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Characterization of a 21 amino acid peptide sequence of the laminin G2 domain that is involved in HNK-1 carbohydrate binding and cell adhesion

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The N-linked HNK-1 carbohydrate expressed by several recognition molecules mediates the adhesion of early postnatal cerebellar neurons to the G2 domain of the terminal globular domain of the laminin α 1 chain (H.Hall *et al.*, submitted). To define this binding site more precisely, G2-derived synthetic peptides were used for binding and competition studies. Peptide 5-G2, comprising the amino acid residues 3431–3451 of G2, inhibited the interaction between the HNK-1-carrying glycolipid and laminin in a concentration-dependent and saturable manner. Peptides which overlap only partially with this sequence interfered less. Peptides comprising other amino acid sequences from G2, and peptides derived from G1 and G3 or a scrambled version of peptide 5-G2, did not show significant effects. Direct binding of peptide 5-G2 to the HNK-1 glycolipid was also demonstrated. Furthermore, peptide 5-G2 interfered in a concentration-dependent and saturable manner with the adhesion of early postnatal cerebellar neurons to laminin. These observations indicate that amino acid residues 3431–3451 of the laminin G2 domain are involved in HNK-1 carbohydrate-mediated cell adhesion.

Key words: cell adhesion/HNK-1 carbohydrate/laminin/nervous system

Introduction

The extracellular matrix of the nervous system plays important roles during development and regeneration (for reviews, see Sanes, 1989; Reichardt and Tomaselli, 1991; Venstrom and Reichardt, 1993). Extracellular matrix molecules have been implicated in cell attachment, spreading and migration, and growth cone guidance. One of the components of the extracellular matrix in the developing and adult central and peripheral nervous system is laminin. Laminin is a self-aggregating, multifunctional glycoprotein consisting of the three polypeptide chains α 1, β 1, γ 1 (Timpl *et al.*, 1979; Martin and Timpl, 1987; Beck *et al.*, 1990, 1991; Yurchenco *et al.*, 1990, 1992; Yurchenco and Cheng, 1993; Burgeson *et al.*, 1994). The β 1 and γ 1 chains are linked by a disulphide bond and associated non-covalently with the α 1 chain forming a coiled-coil structure. The C-terminus of the α 1 chain forms a globular domain consisting of five G domains each comprising ~180 amino

acids. These domains share 25–30% internal homology (Sasaki *et al.*, 1988) and represent independent folding units (Beck *et al.*, 1990, 1991) capable of exerting different functions such as binding of various cell surface receptors (for review, see Edgar, 1989; Mecham, 1991; Hynes and Lander, 1992), promotion of neurite outgrowth (Edgar *et al.*, 1984; Sephel *et al.*, 1989; Lander, 1990; Calof *et al.*, 1994) and recognition of sulphated carbohydrates (Ott *et al.*, 1982; Roberts *et al.*, 1985, 1988; Skubitz *et al.*, 1988, 1991; Kouzi-Koliakos *et al.*, 1989; Tarabozetti *et al.*, 1990). One of these carbohydrates which has been shown to be involved in neural cell adhesion to laminin is the HNK-1 carbohydrate (Mohan *et al.*, 1990; Hall *et al.*, 1993).

The HNK-1 carbohydrate expressed by several neural recognition molecules (Kruse *et al.*, 1984; Schachner, 1989) and two structurally related nervous system-derived glycolipids have been defined by their interaction with the monoclonal antibody HNK-1 directed against a subset of human natural killer cells (Abo and Balch, 1981). The structure was determined as 3' sulphoglucuronylneolactotetraosyl- or hexaosyl-ceramide (Chou *et al.*, 1986, 1991; Ariga *et al.*, 1987). *In vivo* experiments with the monoclonal HNK-1 antibodies have demonstrated that it is involved in the migration of neural crest cells (Bronner-Fraser, 1987). In *in vitro* assays, the HNK-1 carbohydrate has been implicated in migration of neural crest cells and neurons, neurite outgrowth (Künemund *et al.*, 1988; Lallier and Bronner-Fraser, 1991; Lallier *et al.*, 1992) and in short-term cell-to-cell and cell-to-laminin adhesion (Keilhauer *et al.*, 1985; Künemund *et al.*, 1988; Hall *et al.*, 1993). Cell-to-laminin adhesion is mediated by direct binding of the cell surface-expressed HNK-1 carbohydrate to the G2 domain of the terminal globular domain of the laminin α 1 chain (Mohan *et al.*, 1990; H.Hall *et al.*, submitted).

Since it was found that the binding of HNK-1 carbohydrate to laminin was not affected when laminin was treated with urea or reduced and alkylated (H.Hall *et al.*, submitted), we searched for a peptide sequence responsible for binding to the HNK-1 carbohydrate. For this purpose we used a variety of G2 domain-derived synthetic peptides and tested their ability to inhibit the binding. One peptide could be determined which interfered with the binding, bound directly to the HNK-1 carbohydrate and inhibited HNK-1-mediated neural cell adhesion to laminin.

Results

Previous studies defined the HNK-1 carbohydrate binding site on the G2 domain of the terminal globular domain of the laminin α 1 chain (see schematic drawing in Figure 1). Since this binding could not be affected by reduction and alkylation of disulphide bonds or urea denaturation of the laminin E8 fragment (H.Hall *et al.*, submitted), the native conformation of G2

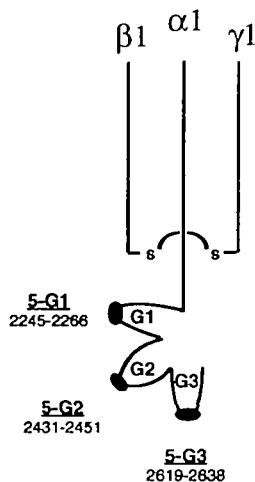


Fig. 1. Schematic structure of the distal portion of the long arm of EHS tumour laminin ($\alpha 1\beta 1\gamma 1$) and the adjacent G domains which are comprised in the proteolytic fragment E8 (Ott *et al.*, 1982). This structure consists of a coiled-coil rod contributed by the C-terminal portions of the $\alpha 1$, $\beta 1$ and $\gamma 1$ chains where the $\beta 1$ and the $\gamma 1$ chains are connected by a disulphide bond (ss) and non-covalently associated to the A chain. The most C-terminal portion of the $\alpha 1$ chain is composed of five similar G domains with only G1, G2 and G3 belonging to the E8 fragment. Positions of peptides 5-G1, 5-G2 and 5-G3 are indicated.

might not be important for binding. We therefore started to search for a peptide which is able to interfere with the binding of the HNK-1 carbohydrate to laminin and which also binds directly to the HNK-1 carbohydrate. Ten different synthetic peptides derived from the G2 domain were studied with respect to the binding of laminin to substrate-immobilized HNK-1-carrying glycolipids (hereafter called HNK-1 glycolipid). The sequences and positions of the peptides within the G domains are indicated in Figure 1 and Table I. One out of these peptides (peptide 5-G2), comprising the amino acid residues 2431–2451 of the laminin $\alpha 1$ chain, showed a pronounced inhibition of binding of $62 \pm 2\%$. Peptide 5-G2 inhibited the binding in a concentration-dependent manner, reaching saturation at $10 \mu\text{M}$

and a maximal inhibition of 65% (Figure 2A). Peptide 4-G2, which overlaps with the six N-terminal amino acids of peptide 5-G2, showed an inhibition of $41 \pm 8\%$ and peptide 6-G2, comprising the 12 C-terminal amino acids of peptide 5-G2, showed a $43 \pm 6\%$ inhibition. Peptide 7-G2 corresponds to the C-terminal heptapeptide of peptide 5-G2 and showed an inhibition of $23 \pm 14\%$. Peptides 1-G2, 2-G2, 3-G2, 8-G2, 9-G2 and 10-G2 did not show a significant inhibition (Table I). Peptides derived from G1 (5-G1) and G3 (5-G3), comprising sequences at positions corresponding to those of peptide 5-G2, did not inhibit the binding of laminin to the HNK-1 glycolipid at a concentration of $166 \mu\text{M}$ ($-21 \pm 13\%$ and $-7 \pm 12\%$, respectively) (Table I and Figure 2B). The scrambled version of peptide 5-G2 (5-G2,sc) did not show any inhibition of the binding ($4 \pm 15\%$). These results indicate that a G2 domain-derived peptide is involved in the binding of laminin to the HNK-1 glycolipid.

To demonstrate that peptide 5-G2 interacts directly with the HNK-1 carbohydrate, binding studies with biotinylated peptides 5-G1, 5-G2 and 5-G3 were performed on substrate-immobilized HNK-1 glycolipid, sulphatides or cerebrosides. Peptide 5-G2 showed concentration-dependent binding to the HNK-1 glycolipid, reaching a saturating concentration at $0.5 \mu\text{M}$ (Figure 3A). Peptides 5-G1 and 5-G3 did not show any binding to the HNK-1 glycolipid (Figure 3A). None of the peptides bound to sulphatides (Figure 3B) or cerebrosides (Figure 3C). These results indicate that peptide 5-G2 binds directly and specifically to the HNK-1 glycolipid. The experiments also exclude the possibility that biotinylation of the peptides alters their functional properties, since the binding specificity corresponded to that obtained from the studies on laminin binding to the HNK-1 glycolipid using non-biotinylated peptides.

Cell adhesion experiments with small cerebellar neurons, 85% of which express the HNK-1 carbohydrate (Wernecke *et al.*, 1985), were performed to investigate whether peptide 5-G2 also interferes with the adhesion of neural cells to laminin. In the presence of peptide 5-G2, cell adhesion was inhibited by $63 \pm 3\%$ at a concentration of $83 \mu\text{M}$ (Figure 4). Control peptides did not ($-3 \pm 8\%$ for 5-G1) or only slightly ($14 \pm 4\%$ for

Table I. List of G2 domain-derived synthetic peptides used to interfere with the binding of HNK-1 glycolipid to laminin

Name	Sequence ^a	Inhibition ^b
1-G2	2370- TWYKIAFQRNRK	-2381 -26 ± 15
2-G2	2394- SDKETKQGETPG	-2405 9 ± 7
3-G2	2419- YVGGGLPHSKAVR	-2430 -8 ± 6
4-G2	2426- SKAVRKGVSSRS	-2437 41 ± 8**
5-G2	2431- KGVSSRSYVGCICKNLEISRST	-2451 62 ± 2***
6-G2	2440- GCIKNLEISRST	-2451 43 ± 6**
7-G2	2445- LEISRST	-2451 23 ± 14*
8-G2	2445- LEISRSTFDLLR	-2456 5 ± 8
9-G2	2451- TFDLLRNSYGVVRK	-2463 -3 ± 6
10-G2	2458- SYGVVRKGCALPEP	-2469 2 ± 9
5-G1	2245- PAVKVTHFKGCMGEAFLNG	-2263 -21 ± 13
5-G2,sc	KVLSCKSTGSRNIRVESGYS	4 ± 15
5-G3	2619- MLKMRTSFHGCIKNVVLDAQ	-2638 -7 ± 12

^aThe sequences of the peptides from 1-G2 to 10-G2 are derived from the G2 domain of the laminin $\alpha 1$ chain. Peptide 5-G1 is derived from G1 and peptide 5-G3 from G3 (see also Figure 1). Peptide 5-G2,sc contains a scrambled version of peptide 5-G2. The numbers in the front and at the end of each peptide indicate the positions of the amino acids within the G1, G2 and G3 domain, respectively, according to the alignment of Sasaki *et al.* (1988). Bold letters represent sequences which are contained in overlapping peptides.

^bValues represent inhibition of binding in % and are normalized setting the control value as 0% inhibition. Values \pm SD represent data from at least three independent experiments carried out in duplicates. Asterisks represent values which are significantly different from the control (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

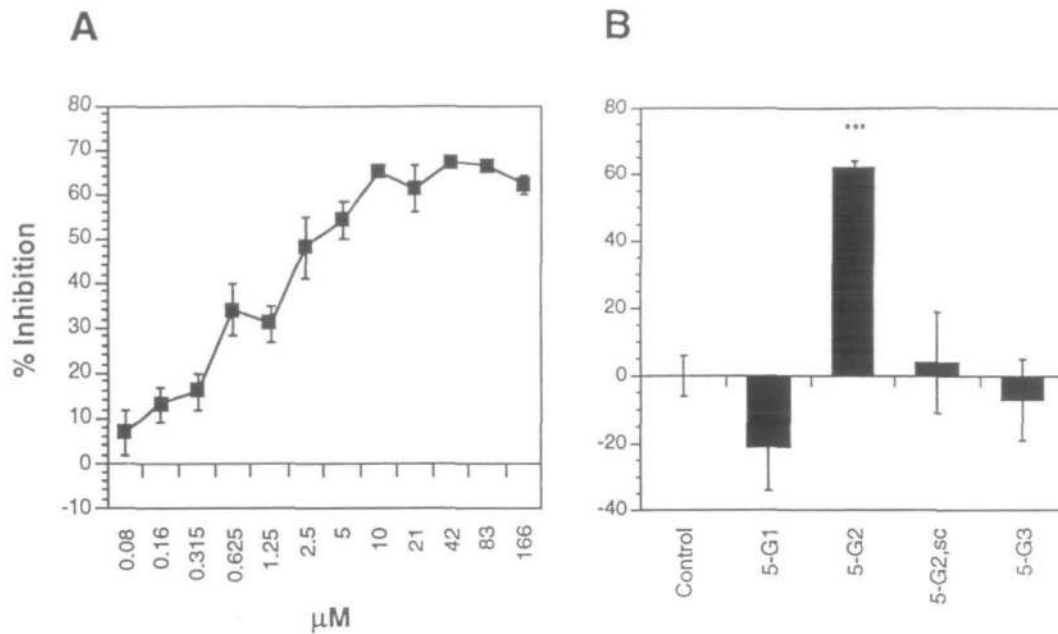


Fig. 2. Binding of laminin to substrate-coated HNK-1 glycolipid in the absence and presence of laminin-derived synthetic peptides. Bound laminin was detected by ELISA with pab LN, followed by goat anti-rabbit HRP-conjugated secondary antibodies. Per cent inhibition was calculated relative to the binding in the absence of additives (= 0% inhibition, control). (A) Per cent inhibition is shown as a function of increasing concentrations of peptide 5-G2: Mean values \pm SDs are from five independent experiments carried out in triplicates. (B) Per cent inhibition in the presence of 166 μ M of each of the peptides 5-G1, 5-G2, the scrambled version of 5-G2 (5-G2,sc) and 5-G3 is shown. Mean values \pm SDs are from at least five independent experiments carried out in triplicates. Asterisks represent values which differ significantly from the control (*** $P \leq 0.001$).

5-G3) interfered with the binding of neurons to laminin (Figure 4). The scrambled version of 5-G2 (5-G2,sc) did not show any inhibition of cell adhesion ($-14 \pm 6\%$) (Figure 4). For control, the same experiments were performed with L-cells, with none of the peptides showing significant inhibitory effects (not shown).

To demonstrate that peptide 5-G2 specifically interferes with the HNK-1 carbohydrate-mediated adhesion of small cerebellar neurons to laminin, adhesion assays were performed in the presence of 5-G2, HNK-1 glycolipid and Fab fragments of the monoclonal antibody (mab) 412 (Figure 5). Inhibition of cell adhesion to substrate-bound laminin in the presence of different concentrations of peptide 5-G2 (83, 33, 16, 1.6 and 0.3 μ M) was dose dependent, with an inhibition of $63 \pm 5\%$ at saturating concentrations of 33 μ M peptide (Figure 5A). Control peptides 5-G1 and 5-G3 were used at a concentration of 83 μ M without significant inhibition (Figure 4). The adhesion of cells to laminin was also inhibited in a concentration-dependent manner in the presence of HNK-1 glycolipid at 20, 5, 2.5, 1.25 and 0.6 μ M (Figure 5B). Saturation of adhesion was achieved at 5 μ M, resulting in $56 \pm 10\%$ inhibition of cell adhesion. Sulphatides and cerebroside used as control glycolipids did not show significant inhibition ($-8 \pm 9\%$ and $-3 \pm 10\%$, respectively) at a concentration of 50 μ M, indicating that the glycolipid backbone is not involved in binding to laminin. Fab fragments of mab 412 were used in the same assay at concentrations of 10, 2, 1, 0.1, 0.06 and 0.02 μ M with a concentration-dependent and saturable inhibition of cell adhesion (Figure 5C). Fab fragments of mab 412 inhibited by maximally $81 \pm 2\%$ at a concentration of 5 μ M. Fab fragments of a polyclonal antibody (pab) NCAM, even at a concentration of 10 μ M, did not show inhibition of cell adhesion ($-7 \pm 6\%$) (not shown). Adhesion of L-cells to laminin in identical assays was not altered by peptide 5-G2, HNK-1 glycolipid micelles

or Fab fragments of mab 412 (not shown). The combined observations show that residues 2431–3451 of the G2 domain of the laminin α 1 chain are involved in the HNK-1 carbohydrate-mediated cell adhesion to laminin.

Discussion

In this study, we define a sequence of 21 amino acids within the α 1 chain of laminin which is involved in the binding of laminin to the HNK-1 carbohydrate. Out of 10 different peptides derived from the G2 domain, which were distributed over the central 100 out of 180 amino acids of the complete G2 domain, only the peptide comprising amino acid residues 3431–3451 was shown to be active in direct binding of the HNK-1 carbohydrate and in interfering with the binding of the HNK-1 carbohydrate to laminin. This peptide has a positive net charge of three and could therefore interact with the negatively charged HNK-1 carbohydrate. This is noteworthy in the context that according to surface probability predictions (Chou and Fasman, 1978; Emini *et al.*, 1985) this sequence of the G2 domain is exposed at the surface of the G2 domain and thus accessible to putative ligands. Similarly, the sequences of the equivalent peptides derived from G1 and G3 are likely to be exposed at the surface of the G domain.

That this peptide is also involved in the adhesion of HNK-1 carbohydrate-carrying neurons was shown by inhibition experiments. For all inhibitors, a concentration dependence and saturation could be demonstrated. However, there are differences in the molar concentrations and the maximal inhibition achieved. HNK-1 glycolipid micelles and the Fab fragments of mab 412 reached saturation of inhibition at ~ 5 μ M. For the inhibitory peptide, a six times higher concentration (~ 33 μ M) was required to achieve saturation of inhibition. A possible explanation could be that the peptide has a lower affinity to the carbohydrate

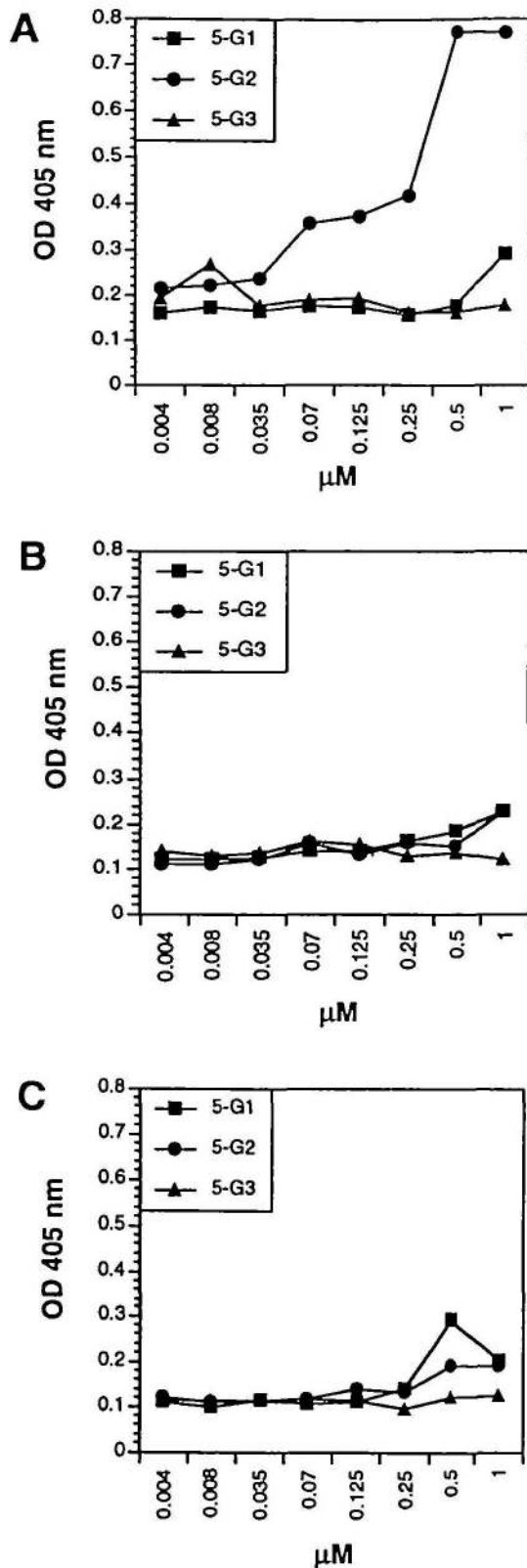


Fig. 3. Concentration dependence of the binding of laminin-derived peptides to different glycolipids. HNK-1 glycolipid (A), sulphatides (B) and cerebrosides (C) were substrate immobilized at a concentration of 1 µM. Bound biotinylated peptides 5-G1, 5-G2 and 5-G3 were detected by ELISA with HRP-conjugated streptavidin. One representative out of three independent experiments is shown. SDs were <5%.

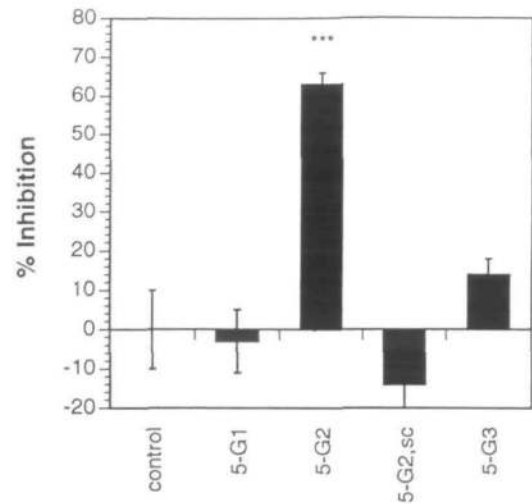


Fig. 4. Inhibition of neural cell adhesion in the absence and presence of different laminin-derived peptides. After pre-incubation of small cerebellar neurons in the absence (control) or in the presence of peptides 5-G1, 5-G2, the scrambled version of 5-G2 (5-G2.sc) and 5-G3 bound cells were recorded. Per cent inhibition was shown in relation to the control value which was determined in the absence of any peptides (= 0% inhibition). Mean values ± SDs are from at least three independent experiments carried out in quadruplicates. Asterisks represent values which differ significantly from the control (***) $P \leq 0.001$.

ligand than the Fab fragments of mab 412. A reduced affinity would thus have to be compensated for by an increased peptide concentration. The slight inhibition of cell adhesion observed in the presence of peptide 5-G3 could be due to the similarity between the peptides 5-G2 and 5-G3, since both share the five central amino acids GCIKN (residues 2440–2445 in peptide 5-G2 and 2628–2633 in peptide 5-G3, respectively).

The question is which molecules on cerebellar neurons may be the carriers of the HNK-1 carbohydrate. Cerebellar neurons express this carbohydrate as a glycolipid from early embryonal stages until adulthood (Schwartz *et al.*, 1987; Prasadarao *et al.*, 1990; Chou *et al.*, 1991). The HNK-1 carbohydrate could also be presented by several neural recognition molecules which are expressed by cerebellar neurons, such as L1 (Kruse *et al.*, 1984, 1985), NCAM (Gordis *et al.*, 1983; Edelman, 1984), TAG-1 (Dodd *et al.*, 1988), integrins (Pesheva *et al.*, 1987) and possibly other molecules. Since it is known for mouse brain L1, the myelin-associated molecule MAG, NCAM and P0 that only subpopulations carry the HNK-1 carbohydrate (Kruse *et al.*, 1985; Faissner, 1987; Poltorak *et al.*, 1987), the expression of the HNK-1 carbohydrate is regulated independently of the protein backbone. Thus, cell interactions may be subject to independent sets of regulatory mechanisms.

With regard to the functional roles of integrins, it is noteworthy that the sequence of 5-G2 overlaps with the nine C-terminal amino acids of another peptide (GD-3, comprising the residues 2443–2463) described as a cell binding and spreading site for human fibrosarcoma cells (Skubitz *et al.*, 1991). Antibodies against the β1 integrin subunit inhibited cell adhesion to this peptide (Skubitz *et al.*, 1991), thus attributing to the GD-3 and possibly also for the 5-G2 peptide sequences an important functional role in integrin–laminin-mediated cell–substrate interactions.

Another candidate for the HNK-1-mediated cell interaction with laminin is Ng-CAM, an L1-like molecule in the chicken,

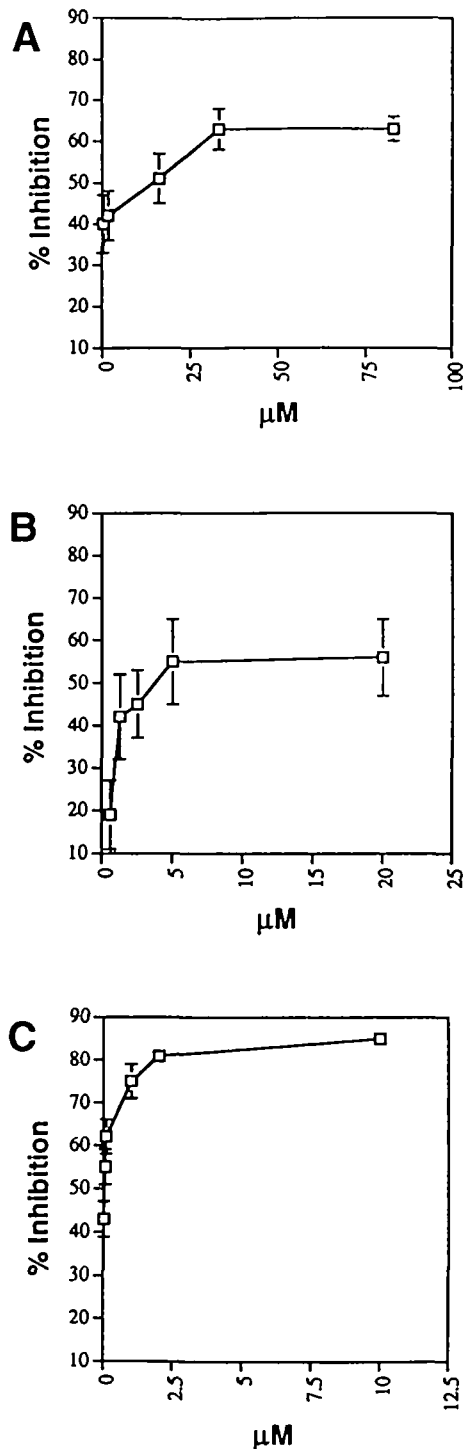


Fig. 5. Concentration dependence of the binding of neural cell adhesion to laminin in the presence of peptide 5-G2 (A), HNK-1 glycolipid (B) and Fab fragments of mab 412 (C). Small cerebellar neurons were pre-incubated either in the presence of peptide 5-G2, HNK-1 glycolipid or Fab fragments of mab 412 at different concentrations before adding this mixture to the substrate. Inhibition of cell adhesion was calculated relative to the binding in the absence of additives (= 0% inhibition). Mean values \pm SDs are from at least three independent experiments carried out in quadruplicates.

which has been reported to bind to laminin (Grumet *et al.*, 1993). Interestingly, the laminin fragment involved in Ng-CAM binding is not fragment E8 comprising the G2 domain, but the P1 fragment which consists of the cross-like region and the truncated short arms of laminin. Thus, it is possible that

HNK-1-dependent interactions with laminin are coupled with HNK-1-independent mechanisms through one ligand, although the binding sites might be localized on distant parts of the laminin molecule. It will now be interesting to determine which adhesion cell surface ligands of cerebellar neurons are involved in binding to laminin and, if there are several, how they contribute separately and possibly even cooperatively to laminin-mediated cell-to-substrate interactions.

Materials and methods

Glycolipids and glycoproteins

The SGGL glycolipids (3'-sulphoglucuronyleolactotetraosyl- and hexaosyl-ceramide), designated as HNK-1 glycolipid, were purified as described by Hall *et al.* (1993) with the exception that the HNK-1 glycolipid was purified over a column of silica gel-60 (Merck no. 15101) instead of a DEAE-Sephadex A-25 ion-exchange column. The HNK-1 glycolipid was eluted with chloroform:ethanol:water (60:35:8 v/v). The purity of the glycolipid was tested by TLC in a chloroform:methanol:water (60:35:8 v/v) system. Sulphatides from bovine brain were purchased from Sigma, cerebroside were obtained from Sigma or Boehringer Mannheim and EHS-tumour laminin from Gibco/BRL or Boehringer Mannheim.

Antibodies

A monoclonal antibody against the HNK-1 carbohydrate (mab 412), a polyclonal antibody against mouse EHS sarcoma laminin (pab LN) and an immunopurified polyclonal antibody against the neural cell adhesion molecule NCAM from mouse brain (pab NCAM) were obtained and purified according to Kruse *et al.* (1984), Pesheva *et al.* (1989), and Martini and Schachner (1986), respectively. Fab fragments of mab 412 and pab NCAM were prepared by proteolytic digestion with papain (Porter, 1959). Horseradish peroxidase (HRP)-conjugated secondary antibodies directed against rat immunoglobulin IgG and IgM or rabbit IgG were purchased from Dianova (Hamburg, FRG).

Synthetic peptides

Peptides derived from the G domains of the laminin $\alpha 1$ chain were obtained by solid-phase peptide synthesis on a Milligen 9050 continuous-flow peptide synthesizer following preparative HPLC purification as described by Knorr *et al.* (1989). The indicated position and the amino acid sequence (Table I) of the acetylated carboxamide peptides correspond to the alignment by Sasaki *et al.* (1988). The synthesis of the following peptides was performed: peptide 3-G2 Ac-YVGGGLPHSKAVR-NH₂, peptide 5-G2 Ac-KGVSSRSYVGCINKLEISRST-NH₂, peptide 6-G2 Ac-GCINKLEISRST-NH₂, peptide 8-G2 Ac-LEISRST-FDLLR-NH₂, peptide 10-G2 Ac-SYGVKGCALPEP-NH₂, peptide 5-G1 Ac-PAVKVTHFKGCMGEAFLNG-NH₂ and 5-G3 Ac-MLKMRTSFHG-CIKNVVDAQ-NH₂. After purification by preparative HPLC, the peptides were >95% pure as determined by analytical HPLC. The ion spray mass spectra (API III, Sciex) showed the expected ion series. Peptide 1-G2 TWYKIA-FQRNRK, peptide 2-G2 SDKETKQGETPG, peptide 4-G2 SKAVRKGVSSR and peptide 9-G2 TFDLLRNSYGVRK were kindly provided by Yoshihiko Yamada, NIH, NIDR, Bethesda, MD, and peptides 7-G2 LEISRST and 5-G2,sc KVILSCKSTGSRNIRVESGYS by Werner Gürr, Department of Immunology, University of Zürich, Switzerland.

Solid-phase binding assays

Inhibition of the binding of laminin to the HNK-1 glycolipid by synthetic peptides. The HNK-1 glycolipid was substrate coated onto 96-well microtitre plates (Falcon, Dietikon, Switzerland) in ethanolic solution at 3 μM by evaporating to dryness. After blocking with 1% fatty acid-free bovine serum albumin (BSA; Sigma catalogue no. A-7030), in phosphate buffered saline (PBS; pH-7.4) for 1 h, the laminin-derived peptides 5-G1, 5-G2, 5-G3 and 5-G2,sc were pre-incubated with the substrates at a concentration of 166 μM for 30 min at room temperature. In another binding assay, peptide 5-G2 was used in a 1:1 dilution series starting at 166 μM in 0.1 M NaHCO₃ containing 10 mM EDTA. Laminin was then added at 12.5 nM in 0.1 N NaHCO₃ supplied with 10 mM EDTA. For control, laminin was incubated with the substrate in the absence of peptides. Bound laminin was detected by incubation with pab LN and HRP-conjugated goat anti-rabbit secondary antibodies. Incubation of antibodies was

carried out at room temperature for 2 and for 1 h, respectively. The generation and detection of the coloured reaction product were performed as described by Horstkorte *et al.* (1993). Per cent inhibition was calculated with reference to the control value obtained in the absence of additives (= 0% inhibition).

Binding of biotinylated synthetic peptides to different glycolipids. Peptides 5-G1, 5-G2 and 5-G3 were biotinylated according to Cole *et al.* (1987) using NHS-LC-biotin (Pierce, Rockford, IL). The efficiency of biotinylation was tested by enzyme-linked immunosorbent assay (ELISA) (Bayer and Wilchek, 1990) and found to be equal for all peptides. HNK-1 glycolipid, sulphatides or cerebroside were substrate immobilized at 1 μM in 96-well microtitre plates as described in the previous paragraph. After blocking with 10% BSA in PBS for 1 h at room temperature, biotinylated peptides 5-G1, 5-G2 or 5-G3 were added in a 1:1 dilution series starting at 1 μM in 0.1 N NaHCO_3 containing 10 mM EDTA for 1 h at room temperature. Bound peptides were detected by HRP-conjugated streptavidin (Sigma catalogue no. S-5512) and the OD determined as described in the previous paragraph.

Cell adhesion assays

Preparation of single-cell suspensions. Single-cell suspensions of small cerebellar neurons (~99% pure) from 6-day-old ICR mice were prepared by mild trypsinization (Schnitzer and Schachner, 1981; Keilhauer *et al.*, 1985). Cells were used for the adhesion assay at a concentration of 1×10^6 cells/ml in basal medium Eagle's (Gibco/BME from BRL). Cells expressed the HNK-1 carbohydrate as tested by indirect immunofluorescence staining (Schnitzer and Schachner, 1981) with mab 412 immediately after preparation.

Mouse fibroblast L-cells (L929; Pantazis and Jensen, 1988) were cultured in BME containing 10% horse serum. Confluent monolayer cells were trypsinized 0.05% trypsin in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} for 5 min at room temperature and used for the adhesion tests at a final concentration of 0.5×10^6 cells/ml.

Cell adhesion to laminin in the presence of different peptides, glycolipids and Fab fragments of mab 412 and pab NCAM. Laminin was substrate immobilized overnight at 8°C in 24-well microtitre plates (NUNC, Nunclon, Switzerland). Coating was performed in 3 μl spots at 25 nM in BME. The coating efficiency was determined to be 15–17% by measuring the protein concentration of the coating solution before and after the coating procedure (Bradford, 1976). The substrate was blocked for 1 h at room temperature with 3% heat-inactivated BSA in PBS. Cells were pre-incubated for 30 min at room temperature either with the peptides 5-G1, 5-G2, 5-G3 or 5-G2,sc at 166 μM or with decreasing concentrations of 5-G2 (83, 33, 16, 1.6 and 0.3 μM) in HBSS without Ca^{2+} and Mg^{2+} . The cells were viable during the time of the assay according to their morphological appearance by phase-contrast microscopy and exclusion of trypan blue for all peptides used. Cells were also pre-incubated for 30 min at room temperature in the presence of Fab fragments of pab NCAM (5 μM) and Fab fragments of the mab 412 at 10, 2, 1, 0.1, 0.06 and 0.02 μM in HBSS without Ca^{2+} and Mg^{2+} . Pre-incubation was also performed with the HNK-1 glycolipid at 20, 5, 2.5, 1.25 and 0.6 μM , and sulphatides or cerebroside at 50 μM . The mixture of cells and inhibitors was incubated with the substrate for 30 min at room temperature. For the control, cells were incubated with laminin in the absence of any additive. Bound cells were washed in HBSS without Ca^{2+} and Mg^{2+} , fixed for 1 h with 2.5% glutaraldehyde and stained with crystal violet (Aumailley *et al.*, 1989). The number of cells per area (0.1 \times 0.06 mm) was determined with the IBAS graphics system (Kontron, Zürich, Switzerland). Data obtained by this procedure were normalized and per cent inhibition was calculated with respect to the control value obtained in the absence of additives (= 0% inhibition).

Statistical analysis

Statistical analysis was performed on non-normalized data by one-way ANOVA and Scheffé's *F* or Fischer's PLSD comparison among the means (Winer, 1971). All values marked with an asterisk differ significantly from the control value at **P* \leq 0.05, ***P* \leq 0.01, ****P* \leq 0.001.

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Abbreviations

BME, basal medium Eagle's; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; mab, monoclonal antibody; OD, optical density; pab, polyclonal antibody; PBS, phosphate-buffered saline; SD, standard deviations.

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