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Characterization of the kinetics of neural cell adhesion molecule homophilic binding

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A solid-phase assay has been developed for the investigation of the kinetics of neural cell adhesion molecule (NCAM) binding. Using this assay we can show that NCAM binds to itself in a time-dependent and saturable manner. Binding constants (K_B values) of 6.9×10^{-8} M and 1.23×10^{-6} M, respectively, were obtained for adult and newborn rat NCAM homophilic binding. Binding is specifically inhibited by Fab' fragments of polyclonal anti-NCAM antibodies but is unaffected by heparin or chondroitin sulphate. This indicates that the NCAM homophilic binding site is separate from and independent of the heparin-binding site and that a developmental modification, probably polysialation, gives rise to marked differences in the adhesive properties of NCAM.

Affinity constant; Homophilic binding; Cell adhesion molecule; (Neural cell)

1. INTRODUCTION

Several cell adhesion molecules have been implicated in cell surface interactions during development of the mammalian nervous system. The neural cell adhesion molecule (NCAM; for review see [1,2]) is currently the most extensively characterized of these. It is expressed on all major neural cell types in the central and peripheral nervous systems [3–5]. It mediates aggregation of single cells [6] and histotypic deployment in neural tissue culture [7]. It has been shown to have a functional role in neuron-neuron, neuron-astrocyte, astrocyte-astrocyte and neural cell-substratum adhesion [8–10] and to be involved in nerve-muscle cell recognition [7].

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Abbreviations: P0 NCAM, neural cell adhesion molecule isolated from newborn rat brains; P40 NCAM, NCAM isolated from rat brains at postnatal day 40; PBS, phosphate-buffered saline; PSA, polysialic acid

NCAM-mediated adhesion appears to occur by a homophilic and possibly polyvalent binding mechanism [1,11,12] in which NCAM on the surface of one cell binds to NCAM on an opposing cell. Studies by Cole and Glaser [10] have demonstrated that cell surface NCAM also participates in cell-substratum interactions in the developing chick nervous system. Cole and coworkers [13] have also presented data indicating that although NCAM-mediated cell-cell and cell-substrate adhesion involves a homophilic binding mechanism, the binding of heparan sulphate may also be required.

The purpose of this study was to determine further the specificity of NCAM homophilic binding and its dependence on heparin, to characterize the kinetics of this interaction and finally, to compare the binding constants (K_B values) for newborn and adult forms of the protein.

2. PROCEDURES

2.1. *Determination of affinity constants*

NCAM purified from newborn (P0 NCAM) or adult rats (P40 NCAM) according to published procedures [14] is

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detritonized using SM2 biobeads [15]. Protein is sonicated for 10 s immediately before use and is then immobilized (2 μg/ml; 1 h at 22°C) on nitrocellulose paper discs (5.5 mm diameter). Excess unreacted binding sites are blocked with 1.0% BSA in PBS. Control discs are coated only with BSA. Increasing concentrations of ^{125}I-labelled NCAM [^{125}I-NCAM] of known specific activity and ranging from 10^{-8} M to 4×10^{-7} M are incubated with paired NCAM- and BSA-coated discs in a total assay volume of 40 μl. After 4 h at 22°C, discs are washed and counted. Specific binding is determined by subtracting unspecific binding (binding to BSA-coated discs) from total binding (binding to NCAM-coated discs). Data are analyzed by non-linear curve fitting using the McPherson 'Radlig' program [16].

NCAM binding to nitrocellulose filters is quantified by incubating discs for 1 h at 22°C with ^{125}I-NCAM (2 μg/ml) of known specific activity. Discs are then washed extensively and the amount of bound NCAM calculated by counting in a gamma counter.

Competition assays are performed by preincubating paired NCAM- and BSA-coated discs for 30 min with competitor (Fab' fragments; heparin; chondroitin sulphate). Incubations are then carried out with standard amounts of ^{125}I-NCAM in the presence of competitor.

3. RESULTS AND DISCUSSION

Both preparations of NCAM bind to nitrocellulose-immobilized protein in a time-dependent (fig.1) and saturable manner. At 22°C, maximal binding occurs after 4 h with a $T_{1/2}$ of 65 min for P40 NCAM and after approx. 20 h and with a $T_{1/2}$ of 130 min for P0 NCAM preparations. The differences in these two figures indicate a slower association constant for P0 NCAM. This is probably attributable to the 3-fold higher amounts of polysialic acid (PSA) found on NCAM isolated from younger animals. Analysis of equilibrium binding data (fig.2) by Scatchard plots [16,17] revealed average binding constants of $6.9 \pm 2.4 \times 10^{-8}$ M and $1.2 \pm 0.9 \times 10^{-6}$ M for P40–P40 and P0–P0 NCAM, respectively (table 1).

B_{max} values, or maximum number of receptor sites available for binding, were estimated to be $0.75 \pm 0.28 \times 10^{-8}$ M for P40 NCAM. Based on an average estimation of 54 ng immobilized

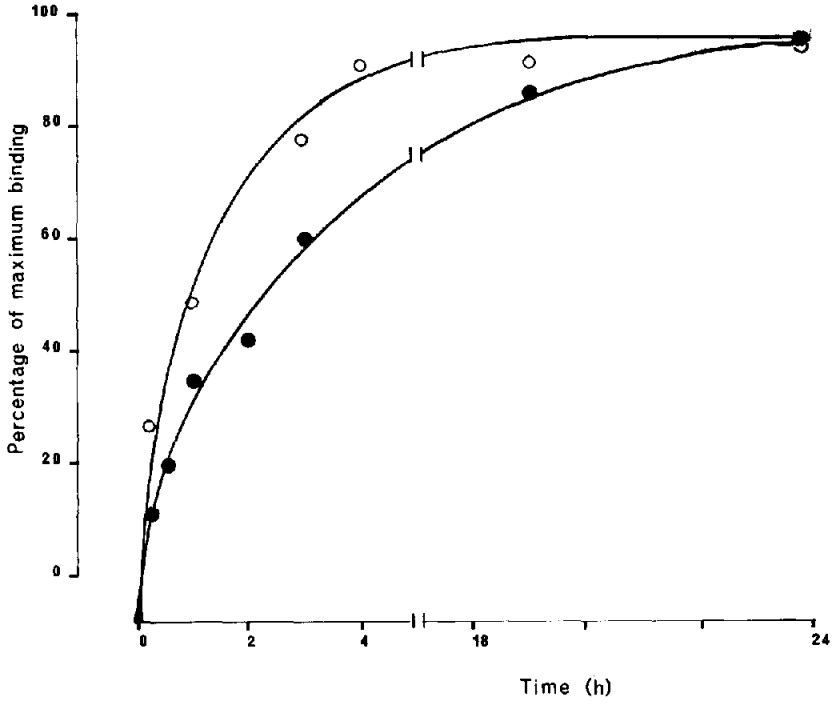

Fig.1. Time course for homophilic binding of P0 (•) and P40 (○) NCAM. Assay conditions are as described in section 2. Assay points represent the mean of two separate determinations.

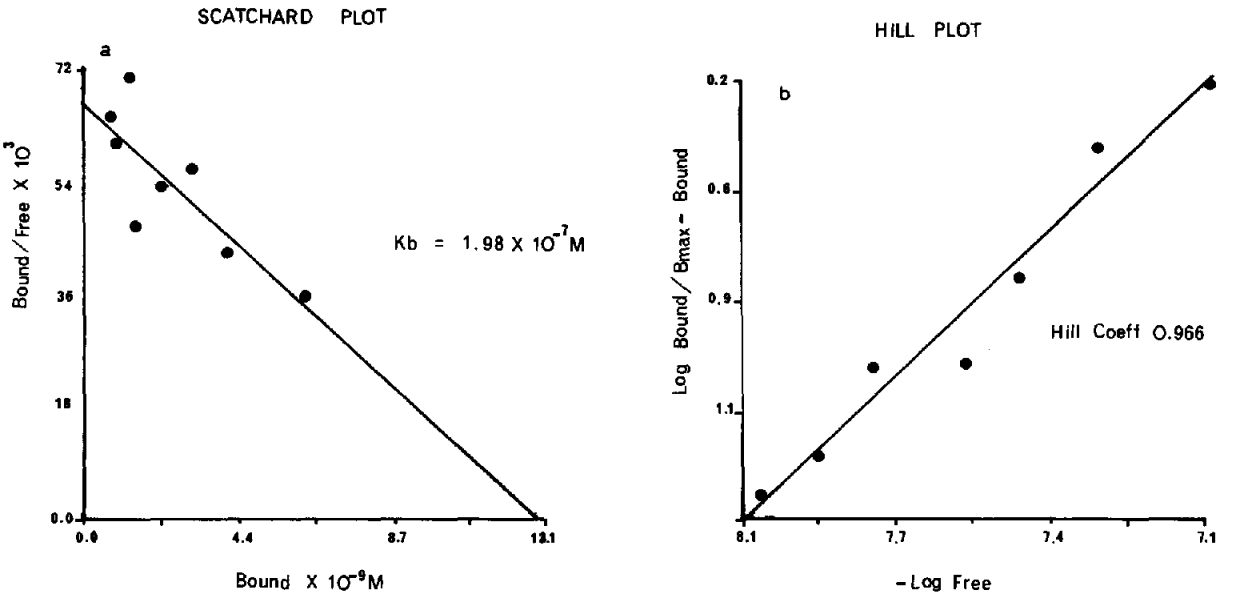

Fig.2. Scatchard (a) and Hill plot (b) analysis of binding data from a representative P40 homophilic binding experiment.

Table 1

Summary of binding characteristics for P0 and P40 homophilic binding

	P0-P0	P40-P40
K_B value	$1.48 \pm 0.63 \times 10^{-6}$ M (7)	$6.92 \pm 2.4 \times 10^{-8}$ M (4)
Correlation coefficient	0.76 ± 0.07 (7)	0.75 ± 0.11 (4)
Hill coefficient	0.92 ± 0.05 (7)	0.88 ± 0.09 (4)

K_B values were obtained from Scatchard analysis of the data using McPhersons (1985) equilibrium binding data analysis (EBDA) program. The correlation coefficient refers to the fit of the Scatchard data. Data are presented as mean ± SE. The number of experiments contributing to the data is shown in parentheses

NCAM per nitrocellulose disc, it could be calculated that there was a 1:1 binding of NCAM molecules to each other. This does not necessarily imply that polyvalent binding of NCAM does not occur. Rather it suggests that equal amounts of labelled and immobilized NCAM bind in doublets or greater aggregates. Hill plot analysis of the data (fig.2; table 1) corroborated this finding, suggesting the existence of only one affinity site (Hill coefficient 0.88 ± 0.09; mean ± SE) and the absence of any cooperativity. Thus, it appears that binding of one NCAM molecule does not enhance or inhibit binding of subsequent molecules implying that although the capability for multiple binding of NCAM molecules to form aggregates does exist [12], such aggregate formation is not encouraged. In any case, the simplest explanation of adhesion in doublets is consistent with the reports of Rutishauser et al. [12], who show that greater than 80% of NCAM molecules in their preparations exist as monomers or dimers.

Fab′ fragments of polyclonal anti-NCAM antibodies were able to interfere with NCAM homophilic binding by up to 50% compared to O-Fab′ fragments (Fab′ fragments prepared from preimmune serum) further verifying the specificity of the NCAM-NCAM interaction (table 2). Heparin or chondroitin sulphate were incapable of interfering with this binding (table 2) implying that the NCAM-heparin binding site and the NCAM-

Table 2

Ability of various agents to inhibit NCAM homophilic binding

Agents	Binding to P40 NCAM as a percentage of control
O-Fab' (1 mg/ml)	100 (4)
Anti NCAM Fab' (1 mg/ml)	47.7 ± 11.7 (4)
Heparin (200 μg/ml)	97.8 ± 3.5 (3)
Chondroitin sulphate (200 μg/ml)	98.5 ± 8.4 (3)

Data are expressed as a percentage of control ± SE. Control values are those determined in the presence of O-Fab'. The number of experiments contributing to the data is shown in parentheses

NCAM homophilic-binding sites are separate and independent. This observation is at variance with the model proposed by Cole et al. [13], in which it is argued that heparin-sulphate proteoglycans cross-link opposing NCAM molecules and permit homophilic binding by inducing a conformational change in the proteins. In these studies we show that NCAM homophilic binding can occur in the absence of heparin-sulphate proteoglycans and that heparin does not affect the amount of binding observed.

The affinity constants determined for the interactions of adult and P0 NCAM are consistent with other reports which suggest that the rates of NCAM-mediated aggregation of brain vesicles or cells is inversely proportional to their sialic acid content [18–20]. The amount of PSA associated with NCAM decreases with postnatal development [19,21]. In this study, we show that the affinity of NCAM homophilic binding increases with age suggesting that the difference is due, at least partially, to differences in sialic acid content. Further studies are necessary to assess the effects of polysialic acid removal and of other post-translational modifications on NCAM homophilic binding.

In conclusion, it has been shown that NCAM binding can be measured in an in vitro binding assay and yields K_B values of approx. 10^{-8} M and 10^{-6} M for P40 and P0 homophilic binding, respectively. Binding follows classical kinetic models for a single affinity-binding site and shows no cooperativity. Heparin and chondroitin sulphate fail to interfere with NCAM homophilic binding implying that the homophilic-binding site is independent of and separate from the heparin-binding site on NCAM.

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REFERENCES

[1] Cunningham, B.A., Hemperley, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R. and Edelman, G.M. (1987) Science 237, 799–806.
[2] Nybroe, O., Linneman, D. and Bock, E. (1988) Neurochem. Int. 12, 251–262.
[3] Noble, M.M., Albrechtsen, C., Møller, C., Lyles, J., Bock, E., Goridis, C., Watanbe, M. and Rutishauser, U. (1985) Nature 316, 725–728.
[4] Langley, O.K., Gombos, G., Hirn, M. and Goridis, C. (1983) Int. J. Dev. Neurosci. 1, 393–401.
[5] Silver, J. and Rutishauser, U. (1984) Dev. Biol. 106, 485–499.
[6] Thiery, J.P., Brackenbury, R., Rutishauser, U. and Edelman, G.M. (1977) J. Biol. Chem. 252, 4234–4238.
[7] Grummet, M., Rutishauser, U. and Edelman, G.M. (1982) Nature 295, 693–695.
[8] Rutishauser, U., Hoffman, S. and Edelman, G.M. (1982) Proc. Natl. Acad. Sci. USA 79, 685–689.
[9] Keilhauer, G., Faissner, A. and Schachner, M. (1985) Nature 316, 728–730.
[10] Cole, G.J. and Glaser, L. (1986) J. Cell Biol. 102, 403–412.
[11] Rutishauser, U., Thiery, J.-P., Brackenbury, R., Sela, B.-A. and Edelman, G.M. (1976) Proc. Natl. Acad. Sci. USA 73, 577–581.
[12] Rutishauser, U., Acheson, A., Hall, A.K., Mann, D.M. and Sunshine, J. (1988) Science 240, 53–57.
[13] Cole, G., Loewy, A., Cross, N.V., Aheson, R. and Glaser, L. (1986) J. Cell Biol. 103, 1739–1744.
[14] Rasmussen, S., Ramlau, J., Axelsen, N.H. and Bock, E. (1982) Scand. J. Immunol. 15, 179–185.
[15] Holloway, P.W. (1973) Anal. Biochem. 53, 304–308.
[16] McPherson, G.A. (1986) Elsevier-Biosoft, Copyright.
[17] Rosenthal, H.E. (1973) Anal. Biochem. 20, 525–532.
[18] Sadaul, R., Hirn, M., Deagostine-Bazin, H., Rougon, G. and Goridis, C. (1983) Nature 304, 347–349.
[19] Hoffman, S. and Edelman, G.M. (1983) Proc. Natl. Acad. Sci. USA 80, 5762–5766.
[20] Rutishauser, U., Watanbe, M., Silver, J., Troy, F. and Vimr, E.R. (1985) J. Cell Biol. 101, 1842–1849.
[21] Rothbard, J.B., Brackenbury, R., Cunningham, B.A. and Edelman, G.M. (1982) J. Biol. Chem. 257, 11064–11069.