

Mechanism of Homophilic Binding Mediated by Ninjurin, a Novel Widely Expressed Adhesion Molecule*

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Ninjurin is a novel protein that is up-regulated after nerve injury both in dorsal root ganglion (DRG) neurons and in Schwann cells. We previously reported that ninjurin demonstrates properties of a homophilic adhesion molecule and promotes neurite outgrowth from primary cultured DRG neurons. We have now found that ninjurin is widely expressed in both adult and embryonic tissues, primarily in those of epithelial origin. Aggregation assays were used to demonstrate that ninjurin-mediated adhesion requires divalent cations and is an energy-dependent process. The critical domain for ninjurin-mediated homophilic adhesion was localized to an 11-residue region (between Pro²⁶ and Asn³⁷) by mutagenesis and by employing synthetic oligopeptides as competitive inhibitors of ninjurin-mediated adhesion. Of particular importance are the Trp residue at position 29 and the 3 arginines in the region. Furthermore, we show that the peptide which inhibits aggregation of Jurkat cells expressing ninjurin is also capable of blocking the ability of ninjurin to promote neurite extension from DRG neurons. Using FISH analysis, the ninjurin gene was localized to human chromosome 9q22. Several genetic diseases of unknown etiology have been mapped to this region, including hereditary sensory neuropathy type 1, self-healing squamous epithelioma, split-hand/foot deformity type 1, and familial dilated cardiomyopathy.

Cell surface adhesion proteins play an important role in embryonic development, in organogenesis, and in tissue regeneration after injury (1–3). In the nervous system, adhesion molecules function as receptors for axonal guidance cues and are important for the development of neuronal connections (4). In the immune system, cell surface molecules mediate signaling mechanisms that are critical for specificity in lymphocyte homing (5). In addition, normal cell-cell interactions are often aberrant in tumors, and there is increasing evidence that impaired cellular interaction due to loss of adhesion proteins plays a role in tumorigenesis, as well as tumor invasion and metastasis (6, 7). Indeed, a gene encoding an adhesion molecule similar to neural cell adhesion molecule (NCAM) and N-cadherin has been identified as a tumor suppressor gene (8, 9).

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In vertebrates, a number of cell surface glycoproteins have been identified as adhesion molecules, including integrins, cadherins, and those containing an immunoglobulin (Ig)¹-like motif (3, 10, 11). The sequence motifs that mediate the adhesive interactions of these molecules have been identified for only a few of these proteins. One of the most well characterized sequence motifs of this type is the tripeptide Arg-Gly-Asp (RGD), which was identified as the sequence within fibronectin that mediates cell attachment (12). Many integrins recognize this RGD motif within their respective binding partners, and these interactions then mediate either cell-substratum or cell-cell interactions. Members of the cadherin family contain multiple copies of the Asp-Arg-Glu (DRE) and Asp-Xaa-Asn-Asp-Asn (DXNDN) sequences (13). Structural analysis of cadherin indicates that these motifs may be situated such that they can form a zipper-like structure that may be critical for cell adhesion (3). Shared sequence motifs for members of the Ig-superfamily of adhesion molecules are less well understood although it has been proposed that a decapeptide sequence (KYSFNYDGSE) in the third Ig-like domain of NCAM is responsible for its homophilic binding interactions (14).

We have identified a cell membrane-associated protein, ninjurin, that possesses homophilic adhesion properties but has no significant homology with other proteins (15). Ninjurin is widely expressed in embryonic and adult rats, predominantly in epithelial cells. To define the mechanism of ninjurin-mediated adhesion, we focused on identifying sequences necessary for the binding interaction. Using a cell aggregation inhibition assay and synthetic peptides corresponding to the predicted ninjurin extracellular domain, we localized the binding region to residues 26 through 37. Based on these results, we tested ninjurin molecules with mutations in this region for their adhesive properties and showed that these ninjurin mutants were incapable of mediating cell aggregation. Furthermore, to demonstrate that ninjurin function is associated with its adhesive properties, we demonstrate that ninjurin-stimulated neurite outgrowth from dorsal root ganglia neurons is reversed by administration of peptides that block ninjurin-mediated cell aggregation.

EXPERIMENTAL PROCEDURES

Analysis of Ninjurin mRNA and Protein Expression Patterns—Total RNA was prepared from adult rat tissues and samples (10 µg) were electrophoresed on 1% agarose-formaldehyde gels and blotted onto nylon membranes as described previously (16). Membranes were probed with a ³²P-labeled fragment of ninjurin cDNA. For *in situ* hybridization analysis of embryos, timed pregnant rats of the indicated gestational age were sacrificed, and the embryos were immediately frozen. *In situ* hybridization was performed using ³³P-labeled antisense or sense RNA

¹ The abbreviations used are: Ig, immunoglobulin; DRG, dorsal root ganglia; NCAM, neural cell adhesion molecule; CMTMR, 5-(and-6)-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; CMFPA; 5-chloromethylfluoresceindiacetate; nt, nucleotides; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; Cy3, indocarbocyanine; FCS, fetal calf serum.

probes transcribed from the ninjurin cDNA (nt 518–1026) on fresh frozen tissue samples as described previously (17). For immunohistochemical analysis of adult rat tissues, male Sprague-Dawley rats (200–300 g) were anesthetized and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). The analysis was performed on 15 μ m cryostat sections using standard methods. The purified anti-ninjurin antiserum was used at a 1:2500 dilution, and specific staining was detected with indocarbocyanine (Cy3)-conjugated anti-rabbit IgG (Jackson Immunological Laboratories). All the tissue samples were stored at -70°C . For immunofluorescent staining of live cultured cells, Chinese hamster ovary (CHO) cells expressing ninjurin were washed 3 times with PBS containing 0.5% Triton X-100. The cells were then incubated with a mixture of rabbit anti-ninjurin antiserum and a mouse anti- β -actin monoclonal antibody (1:500 dilution, Sigma) for 30 min at 4°C and then fixed with 4% paraformaldehyde in PBS. Immunoreactivity was visualized with either Cy3-conjugated anti-rabbit IgG (ninjurin) or fluorescein-conjugated anti-mouse IgG (β -actin). Immunoblotting using the anti-ninjurin antibodies was performed as described previously (18).

FISH Localization—A ninjurin genomic probe was labeled with digoxigenin (Random Primed DNA Labeling Kit, Boehringer-Mannheim). The labeled probe was used for *in situ* hybridization of human chromosomes derived from methotrexate-synchronized normal peripheral lymphocyte cultures. The conditions of hybridization, the detection of hybridization signals, digital-image acquisition, processing and analysis as well as the procedure for direct visualization of fluorescent signals to banded chromosomes were performed as described previously (19, 20).

Peptide Preparation—Peptides were synthesized using an Applied Biosystems peptide synthesizer. Lyophilized crude peptides were purified by reverse phase-high performance liquid chromatography using an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. The purity and composition of the purified peptides were verified by mass spectrometry. Purified peptides were dissolved in distilled water and stored at -70°C .

Plasmids and Mutagenesis—The ninjurin expression vector (pCMV-ninjurin) has been previously described (15). Expression vectors containing ninjurin mutants pCMV-ninjurin(W29A), which replaces the Trp with Ala at position 29, and pCMV-ninjurin(R32,34N), which replaces the Arg at positions 32 and 34 with Asn, were prepared by introducing the indicated mutations into synthetic oligonucleotides (5'-GCTCTCCGCGCGCTCTTCTTGTTGGCGTAATGGTTTACATTGATGGGCCGGTTCCTCAAACCCGCGCGGGGTGG for W29A and 5'-GCTCTCCGCGCGCTCTTCTTGTTGGCGTAATGGTTTACATTGATGGTGTGTTCTCAAACCCGCGGGGTGG for R32,34N). PCR reactions using the pCMV-ninjurin plasmid as template were performed in which the mutagenic primer was paired with a primer corresponding to nt 840–860 of the pCMV vector. The amplified products were digested with *Bgl*II and *Sac*II, and the fragments containing the mutations were ligated into pCMV-ninjurin digested with *Bgl*II and *Sac*II so as to replace the corresponding wild-type fragment and create pCMV-ninjurin(W29A) and pCMV-ninjurin(R32,34N).

Cell Culture and Transfection—CHO and Jurkat cells were grown as described previously (15). The expression vectors pCMV-ninjurin, pCMV-ninjurin(W29A) and pCMV-ninjurin(R32,34N) were transfected into CHO cells via calcium phosphate precipitation or into Jurkat cells via electroporation. Stable transfectants were selected by growing in medium containing G418 (0.4 mg/ml).

Primary cultures of neurons from dorsal root ganglia (DRG) were prepared from E17 rat embryos and dissociated neurons were grown on confluent monolayers of either native CHO cells or CHO cells expressing ninjurin in 24-well plates. Cultures were grown in a medium that consisted of 90% Eagle's minimal essential medium (Life Technologies, Inc.), 10% FCS, 20 μ g/ml each fluorodeoxyuridine and uridine, and 50 ng/ml mouse nerve growth factor (gift from E. Johnson, Washington University). Synthetic peptides were added at a concentration of 0.16 mM to wells where indicated. Six h after plating, the cells were fixed in 4% paraformaldehyde in PBS and immunohistochemistry was performed using anti-neurofilament H antibody. The length of the neurites extended by the DRG neurons was measured as described previously (21).

Cell Aggregation Assays—Aggregation assays were performed in 96-well microtiter plates as described previously (22) using Jurkat cells stably transfected with ninjurin expression vectors (either wild type or mutant). Results of the assays were quantitated after 1 h unless otherwise indicated. Reagents and peptides were added to the cell suspension prior to performing the assays at the indicated concentrations. The requirement for divalent cations was tested in Hank's balanced salt solution without calcium and magnesium containing 10% dialyzed fetal bovine serum. Cell quantitation was performed as described previously

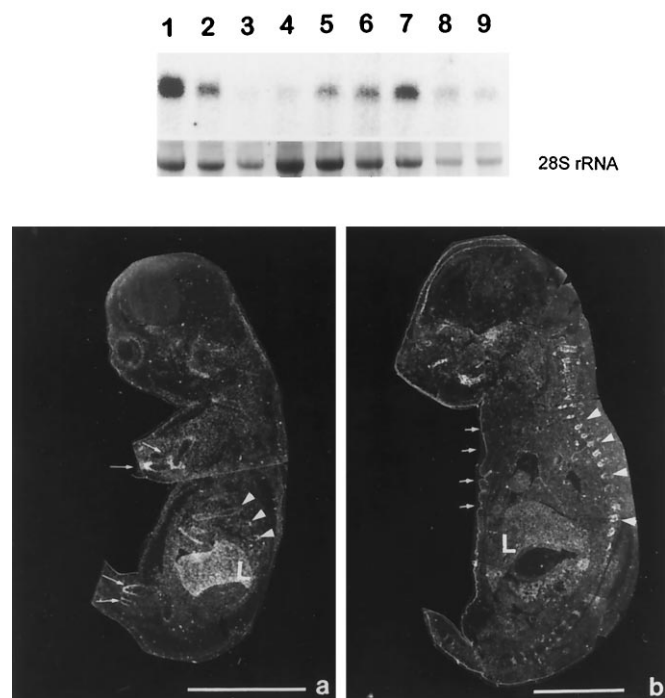


Fig. 1. Ninjurin RNA analysis in adult and embryonic rat tissues. Top panel, samples of total RNA (10 μ g) from liver (lane 1), heart (lane 2), lung (lane 3), gut (lane 4), thymus (lane 5), spleen (lane 6), adrenal (lane 7), DRG (lane 8), and brain (lane 9) were electrophoresed, blotted, and hybridized with ^{32}P -labeled ninjurin cDNA probe. The ethidium bromide staining of 28S ribosomal RNA is shown to demonstrate the quantity of RNA loaded. Bottom panel, sagittal sections of E17 (a) and E19 (b) rat embryos were hybridized with ^{32}P -labeled ninjurin RNA probe. Note the intense expression of ninjurin mRNA at E17 in the liver (L), growing limb (arrows), and costal bones (arrowheads) and at E19 in the liver (L), adrenal (A), spleen (S), vertebrae (arrowheads) and skin (arrows). Ninjurin mRNA is not detected in the brain at either E17 or E19. Scale bars = 5 mm.

(22). Briefly, photographs of the cells were taken at the indicated time points, and the total number of cells as well as the number of cells incorporated into aggregates were counted and a ratio was determined. At least 4 assays were performed for each set of experimental conditions. Statistical analysis was performed by SigmaPlot (version 3.0).

For aggregation experiments performed using a mixture of cells, cells expressing wild-type ninjurin were stained red with 1 mM CMTMR, and cells expressing mutant ninjurin were stained green with 1 mM CMFTA as described in the manufacturer protocol (Molecular Probes). The cells were resuspended to 1×10^6 cells/ml, and 3 ml of the mixed cell suspension was allowed to form aggregates in 6-well culture plates. The composition of the aggregates was monitored by fluorescence microscopy.

RESULTS

Ninjurin Is Expressed in a Variety of Tissues during Embryogenesis and Adulthood—In previous work, we showed that ninjurin is expressed in the nervous system, particularly after nerve injury (15). To further examine the expression pattern of ninjurin in the adult rat, we performed RNA blot analysis on samples isolated from a variety of tissues. The highest levels of ninjurin mRNA were found in the liver. In addition, thymus, heart, adrenal gland and spleen also had significant levels of ninjurin transcripts, whereas brain and DRG had low levels of expression (Fig. 1). To examine ninjurin expression during development, *in situ* hybridization was performed on rat embryos sacrificed at embryonic days 17 and 19. Ninjurin expression is observed in a variety of embryonic tissues, with the most abundant expression observed in tissues where it is highly expressed in the adult (e.g. liver, adrenals, and spleen) (Fig. 1). Ninjurin was also detected in the vertebra and limbs, where its expression increased with increasing embryonic age. The signal was detected primarily in regions of active ossification,

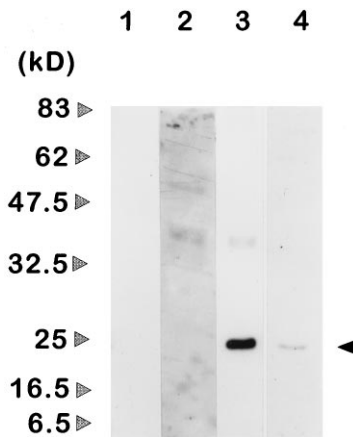


FIG. 2. Protein blot analysis of ninjurin. Proteins from lysates prepared from native CHO cells (lane 1), CHO cells stably transfected with a CMV-ninjurin expression construct (lanes 2 and 3) or adult rat liver (lane 4) were electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with affinity purified anti-ninjurin rabbit antibodies. Ninjurin (denoted by arrowhead) was visualized using enhanced chemiluminescence. In lane 2, the antiserum was preadsorbed with an excess amount of the peptide used as the immunogen to demonstrate the specificity of the antibodies.

such as the terminal areas of the limb and vertebral bones. The most intense signal was observed over layers of dividing chondrocytes, rather than over regions that were already ossified. Ninjurin was also highly expressed in the skin, where it was primarily detected in the epithelium. In the central nervous system, ninjurin expression was very low throughout the embryonic period examined. Tissues hybridized with a ninjurin sense probe or tissues treated with ribonuclease prior to hybridization showed no appreciable signal (data not shown).

Ninjurin Is Expressed Predominantly in Epithelial Cells—To further characterize the ninjurin protein, we prepared a polyclonal antiserum against a peptide derived from the N terminus (amino acids 1–15). Anti-ninjurin antibodies were then purified by immunoaffinity chromatography. These antibodies recognized a 22-kDa protein that was present in rat liver and in CHO cells stably transfected with a ninjurin expression vector, but not in native CHO cells (Fig. 2).

After verifying their specificity for ninjurin, these antibodies were used to examine cell type-specific ninjurin expression via immunohistochemistry. This analysis confirmed that ninjurin is expressed in a wide variety of tissues in the adult rat, including the liver, kidney, thymus, uterus, adrenal gland, retina, and dorsal root ganglia (Fig. 3). In the dorsal root ganglia, ninjurin was not detected in neurons but rather was expressed in the satellite cells that ensheath the neuronal cell bodies. In the adrenal gland, ninjurin is expressed throughout the cortex and appears to be on the surface of the cortical cells. Ninjurin immunoreactivity was also observed on the surface of the hepatocytes of the liver. In the kidney, ninjurin was detected in the podocytes and/or mesangial cells of the glomerulus, but other renal cell types were negative. In the thymus, scattered cells stained intensely. These ninjurin-expressing cells could represent a specific subset of thymocytes or the thymic epithelial cells, which provide the proper microenvironment for lymphocyte maturation. Flat cells located on the surface of the thymic cortex and in the area adjacent to the blood vessels also expressed ninjurin. Staining for ninjurin was also observed in the uterus, where ninjurin-positive cells were found in the myometrium. These cells were distinct from smooth muscle cells, and their distribution and morphology was consistent with fibroblasts of the associated connective tissues. In the retina, ninjurin was detected in the Muller cells

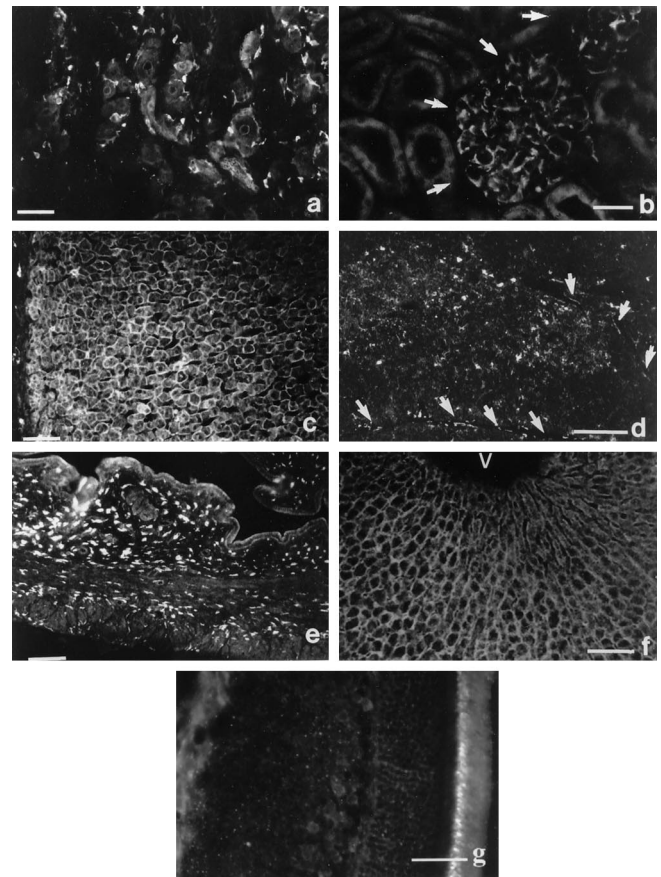


FIG. 3. Localization of ninjurin by immunohistochemistry. Affinity purified anti-ninjurin antibodies were used to detect ninjurin in a variety of adult rat tissues by immunohistochemistry: DRG (a), kidney (b), adrenal gland (c), thymus (d), uterus (e), liver (f); and retina (g). Note that arrows in panel b denote a glomerulus, and in panel d, they denote blood vessels on the cortical surface of the thymus. In panel f, v denotes the central vein of the liver. Scale bars in panels a, c, e, and f = 100 μ m; in panel b, scale bar = 50 μ m; and in panel d, scale bar = 200 μ m.

near the apical ends, which project into the interphotoreceptor matrix. The brush-like staining pattern strongly indicates that the intense immunoreactivity is restricted to the apical microvilli of the Muller cells. Taken together, these results demonstrate that ninjurin immunoreactive cells are found in a wide variety of tissues.

The pattern of staining we observed suggests that ninjurin is associated with the cell surface, consistent with its role in mediating cell adhesion and with the cell surface labeling experiments previously reported (15). To provide further confirmation of its surface localization, immunostaining of live cells was performed. CHO cells stably expressing ninjurin were incubated with anti-ninjurin antibodies at 4 $^{\circ}$ C prior to fixation, conditions during which antibodies are excluded from penetrating the cell. The ninjurin immunoreactivity was intense and appeared to be restricted to the cell surface (Fig. 4). When antibodies to the intracellular protein β -actin were used to stain the cell under similar conditions, no immunoreactivity was observed. These results confirm that the antibodies were unable to enter the cell under these staining conditions and clearly indicate that the N-terminal region of the ninjurin molecule (to which the antibodies were raised) is located extracellularly.

Chromosomal Localization of the Human Ninjurin Gene—To obtain further clues regarding the function of ninjurin and its possible association with human disease, the chromosomal localization of the human ninjurin gene was determined. In two

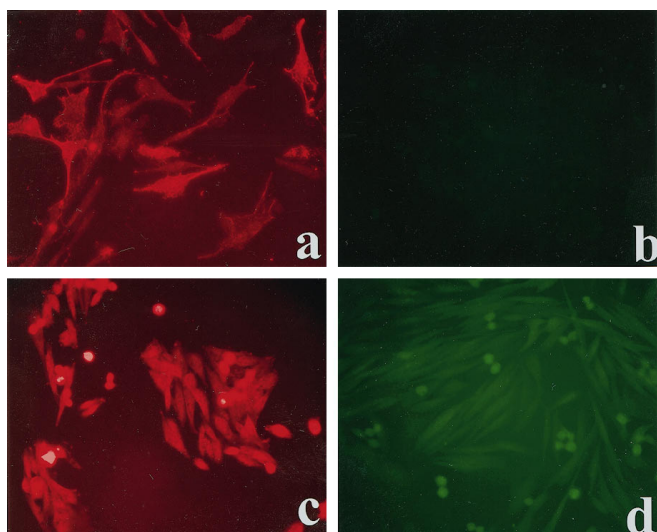


FIG. 4. Immunostaining of live cells demonstrates ninjurin is located extracellularly. CHO cells expressing ninjurin were incubated with a mixture of rabbit anti-ninjurin and mouse anti- β -actin antibodies under conditions that preclude panels *a* and *b* or allow panels *c* and *d* antibodies access to the interior of the cell. Ninjurin was visualized (*a*, *c*) using Cy3-conjugated anti-rabbit IgG, and β -actin was visualized (*b*, *d*) using fluorescein isothiocyanate-conjugated anti-mouse IgG.

experiments with lymphocytes from different individuals, 80% of the chromosome spreads had specific fluorescent signals at identical sites on both chromatids of the long arm of chromosome 9. From a total of 190 metaphases examined, 175 had fluorescent signals on both chromosomes 9 (Fig. 5A). The location of the fluorescent signal was determined directly in 50 metaphases with 4',6-diamidino-2-phenylindole-enhanced G-like banding at region 9q.22.1-q22.3 where we assign the locus of the ninjurin gene (Fig. 5B). Several diseases of unknown pathogenesis have been linked to this region, including hereditary sensory neuropathy type 1 (23), self-healing squamous epithelioma (24), split-hand/foot deformity type 1 (25), and familial dilated cardiomyopathy (26).

Properties of Ninjurin-mediated Adhesion—We had previously used a cell aggregation assay to demonstrate that Jurkat T-cell leukemia cells expressing ninjurin showed increased adhesiveness compared with native Jurkat cells (15). Additional studies demonstrated that ninjurin adhesion was mediated via homophilic interactions. Inspection of the ninjurin sequence did not reveal any motifs present in other adhesion molecules that might mediate homophilic adhesion. To characterize the adhesive properties of ninjurin, cell aggregation assays using ninjurin-expressing Jurkat cells were performed under various conditions (Fig. 6). Ninjurin-mediated adhesion was completely inhibited by prior addition of 20 μ M cytochalasin B which depolymerizes actin filaments, as well as by 0.02% sodium azide, an inhibitor of oxidative phosphorylation. Aggregation was also inhibited when the assays were conducted at 4 $^{\circ}$ C. These results demonstrate that ninjurin-mediated adhesion requires a functional cytoskeleton and is an active, energy-dependent process. We also examined the pH and divalent cation requirements of the interaction. Minimal aggregation of ninjurin-expressing Jurkat cells was observed when assays were performed in Ca^{2+} - and Mg^{2+} -free medium, or in normal medium containing greater than 5 mM EDTA. Aggregation could be restored by the addition of either CaCl_2 or MgCl_2 , indicating that ninjurin-mediated adhesion requires divalent cations. Ninjurin-expressing Jurkat cells showed the same degree of aggregation between pH 7 and 11, whereas aggregation was inhibited at a pH less than 6.

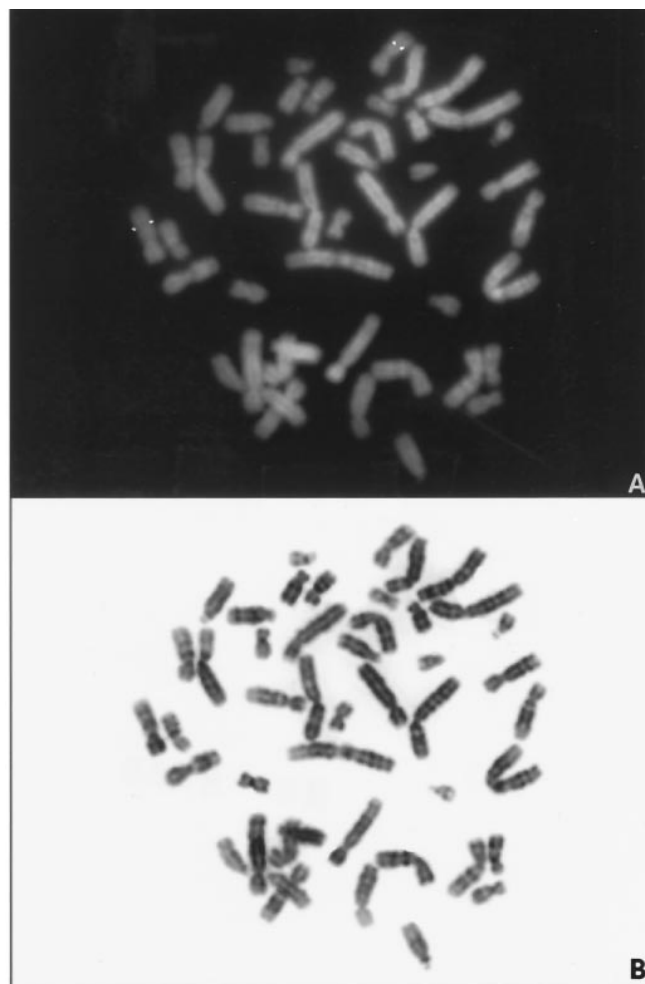


FIG. 5. FISH localization of the ninjurin gene to human chromosomes. A, digital image of a metaphase chromosome spread derived from methotrexat- synchronized normal human peripheral leukocytes after hybridization with a digoxigenin-labeled ninjurin genomic fragment probe. Two chromosomes have symmetrical signals on sister chromatids. B, the digital image of the 4', 6-diamidino-2-phenylindole-counterstained chromosomes of the same metaphase spread that was contrast-enhanced and look-up-table-inverted to obtain G-like bands. The banding resolution of individual chromosomes permits direct localization of the hybridization signal to band 9q22.1-q22.3, the assigned location of the human ninjurin gene. To confirm the identity of chromosomes, preparations were rehybridized with chromosome-specific painting probes (33), and previously observed labeled metaphases were re-recorded.

Characterization of Ninjurin Adhesion Domain—Analysis of the ninjurin sequence predicted that it contains two transmembrane domains (between residues 72 and 100 and between 118 and 139) (15). This analysis further suggested that the amino terminus of the molecule is likely to be located extracellularly. To directly investigate the hypotheses that the N-terminal hydrophilic region is located extracellularly and is responsible for homophilic adhesion, we tested whether peptides from this region could inhibit ninjurin-mediated aggregation (27) (Fig. 7). Partially overlapping synthetic peptides whose sequences are derived from the predicted ninjurin extracellular domain were tested for their ability to inhibit ninjurin-mediated adhesion (Fig. 8A). Peptides 1 and 2 inhibited aggregation in a dose-dependent manner. Aggregation was completely abolished by peptide 2 at concentrations above 0.33 mM, whereas peptide 1 inhibited aggregation less efficiently. Peptide 4 had no effect on aggregation, and we were unable to test peptide 3 due to its very poor solubility. These results indicated that the nin-

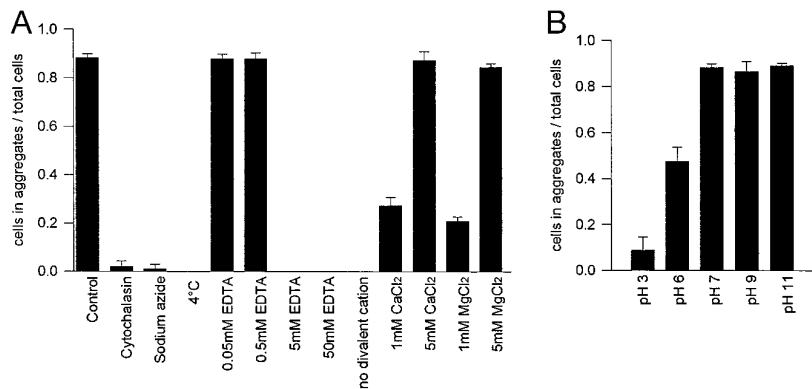


FIG. 6. **Characterization of ninjurin-mediated adhesion.** A, aggregation assays using ninjurin-expressing Jurkat cells were performed in RPMI 1640 with 10% fetal calf serum (FCS) containing 20 μ M cytochalasin B, 0.02% sodium azide, or the indicated concentration of EDTA. Assays were also conducted at 4 $^{\circ}$ C. Assays were also conducted in Hanks' balanced salt solution with 10% dialyzed FCS containing the indicated divalent cation. B, assays were conducted in RPMI 1640 with 10% FCS at the indicated pH. Aggregation assays were performed in flat bottomed 96-well microtiter plates. In each assay, 100 μ l of cell suspension at 2×10^6 cells/ml was added per well, and the ratio of the number of cells in the aggregates to the total number of cells was determined after 1 h. Data represent the mean \pm S.D. of four independent experiments

	1	10	20	30	40	50	60	70	80	90	100
	MDPGTEEYEL	NGDLRPGSPG	SPDASPPRWG	LRNRPINVNH	YANKKSAAES	MLDIALLMAN	ASQLKAVVEQ	GNEFAFFVPL	VVLISISLVL	QIGVGVLILF	
P1	MDPGTEEYEL	NGDLRPGSPG	SPDASPPRWG								
P2		PGSPG	SPDASPPRWG	LRNRPINVNH	YANKK						
P3				LRNRPINVNH	YANKKSAAES	MLDIALLMAN					
P4					SAAES	MLDIALLMAN	ASQLKAVVEQ	GNEFA			
P5				PPRWG	LRNRPINVNH	YANKKSAAES					
P6				PPRWG	LRNRPIN						
P7				RNRPINVNH	YAN						
P8					VNH	YANKKSAAES					
				26	37						
M1(R28N)				PPN	WG LRNRPIN						
M2(W29A)				PPRA	G LRNRPIN						
M3(G30A)				PPRW	A LRNRPIN						
M4(L31N)				PPRWG	NLRNRPIN						
M5(R32N)				PPRWG	LNRNRPIN						
M6(N33L)				PPRWG	LRLRPIN						
M7(R34N)				PPRWG	LRNNRPIN						

FIG. 7. **List of synthetic peptides used in competition experiments.** The N-terminal 100 amino acids of rat ninjurin are shown, with the residues critical for adhesion indicated in **bold**. The names and sequences of the peptides are indicated, with the mutated residues denoted by **underlined bold** characters.

jurin N terminus was indeed located extracellularly, and that residues corresponding to peptide 2 were critical for binding.

To further delimit the site of interaction, we tested four additional peptides (peptides P5–P8; Fig. 7) for their ability to inhibit ninjurin-mediated aggregation. These results demonstrated that a peptide (P6), corresponding to ninjurin residues 26–37, showed inhibitory activity comparable with peptide 2 (Fig. 8B, data not shown for P5, P7, and P8). Aggregation was inhibited at P6 concentrations greater than 0.02 mM and was completely abolished at concentrations above 0.4 mM. To identify the amino acids most critical for ninjurin-mediated adhesion, we synthesized a series of peptides (M1–M7) extending from residue 26 to 37, each containing a single amino acid mutation (Fig. 7). The ability of each of these peptides to inhibit ninjurin-mediated adhesion in a dose-dependent manner was examined (Fig. 8B). We found that mutation of the Trp or any of the Arg residues resulted in a loss of the ability to inhibit aggregation, implying that these 4 residues play an important role in ninjurin-ninjurin interactions. Mutations made to the non-charged residues including Gly³⁰ and Leu³¹ did not alter the ability to inhibit aggregation, indicating that these residues are not critical to the interaction. One of the mutations (Asn³³ to Leu) resulted in a peptide with greater inhibitory activity than wild

type, suggesting that it interacts with ninjurin very strongly.

Ninjurin Mutants Fail to Support Aggregation—Aggregation inhibition experiments using synthetic peptides revealed that residues 26–37 (the ninjurin adhesion motif) are responsible for ninjurin-mediated homophilic cellular adhesion. To confirm the importance of this motif, two Jurkat cell lines expressing ninjurin mutants were generated. One was designed to express ninjurin(W29A), in which Trp²⁹ was mutated to Ala; and the other expressed ninjurin(R32,34N), in which Arg³² and Arg³⁴ were both mutated to Asn. Protein blot and immunohistochemical analyses of these cell lines confirmed that the expression levels for wild type and mutant ninjurin molecules were comparable and that the ninjurin mutants were expressed on the cell surface (Fig. 9). Aggregation assays with these ninjurin transfected cell lines revealed that Jurkat cells stably expressing ninjurin(W29A) showed no increase in aggregation over native Jurkat cells, whereas cells expressing ninjurin(R32,34N) showed greater aggregation than native Jurkat cells but significantly less than Jurkat cells expressing wild-type ninjurin (Fig. 10), a result consistent with that of the peptide competition assay. To determine whether the mutated ninjurin molecules could interact with wild-type ninjurin, we performed aggregation assays on mixtures of wild type and mutant nin-

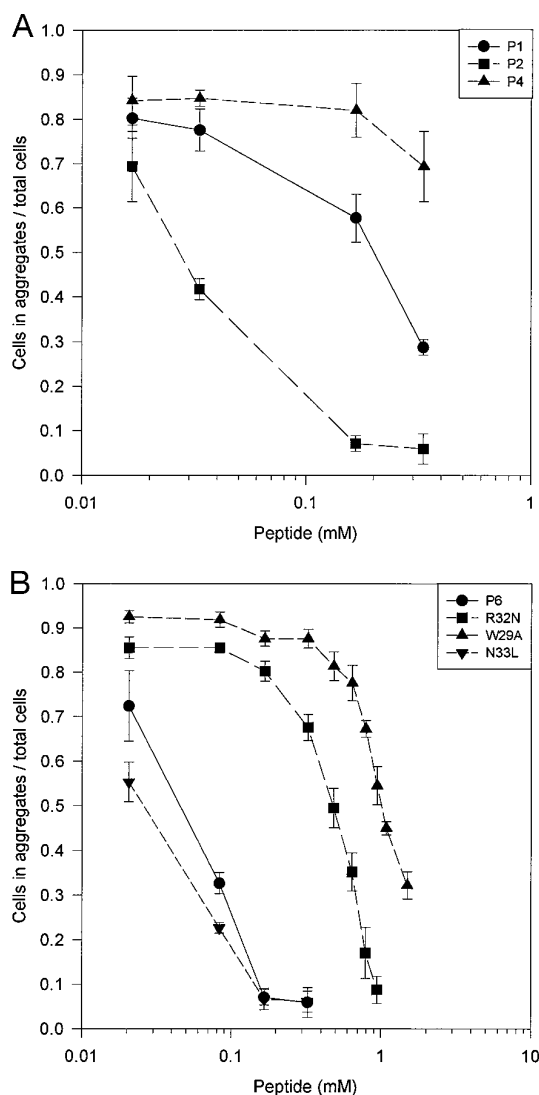


FIG. 8. Identification of residues responsible for ninjurin-mediated adhesion. A, aggregation assays using ninjurin-expressing Jurkat cells were performed in the presence of each of the indicated peptides. The number of cells incorporated into aggregates was determined after 1 h, and the ratio of cells in aggregates to total cells was calculated and plotted *versus* peptide concentration. Data represent the mean \pm S.D. of four independent experiments. B, aggregation assays were performed as above with each of the mutated peptides (M1-M7) listed in Fig. 7. Each peptide was tested at concentrations ranging from 0.02 to 1.6 mM, and the aggregation ratio was plotted as above. Results using G30A and L31N were not statistically different from those obtained using the native peptide (P6). Results using R32N were identical to those obtained with R34N and R28N. All the data represent the mean \pm S.D. of four independent assays.

jurin molecules, which were differentially stained by fluorescent dyes. When equal numbers of cells expressing wild-type ninjurin (colored *red*) and ninjurin(W29A) (colored *green*) were mixed together, aggregates contained predominantly wild-type ninjurin-expressing cells. Aggregates containing both types of cells were not observed, indicating that the mutant molecules are incapable of interacting with wild-type ninjurin. A similar experiment conducted with cells expressing wild-type ninjurin (colored *red*) and ninjurin(R32,34N) (colored *green*) resulted in an increased number of aggregates. Some of the aggregates showed a mosaic pattern, indicating that this ninjurin mutant could interact with wild-type ninjurin. Taken together, these results are consistent with those obtained using the peptide competition assays and indicate that residues 26 to 37 are critical for ninjurin-mediated adhesion.

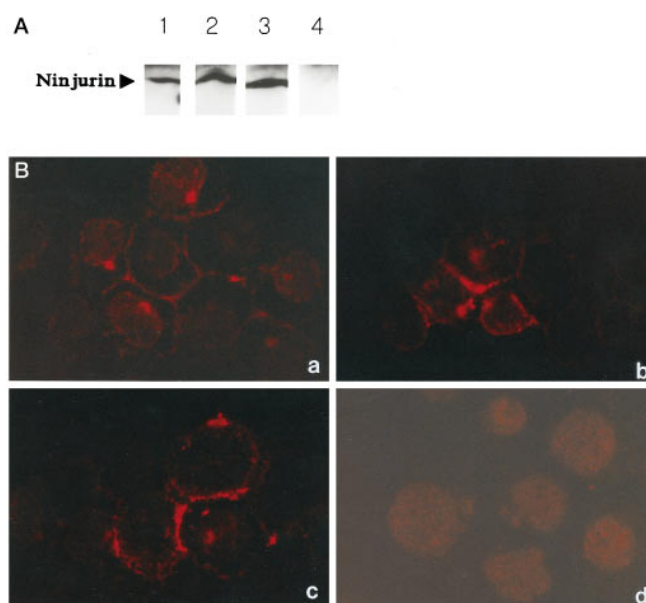


FIG. 9. Wild-type and mutant ninjurin molecules are expressed on the cell surface at comparable levels. A, immunoblot analysis of native and mutant ninjurin protein expression in stably transfected Jurkat cells. Lysates of Jurkat cells expressing wild-type ninjurin (lane 1), ninjurin(W29A) (lane 2), ninjurin(R32,34N) (lane 3), and native Jurkat cells (lane 4) were electrophoresed on a 12% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and incubated with affinity purified anti-ninjurin antibodies. Ninjurin (denoted by arrow) was visualized by enhanced chemiluminescence. B, immunohistochemical analysis of wild type and mutant ninjurin protein expression in stably transfected Jurkat cells. Affinity-purified anti-ninjurin antiserum was used to detect ninjurin in Jurkat cells expressing wild-type ninjurin (a); ninjurin(W29A) (b); ninjurin(R32,34N) (c); and wild type Jurkat cells (d). Cell preparations were made using Cytospin.

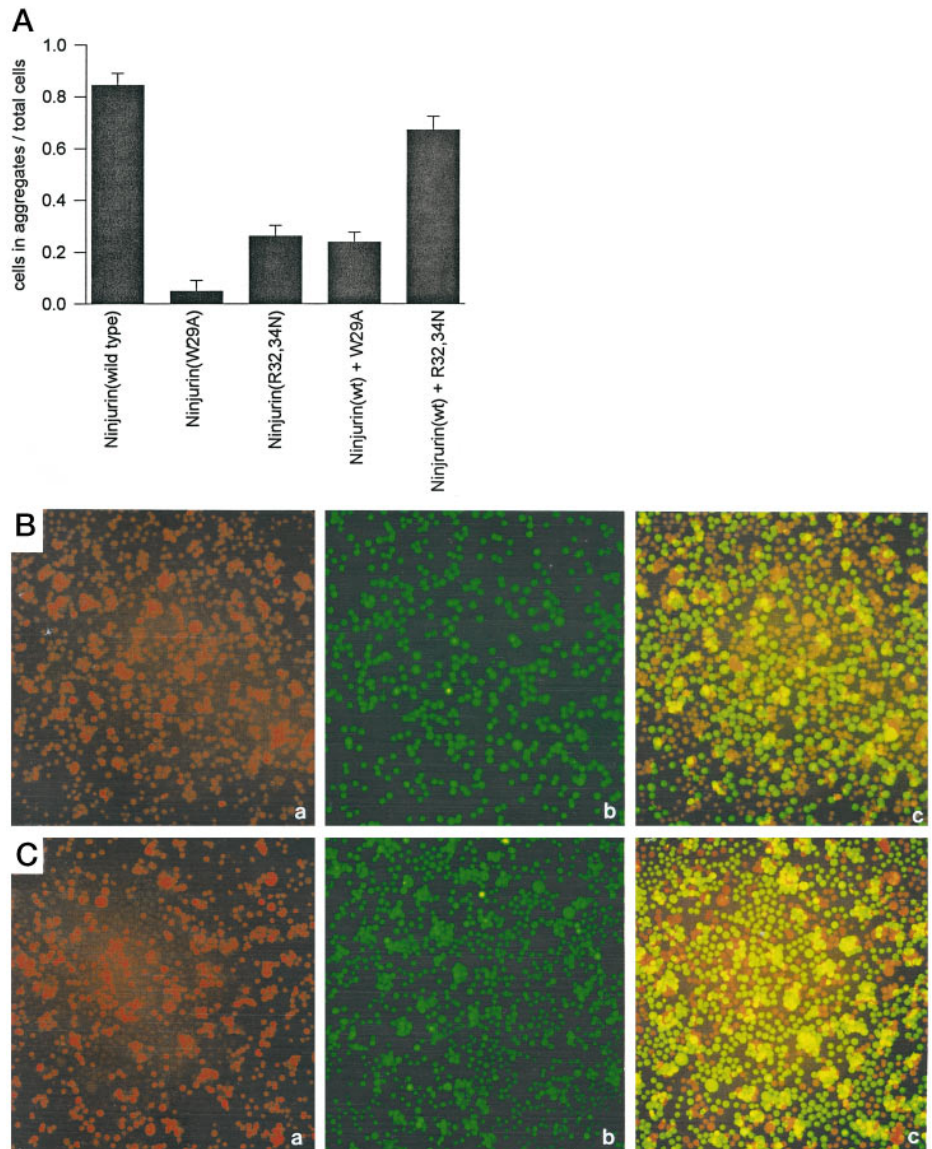
Ninjurin-stimulated Neurite Outgrowth Is Mediated through the Adhesion Motif—Using ninjurin-expressing CHO cells, we have previously shown that ninjurin promotes the extension of neurites from primary cultured DRG neurons. To determine whether this phenomenon occurs via adhesive properties of ninjurin, we tested the ability of peptides that blocked ninjurin aggregation to inhibit neurite outgrowth. DRG neurons from E17 rat embryos were dissociated and seeded onto confluent monolayers of either native or ninjurin-expressing CHO cells. The neuronal cultures were treated with either P6 (wild type) or M2 (W29A) peptide at a concentration of 0.16 mM, and neurite extension was monitored (Fig. 11). As previously documented, neurites from neurons grown on CHO cells expressing ninjurin showed increased neurite outgrowth ($569 \pm 134 \mu\text{m}$) compared with those grown on native CHO cells ($297 \pm 104 \mu\text{m}$) (15). However, the promotion of neurite outgrowth by ninjurin was inhibited ($317 \pm 83 \mu\text{m}$) when the cultures were treated with the peptide of native sequence (P6), whereas the peptide (M2) containing the W29A mutation had no significant inhibitory effect on neurite extension by DRG neurons ($638 \pm 209 \mu\text{m}$) (Fig. 11). Neither of the peptides affected the neurite extension when neurons were cultured on native CHO cells (data not shown). These results indicate that the residues identified as important for adhesion using the Jurkat cell aggregation assay are also required for ninjurin-stimulated neurite outgrowth, suggesting that the adhesive properties of ninjurin play an important functional role in this process.

DISCUSSION

Ninjurin was first identified as a molecule that is up-regulated in Schwann cells and neurons after peripheral nerve injury. Subsequent analysis of ninjurin function revealed that

FIG. 10. Aggregation assays using Jurkat cells expressing either wild type or mutant ninjurin molecules.

A) Jurkat cells stably transfected with wild-type ninjurin, ninjurin(W29A), and ninjurin(R32,R34N), either alone or mixed in a 1:1 ratio with cells expressing wild-type ninjurin, were used to perform aggregation assays. The ratio of cells in aggregates to total cells was determined after 1 h. The data represent the mean \pm S.D. of four independent experiments. **B,** aggregation assays were performed with 1:1 mixtures of cells expressing wild-type ninjurin (stained with red fluorescent dye) and those expressing ninjurin-(W29A) (stained with green fluorescent dye). The same field was exposed three times to show the distribution of wild-type ninjurin-transfected cells only (*a*), ninjurin(W29A) transfected cells only (*b*), and both kinds of cells (double exposure in *c*). Note that the green cells do not aggregate to themselves or form clusters with cells expressing wild-type ninjurin. **C,** aggregation assays were performed with a 1:1 mixture of cells expressing wild-type ninjurin (stained with red fluorescent dye) and those expressing ninjurin-(R32,34N) (stained with green fluorescent dye). The same field was exposed three times as in panel *B*. Note that green cells are present in clusters with red cells, resulting in a yellow color in the double exposure.



it is a cell surface molecule that promotes cell aggregation and stimulates neurite outgrowth, suggesting that it may play an important role in nerve regeneration (15). The mechanism by which ninjurin promotes aggregation has been unclear, since it does not contain sequence motifs found in other adhesion molecules. Mutation analysis has enabled us to identify a region in ninjurin that functions as a homophilic adhesion motif that bears no resemblance to previously identified adhesion motifs. Using aggregation inhibition assays and synthetic peptides containing mutated residues within this region, we found that a Trp and three Arg residues within this 10 amino acid region are important for ninjurin-mediated recognition. To verify the critical role of this adhesion motif in ninjurin-mediated functions, peptides that inhibited ninjurin-mediated cell aggregation were used to show that ninjurin-stimulated neurite outgrowth is dependent on its adhesive properties.

The ninjurin adhesion motif (residues 26–37) contains a tryptophan and a cluster of arginine residues. None of the previously reported homophilic adhesion molecules contain this peptide motif or a combination of tryptophan and arginines as functionally relevant residues (28). Competition experiments with peptides derived from this region demonstrated that replacement of tryptophan by alanine dramatically decreased the ability to inhibit aggregation, suggesting that this

tryptophan residue plays a major role in ninjurin-ninjurin interactions. The importance of the tryptophan residue was also confirmed by mixed aggregation assays using Jurkat cells expressing ninjurin(W29A), which demonstrated that these cells do not adhere to themselves or to cells expressing wild-type ninjurin. This tryptophan residue may be directly involved in the physical interaction between ninjurin molecules, or it may be necessary for the overall structure of the domain. Replacement of the arginines showed significant, but less dramatic effects on ninjurin adhesion. Interestingly, even though ninjurin binding is dependent on divalent cations, the ninjurin adhesion motif does not contain acidic residues commonly associated with cation binding motifs. Overall, ninjurin contains 13 Glu and Asp residues, with 6 of these residues located within the amino-terminal 23 residues. Perhaps this acidic region is related to the cation dependence of ninjurin and plays a role in the formation of the functional ninjurin adhesion domain.

The demonstration that the inhibitory peptide we identified via cell aggregation assays was able to reverse the ninjurin-stimulated neurite outgrowth from neurons indicates that the biological function of ninjurin is related to its adhesive properties. We also observed that the basal adhesion observed for wild-type Jurkat cells was abolished in the presence of peptides containing the adhesion motif of ninjurin (data not shown).

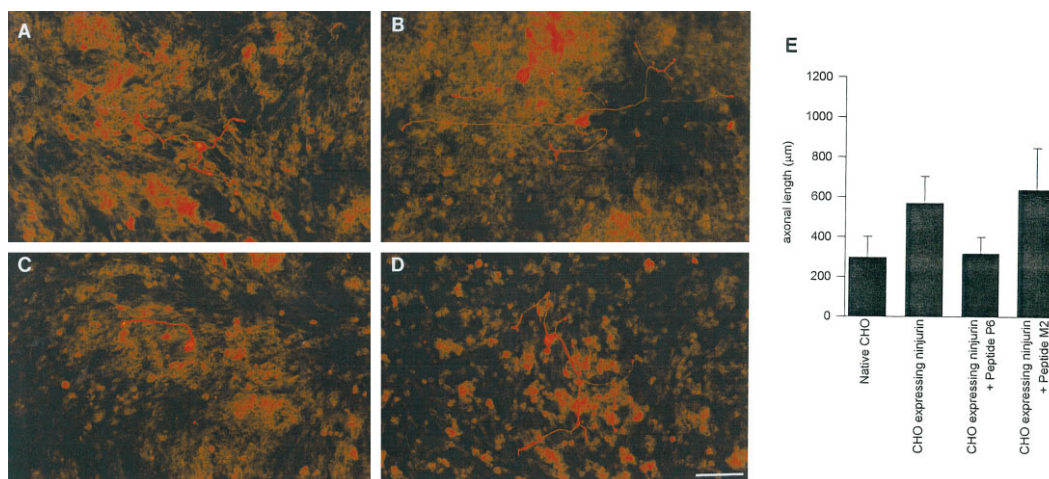


FIG. 11. Axonal growth promoted by ninjurin is reversed by a peptide that inhibits aggregation. DRG neurons from E17 rat embryos were seeded onto monolayers of native CHO cells (A) or CHO cells expressing ninjurin (B-D). Cultures were maintained with peptide P6, in panel C, or M2, which contains the W29A mutation (D). Six h later, the cells were fixed and neurites were visualized by immunohistochemistry with anti-neurofilament H antibodies. Scale bar = 200 μ m. Neurite length shown in panel E was quantitated by measuring neurites from approximately 50 neurons grown under each condition. The data represent the mean \pm S.D. of three independent experiments.

This suggests that other molecule(s) expressed by Jurkat cells possibly share this motif. If other molecules with the ninjurin-like adhesion motif exist, then the possibility that ninjurin may participate in heterophilic as well as homophilic interactions must be considered. Clearly, the ability of ninjurin to participate in heterophilic binding would greatly extend the number of interactions, and potentially the number of functions, in which ninjurin is involved.

In addition to its expression by Schwann cells and neurons after nerve injury (15), we found that ninjurin is present in a wide variety of tissues (e.g. thymus, kidney, liver, adrenal gland), both during development and in adulthood. Ninjurin is predominantly expressed in epithelial cells, suggesting that it is important in the normal development and/or function of a number of tissues, in addition to its role in promoting nerve regeneration. For instance, its presence in the thymus may indicate a role in thymocyte development as adhesion molecules on thymic epithelial cells are thought to be important in this process (29). Ninjurin may also be important in the formation of cellular polarity as it is located in a restricted region in Muller cells, the highly polarized cells of the retina. Within this region, ninjurin may be present in a specific intercellular adhesion apparatus, or it may be interacting with specific cytoskeletal components.

The mapping of the ninjurin gene to human chromosome 9q22 is intriguing as several diseases of unknown pathogenesis have been linked to this region and because mutations in another neural cell adhesion molecule, L1, have been associated with a number of severe genetic disorders, including X-linked hydrocephalus, MASA syndrome (Mental retardation, Aphasia, Shuffling gait, and Adducted thumbs), and spastic paraplegia type 1 (30, 31). The role of ninjurin in promoting nerve regeneration and its expression in sensory neurons of the DRG makes it a reasonable candidate gene for hereditary sensory neuropathy type 1, a disease characterized by progressive degeneration of DRG and motor neurons followed by sensory loss, muscle weakness, and neural deafness (23). The linkage to familial dilated cardiomyopathy is also interesting as there is evidence suggesting that an autoimmune mechanism directed against cell surface adhesion molecules could be involved in this disease (26, 32). The expression pattern of ninjurin, its identification as a new adhesion protein, and its potential linkage to a number of hereditary disorders suggest that it plays an important role in the proper development and function of a variety of tissues.

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