B lymphocytes to the antibody-producing cells.

Ninjurin2 expression in mature sensory and enteric neurons clearly distinguishes this molecule from ninjurin1 and other cell surface adhesion molecules. In mouse DRG, many neurons are generated between E10 and E13 in a neurotrophin-3-dependent manner (Farinas et al., 1996, 1998). In the enteric nervous system, submucosal neurons are generally born later than myenteric neurons, lack ninjurin2 expression in A. Scale bar, 100 μm. C–F, BrdU and either ninjurin2 (C, D) or NSE (E, F) were visualized by Cy3-conjugated secondary antibody, and proliferating cells were visualized by FITC-conjugated anti-BrdU immunohistochemistry (D, F). The ninjurin2-positive myenteric ganglia lack BrdU staining (arrows in C and D), whereas the NSE-positive cells in the submucosal ganglia indicated by arrows in E and F are BrdU-positive. Note that C and D represent the same section as do E and F. Scale bars, 50 μm.
include interaction between neurons and peripheral sensory organs that express ninjurin2 or other unidentified heterophilic partners of ninjurin2.

Expression of ninjurin2 in the DRG neurons is not uniform; there are a very small number of neurons in which ninjurin2 immunoreactivity is weak or absent. Such neurons are more clearly observed after axonal injury when most DRG neurons increase ninjurin2 expression. Coimmunostaining of ninjurin1 and ninjurin2 showed that ninjurin1 is also not induced in neurons that lack ninjurin2 expression (data not shown). This result suggests that some DRG neurons are dependent on surface molecules other than ninjurins for axonal regeneration or other functions.

Although ninjurin1 and 2 are differentially expressed in the PNS, ninjurin2 is likely to play a role in nerve regeneration via homophilic interaction, just like ninjurin1. The time course of upregulation of ninjurin2 after nerve injury is similar to that of ninjurin1, and the level of neurite outgrowth enhancement in coculture experiments with DRG neurons and ninjurin2-expressing CHO cells was comparable with the ninjurin1-expressing CHO cells (Araki and Milbrandt, 1997). During peripheral nerve regeneration, ninjurin2 may have additional or synergistic effect on the neurite outgrowth-promoting effects of ninjurin1 and other adhesion molecules, including N-CAM and L1 (Bixby et al., 1988; Seilheimer and Schachner, 1988).

In the human chromosome region 12p13 in which ninjurin2 is located, several diseases of unknown etiology have been mapped (Pfeiffer et al., 1992; Satsangi et al., 1996; Duerr et al., 1998). Diseases with neurological defects, such as acrocallosal syndrome, are of particular interest because mutations in another adhesion molecule, L1, result in severe neurological defects, including MASA (mental retardation, aphagia, shuffling gate, and adducted thumbs) syndrome (Vits et al., 1994). Possible association of ninjurin2 with inflammatory bowel disease is also interesting from both immunological and neurological perspectives. From the immunological perspective, differences in lymphocyte homing capability between inflammatory bowel disease and normal intestine have been reported previously (Panes and Granger, 1998). Surface adhesion molecules like ninjurin2 expressed on lymphocytes could be important for the pathogenesis of local and/or systemic inflammatory disorders. On the other hand, a recent report showed that moderate degeneration of myenteric neurons could cause severe inflammation in the gut (Bush et al., 1998). Thus, ninjurin2 mutations could also affect gut physiology by altering enteric neuron activities, such as regulation of mucosal blood flow.

REFERENCES


