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Biochimica et Biophysica Acta 1330 (1997) 138–144

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Cellular determinants of the lateral mobility of neural cell adhesion molecules

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Received 16 December 1996; revised 20 May 1997; accepted 20 May 1997

Abstract

The lateral mobility of the neural cell adhesion molecule (NCAM) was examined using fluorescence recovery after photobleaching (FRAP). Various isoforms of human NCAM, differing in their ectodomain, their membrane anchorage mode or in the size of their cytoplasmic domain, were expressed in NIH 3T3 cells and C2C12 muscle cells. When the various isoforms were compared in 3T3 cells, FRAP studies showed both GPI-anchored and transmembrane isoforms diffused rapidly and only small differences in either the diffusion coefficients (D) or the mobile fractions (mf) were measured, suggesting the importance of the ectodomain in regulating lateral diffusion. However, the mobility of all NCAM isoforms was greatly reduced in regions of cell–cell contact, presumably due to homophilic *trans* interactions between NCAMs on adjacent cells. NCAM isoforms transfected into C2C12 cells which express NCAM naturally usually displayed a significantly lower D compared to the same isoforms transfected into 3T3 cells. Thus, NCAM lateral mobility is modulated in regions where cells interact and by the structure of the host cell membrane. © 1997 Elsevier Science B.V.

Keywords: Cell adhesion protein; Neuron; Membrane mobility; Diffusion coefficient; Cell-cell interaction; Splicing

1. Introduction

The nearly 30 members of the neural cell adhesion molecule (NCAM) family arise from a single gene that is alternatively spliced during transcription [1–7]. Different isoforms result in various forms of membrane anchorage, including membrane spanning peptides with different sized cytoplasmic domains or a glycosylphosphatidyl-inositol (GPI) linkage. Other isoforms have ectodomain variations that are cell type specific; these include a 37 amino acid insert, called the muscle specific domain (MSD) [7], and a 10 amino acid insert derived from the VASE exon [4,8]. While NCAM has an important role in mediating cell–cell adhesion, it is also involved in regulating neurite outgrowth [1–5]. Compared to the 180 kDa large cytoplasmic domain isoform, the GPI-linked 120 and the 140 kDa isoform, containing a small cytoplasmic domain, are much more effective at promoting neurite outgrowth [5].

Considering the various functions of NCAM and the many isoforms, it was of interest to determine the lateral mobility of NCAM isoforms expressed by transfection in various cell lines using FRAP. Mea-

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PII S0005-2736(97)00128-4

surements were conducted in two cell types: 3T3 cells in which NCAM is foreign and C2C12 muscle cells which naturally express NCAM. Since NCAM isoforms with the same or similar ectodomain have very different modes of membrane anchorage, these measurements allowed a further test of the hypothesis that, for at least certain membrane proteins, the mode of membrane anchorage does not affect lateral mobility [9,10]. The FRAP data largely support this hypothesis and suggest that for NCAM the ectodomain plays a more important role in regulating lateral mobility. However, the lateral mobility of these proteins depends on the cellular background: NCAM exhibits a larger diffusivity in 3T3 cells where it is foreign compared to C2C12 cells where it is expressed naturally.

Since 3T3 cells expressing NCAM serve as a substrate for neurite outgrowth, and NCAM on adjacent cells must homophilically interact for such activation to occur, it could be argued that NCAM lateral mobility is rate limiting for the initial interaction that triggers subsequent signal transduction. However, these experiments demonstrated that the potential of a given isoform expressed in the feeder cell layer to promote neurite outgrowth and its lateral mobility in these cells was not correlated.

2. Materials and methods

2.1. Cells and reagents

C2C12 mouse muscle cells and NIH 3T3 fibroblasts were transfected with various isoforms of human NCAM as described elsewhere [1,11]. Cells were routinely cultured in DMEM-H [Dulbecco's Modified Eagle Medium, with D-glucose, L-glutamine and sodium bicarbonate; H for high glucose (9 g l^{-1})], supplemented with 10% fetal bovine serum, and plated on 12 mm × 12 mm coverslips for the FRAP measurements two days prior to experiments.

The anti-human NCAM (ERIC-1) is a mouse mAb IgG1 [12]; it was obtained from an ascites (Vector Labs, UK) and purified by ammonium sulfate precipitation followed by a protein G column using the mAb Trap G11 Kit (Pharmacia). Fab fragments were produced by digestion with immobilized papain using a kit (Pierce Biochemicals, Rockford, IL) with the modification that a Protein G column was employed for removal of undigested IgG and Fc fragments. Fluorochrome conjugation to IgG and Fab fragments was carried out by dialyzing IgG/Fab (3 mg ml^{-1}) into 100 mM Na carbonate/bicarbonate buffer at pH 9.3. Rhodamine B isothiocyanate was added at a ratio of 10 mg/mg protein and stirred at 4°C for 16 h. A Sephadex G10 column was used to remove the majority of free dye followed by extensive dialysis against PBS. The conjugated ERIC 1 was tested for activity by immunofluorescence titration against the human NCAM expressed in transfected 3T3 cells and compared to the original unconjugated IgG. No difference in activity was observed indicating the reagent was not affected by the conjugation.

2.2. FRAP studies

Labelling for the FRAP measurements was accomplished by washing the cells twice in HBSS(+) [Hank's balanced salts solution with calcium and magnesium] and then incubating the cells in the antibody solution (diluted with HBSS(+)) for 10 min at room temperature. Coverslips were mounted on a metal slide in HBSS(+) as previously described [13]. FRAP measurements were made on a Leitz Ortholux microscope employing a Leitz 40 × oil objective lens (NA 1.3). The focused Gaussian laser beam radius was calculated to be about 1 mm. This calculation was confirmed by quantitative fluorescence microscopy using digitized image processing. The 514 nm line of a Spectra-Physics 164-07 argon 107 laser (Mountain View, CA) was used. Spots on the cell were bleached for 60–100 ms using a laser power of 40–50 mW. After labelling the cells with rhodamine-conjugated anti-NCAM IgG or Fab fragments, FRAP measurements were performed at room temperature, periodically interrogating the fluorescence recovery (pulse duration 0.5 s, time between pulses 2 s for 3T3 studies; pulse duration 1 s, time between pulses 2 s for C2C12 cells; the instrumentation monitors recovery within each interrogation pulse). A 350 micron pinhole in the intermediate image plane was used to limit the collection depth of the photomultiplier. A single curve was recorded per transfected cell, and each coverslip was used for less than 1 h after antibody labelling. Fluorescence recovery curves were analyzed using software developed

by Gordon et al. [14]. This approach requires the user to specify the prebleach fluorescence value, the final fluorescence value (after recovery is complete) and the baseline value for the recovery curve.

We had previously discussed reasons for the upward drift of lateral diffusion coefficients reported by this laboratory [9]. We have now completed investigation of those factors. Control experiments indicate that the major source of the increase in D is due to a switch in technology in 1986 for attenuating the laser beam after photobleaching for the measurement phase of the FRAP experiment. The neutral density filters, polished to near parallelism, were exchanged for a beam splitter device which insures, when properly aligned, that the measuring beam is perfectly coincident with the photobleaching laser beam [15]. The combination of factors previously enumerated, full curve fitting, and this prior improvement in beam attenuation produces an ca. 2-fold increase in the D values calculated from the FRAP curves when compared to the pre-1986 analysis. Since most of the biological conclusions are derived from comparisons

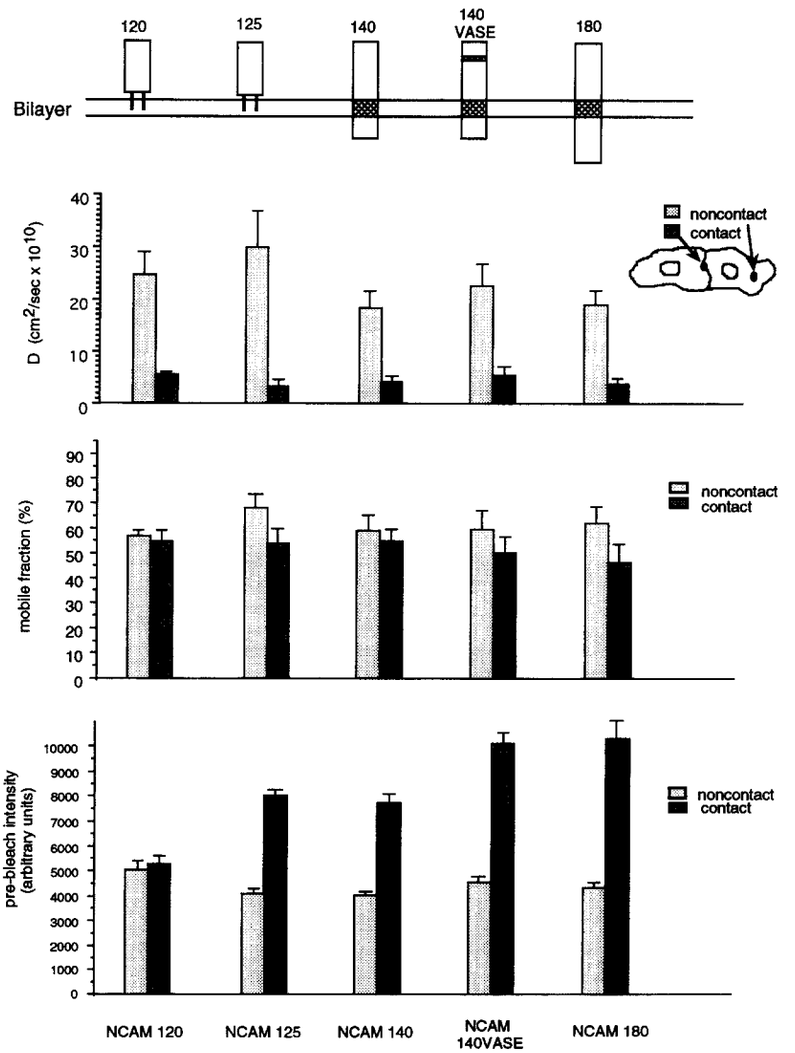

Fig. 1. The lateral mobility of NCAM isoforms expressed in 3T3 cells in regions of cell–cell contact as compared to other cell regions. Contact and non-contact regions were tested in separate experiments. Top panel, linear structures of the isoforms tested; middle panels, diffusion coefficients and mobile fractions; bottom panel, pre-bleach fluorescence intensity excited by the FRAP laser beam in non-contact vs. contact zones. Values ($\pm$ SEM).

of data taken under the same conditions, they are not affected by this upward revision of our D values.

3. Results

3.1. Lateral mobility of NCAM isoforms expressed in 3T3 cells

Various human NCAM isoforms (Fig. 1) were expressed by gene transfer in 3T3 cells, which lack endogenous NCAM. These NCAM isoforms exhibited similar diffusion coefficients D and mobile fractions (Table 1). In particular, the mode of membrane anchorage [GPI-anchored (NCAM 120 and 125), or anchored by a small (NCAM 140) or large (NCAM 180) cytoplasmic domain] or the presence of the VASE insert in the ectodomain did not affect lateral mobility significantly. All transfected isoforms tested also displayed a greatly reduced lateral diffusivity in contact regions of 3T3 cells, compared to other parts of the cells (Fig. 1, top data panel). The mobile fractions, however, remained unaffected by such contact (Fig. 1, middle data panel). For all isoforms except NCAM 120 the intensity in the contact zone was roughly doubled (Fig. 1, bottom data panel). We attribute this to the simple fact that two cell surfaces are measured in contact zones as a similar effect was measured for the glycoprotein, pgp1 [CD44] [13]. However, we cannot rule out some increase in the surface density of NCAM in such regions.

Control experiments indicated that the measured lateral mobility of all NCAM isoforms expressed in 3T3 cells did not depend significantly on whether intact IgG or Fab fragments were employed as labels for FRAP measurements (Fig. 2).

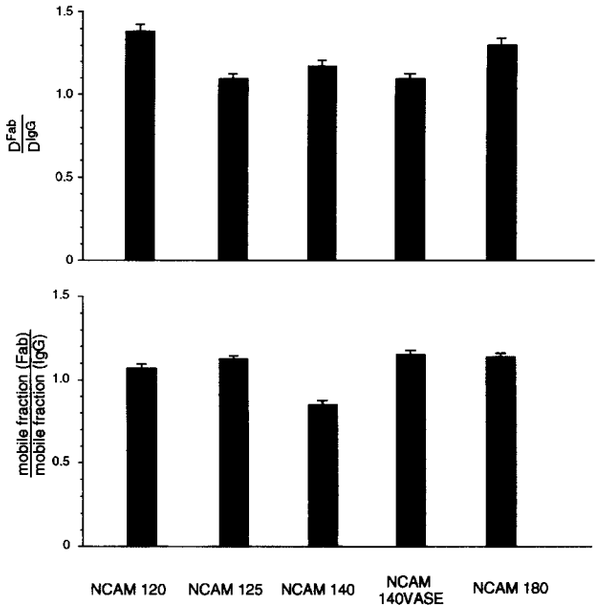

Fig. 2. Relative lateral mobility of various NCAM isoforms in 3T3 cells using NCAM IgG or Fab fragments for the FRAP measurement. Top panel, relative diffusion coefficients expressed as the D^{Fab}/D^{IgG}; bottom panel, relative mobile fractions expressed as the ratio mobile fraction(Fab)/mobile fraction(IgG).

3.2. Lateral mobility of NCAM isoforms expressed in C2C12 muscle cells

Human NCAM isoforms were also expressed, by gene transfer, in C2C12 muscle cells which possess an endogenous NCAM complement. These NCAM isoforms exhibited a 2- to 8- fold lower diffusivity in the C2C12 cells as compared to the 3T3 cells (Table

Table 1
Lateral mobility of human NCAM expressed by gene transfer in 3T3 and muscle cells [a]

Isoform/cell type	Human NCAM expressed in 3T3 cells			Human NCAM expressed in C2C12 cells		
	D ($\times 10^{10}$ cm^2 s^{-1})	Mobile fraction (%)	N	D ($\times 10^{10}$ cm^2 s^{-1})	Mobile fraction (%)	N
120	24.3(±4.2)	57.5(±3.3)	19	10.9(±1.9)	44.3(±4.0)	22
125	30.2(±6.6)	67.7(±5.0)	18	3.7(±0.4)	69.1(±3.7)	20
125 VASE	–	–	–	4.5(±0.8)	57.2(±3.5)	22
140	18.0(±3.7)	60.9(±4.1)	21	4.7(±0.5)	71.1(±4.7)	23
140 VASE	22.4(±3.6)	60.4(±4.4)	23	–	–	–
180	18.3(±3.1)	61.4(±4.7)	20	–	–	–

[a] Values (±SEM).

Table 2
Lateral mobility of endogenous NCAM in C2C12 muscle cells at various stages of differentiation

Stage of differentiation	D ($\times 10^{10}$ cm^2 s^{-1})	Mobile fraction (%)	Number of samples
Myoblasts [a]	6.9(±0.2) [b]	50.8(±5.4)	26
Midfusion	5.6(±0.2)	45.2(±4.1)	32
Myotubes [c]	5.3(±0.2)	58.0(±4.5)	28

[a] NCAM 140 predominantly expressed.
[b] Mean (±SEM).
[c] NCAM 125 (GPI-anchored) predominantly expressed.

1). In the 3T3 cells, all transfected isoforms tested displayed diffusion coefficients between 18×10^{-10} and 30×10^{-10} cm^2 s^{-1}, whereas D ranged between 4×10^{-10} and 11×10^{-10} cm^2 s^{-1} in the muscle cells (Table 1). Mobile fractions were generally in the range of 50–70% (Table 1). The differences between the 120 and 125 kDa muscle isoforms for both D ($p < 0.01$) and mobile fraction ($p < 0.01$) are significant but at this juncture we have no convincing explanation for them.

The lateral mobility of endogenous NCAM was measured by FRAP in C2C12 cells in the myoblast, midfusion and the postfusion myotube state (Table 2). The distribution of isoforms of the endogenous NCAMs expressed in the muscle cell changes during the transition from myoblasts to myotubes. The predominant NCAM expressed in the myoblast stage is the transmembrane 140 kDa isoform whereas the 125 kDa isoform is more prevalent in myotubes [16]. Lateral diffusion was slightly more rapid in the myoblasts as compared to the myotubes with values for midfusion cultures falling in between (Table 2). The lateral diffusion coefficients were lower for the transfected isoforms as compared to the corresponding endogenous NCAM, but the mobile fractions were somewhat higher for the transfected isoforms (compare Tables 1 and 2).

4. Discussion

The present studies indicate that NCAM diffusivity on the micron distance scale as measured by FRAP is, in general, similar for different isoforms within the same cell background, but the diffusivity does depend on the cell type. Furthermore, where tested, the lateral transport properties measured by FRAP for endogenous isoforms were similar to those for NCAM isoforms expressed by gene transfer indicating the latter methodology was not introducing a significant artifact.

Transfection of several NCAM isoforms into different cell lines allowed comparison of NCAM mobility in cells where it is a completely foreign protein (3T3 cells) to cells that do express endogenous isoforms of NCAM (C2C12). With the exception of NCAM 120 expressed in C2C12 cells, FRAP studies show that different NCAM isoforms, all having the same or similar ectodomains, have similar diffusivities and mobile fractions in a given cell type, regardless of the type of membrane anchorage or the size of the cytoplasmic domain. These results indicate the importance of the ectodomain as a determinant of NCAM lateral mobility, extending a conclusion reached by Zhang et al. [9] for various mutant membrane proteins derived from the VSV G glycoprotein.

It may be postulated that the intrinsic lateral diffusivity of membrane proteins, determined by an effective bilayer viscosity, is further reduced by an effective 'viscosity' of the pericellular matrix, through which the ectodomain must move [17,18], as well as by transient interactions with slowly diffusing or immobile structures [9,10]. The large and similar diffusion coefficients characterizing all NCAM isoforms in 3T3 cells suggest that the bilayer viscosity, acting on the membrane anchorage domains, and the pericellular matrix viscosity, acting on very similar ectodomains, determine NCAM diffusivity in these cells.

Indeed, a number of diverse studies suggest that ectodomain interactions, acting to produce viscous drag or indirectly by activating additional intramembrane or cytoskeletal interactions, are a key determinant of lateral mobility. For example, reducing the effective size of the ectodomain, including molecules complexed to it, will act to increase the lateral mobility. Genetic removal of the three oligosaccharides from a Class I MHC antigen increased its lateral diffusion coefficient 3 fold compared to the wild type antigen [19]. Zhang et al. [20] measured a 3 fold increase in the lateral diffusion coefficient of Thy-1, a GPI-anchored glycoprotein, comparing rhodamine IgG to rhodamine conjugated Thy-1 as labels for

FRAP studies. Modification of the extracellular matrix can reduce the effective viscous drag that this milieu exerts on the diffusing entity. In this regard, trypsin treatment of transformed and normal cells increased the lateral mobility of succinyl–Con A receptor(s) conjugates by 2- and 4-fold, respectively [21], suggesting that removal of extracellular matrix components can increase lateral mobility.

The diffusivity of a number of transfected NCAM isoforms in contact regions of 3T3 cell cultures is much lower than in non-contact regions. This result can be interpreted in terms of the homophilic binding of NCAM expected to occur between cells which are in contact. This interpretation is supported by the fact that the cell surface glycoprotein pgp-1 [13] does not experience a reduction in its lateral mobility in regions of cell–cell contact. This molecule is not expected to undergo homophilic *trans* interactions across cell–cell contacts. The reduction in mobility does not necessarily involve direct interaction of NCAM with the cytoskeleton as it occurs with GPI-anchored isoforms as well.

It is conceivable that NCAM surface density is increased in the region of cell contact thereby reducing its mobility and enhancing its potential for *trans* interactions between adjacent cells [22]. The fluorescence intensity is approximately doubled in contact zones (Fig. 1) suggesting at most a two fold increase in NCAM surface density. However, a similar doubling of intensity is seen for overlapping regions of cells expressing the surface glycoprotein, pgp1, but no change in lateral mobility was measured in contact vs. non-contact zones [13]. We are thus inclined to interpret the increase in fluorescence to simple morphological effects, a doubling of the edges or surfaces interrogated by the FRAP measuring beam, as opposed to pronounced accretion of NCAM in the contact zones.

All transfected NCAM isoforms exhibit a significantly reduced lateral mobility in C2C12 cells as compared to 3T3 cells, whereas the mobile fractions remain basically unchanged. One possibility for this difference in diffusivity could be *cis* (or lateral) interactions of the diffusing NCAM with other membrane components, which may include endogenous NCAM or glycolipids in the C2C12 cells. Such putative interactions could be caused, in part, by differential glycosylation of NCAM in muscle cells and/or the presence of NCAM 'binding' proteins in the muscle cells. The latter includes cytoplasmic components binding to the cytoplasmic domain of NCAM which could reduce lateral mobility [23].

Pollerberg et al. [6] found that NCAM expressed endogenously in cultured mouse neuroblastoma cells also exhibited lower lateral diffusion coefficients [$(1-2) \times 10^{-10}$ cm^2 s^{-1} at 4°C] than we found for human NCAM expressed in 3T3 cells. As the cells differentiate, the predominant NCAM isoform expressed changes from 140 to 180 kDa; concomitant with this change in expression, the mobile fraction drops markedly. In contrast, the differentiation of myoblasts to myotubes that occurs with a shift of expression from NCAM 140 to 125 as the predominant isoform [16] does not appreciably change the measured lateral mobility values (Table 2).

Since NCAM-120 and NCAM-140 when expressed in 3T3 cell monolayers demonstrate an ability to enhance the outgrowth of neurites relative to the 140 VASE and the 180 isoforms of NCAM [1,4,5], but all isoforms have similar lateral mobility in 3T3 cells, it is clear that the long range (micron scale) lateral mobility of these NCAM isoforms is not correlated with the ability of a particular isoform to promote neurite outgrowth.

Acknowledgements

This work was supported by NIH grant GM 41402 (KJ) and the Muscular Dystrophy Group of Great Britain and the Wellcome Trust (FSW and PD). The authors thank B.G. Barisas and M. Edidin for helpful discussions on the source of systematic errors in FRAP measurements and Michael Edidin for providing VA2 cells and antibody as a check on our FRAP setup.

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