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Direct demonstration of NCAM *cis*-dimerization and inhibitory effect of palmitoylation using the BRET^2 technique

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ABSTRACT

Biological activity of the neural cell adhesion molecule (NCAM) depends on both adhesion and activation of intra-cellular signaling. Based on in vitro experiments with truncated extra-cellular domains, several models describing homophilic NCAM *trans*- and *cis*-interactions have been proposed. However, *cis*-dimerization in living cells has not been shown directly and the role of the cytoplasmic part in NCAM dimerization is poorly understood. Here, we used the bioluminescence resonance energy transfer (BRET^2) technique to directly demonstrate that full-length NCAM *cis*-homodimerizes in living cells. Based on BRET^{2}50 values we suggest that the intra-cellular part of NCAM inhibits *cis*-dimerization, an effect mainly dependent on the palmitoylation sites.

Structured summary:
MINT-8071463: *NCAM140* (uniprotkb:P13591-1) *physically interacts* (MI:0915) with *NCAM140* (uniprotkb:P13591-1) by *bioluminescence resonance energy transfer* (MI:0012)
MINT-8071506: *NCAM180* (genbank_protein_gi:119587605) *physically interacts* (MI:0915) with *NCAM-180* (genbank_protein_gi:119587605) by *bioluminescence resonance energy transfer* (MI:0012)
MINT-8071483: *NCAM140* (uniprotkb:P13591-1) *physically interacts* (MI:0915) with *NCAM140* (uniprotkb:P13591-1) by *competition binding* (MI:0405)

1. Introduction

The neural cell adhesion molecule (NCAM) is a cell surface glycoprotein belonging to the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs). NCAM is predominantly expressed in the nervous system and regulates neuronal and glial development and regeneration, synaptic plasticity, and learning [1–3]. These effects depend on NCAM-mediated cell adhesion and intracellular signaling and involve multiple homo- and heterophilic interactions [1,2,4–6].

NCAM exists in three major isoforms, resulting from alternative splicing of a single gene, and include two transmembrane isoforms, NCAM-140 and -180, with intracellular domains (ICDs) consisting of 120 and 386 amino acids, respectively, and NCAM-120, lacking an ICD and linked to the membrane by a glycosylphosphatidylinositol (GPI) anchor. All three isoforms share the same extracellular domain (ECD) comprising five Ig-like (Ig) modules and two membrane-proximal fibronectin type 3 (Fn3) modules [7,8].

Abbreviations: BRET, bioluminescence resonance energy transfer; CAM, cell adhesion molecule; ECD, extracellular domain; Fn3, fibronectin type 3; GFP, green fluorescence protein; GPI, glycosylphosphatidylinositol; ICD, intracellular domain; Ig, immunoglobulin; NCAM, neural cell adhesion molecule; Rluc, Renilla luciferase; TMD, transmembrane domain

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The mode of NCAM homophilic interactions has been debated, and different Ig modules have been implicated in binding [6]. According to a recent model, NCAM is always present on the cell surface as *cis*-dimers, formed by interactions between IgI and II modules, and the *trans*-homophilic interactions of the NCAM *cis*-dimers cause NCAM-mediated cell adhesion [6,9] (Fig. 1). However, this model is based on the crystal structure of a truncated ECD, and the precise role of *cis*-dimerization is still unknown because the formation of *cis*-dimers has not been directly demonstrated in living cells.

Additionally, the importance of the ICD for NCAM-140 and -180 *cis*-dimerization has not been investigated. Intracellular domains may either enhance dimerization via putative dimerization sites or constitute a steric hindrance inhibiting dimerization. Furthermore, the ICD has binding sites for several intracellular proteins that may affect dimerization. Thus, NCAM interacts with, for example, non-receptor tyrosine kinase Fyn via the receptor protein tyrosine phosphatase RPTPα, growth-associated protein 43, and spectrin [4,10–14]. Such intracellular binding partners may affect dimerization by localizing NCAM to distinct plasma membrane regions. Furthermore, the ICD of NCAM contains a palmitoylation site (PS) consisting of four cysteins, and palmitoylation of this site targets NCAM-140 and -180 to lipid rafts [4,15,16]. Whereas NCAM-120 is almost exclusively present in rafts as a consequence of its GPI anchor, NCAM-140 and -180 are found in both raft and non-raft membrane fractions [8,17–21].

The present study addressed the question whether NCAM *cis*-dimerizes in living cells and whether the ICDs of NCAM influence this dimerization. This was investigated directly using the bioluminescence resonance energy transfer^2 (BRET^2) technique. We report that the interactions between NCAM ECDs are sufficient to allow *cis*-dimerization in living cells and that the ICD attenuates NCAM dimerization mainly via palmitoylation sites.

2. Materials and methods

2.1. Expression vectors

cDNA fragments were synthesized by polymerase chain reaction with MluI/SacII sites flanking human NCAM-180 (residues 1-1119, NCBI accession code EAW67201) and the following fragments of human NCAM-140 (Uniprot code:P13591-1): NCAM-140 (full length; residues 1–848), NCAM-PS-trunc (truncated after intracellular PSs; residues 1–755), and NCAM-trunc (truncated after the transmembrane domain [TMD]; residues 1–737). The plasmids used as templates were described previously [22,23]. The amplified cDNA fragments were subcloned into pGFP^2-N3 and pRluc-N3(h) (Perkin–Elmer). As a result of cloning, the linker PRARDPPVAT from the multiple cloning site of the vectors is present between NCAM fragments and GFP^2/Rluc. Untagged NCAM constructs were produced by mutagenesis introducing a stop codon after the linker in the GFP^2-tagged NCAM constructs. All cDNA clones were verified by sequencing, and membranous localization of all GFP^2-fused constructs was shown by fluorescence microscopy (Suppl. Fig. 1A–D).

To avoid interference between GFP^2/Rluc moieties and the extracellular dimerization of NCAM as well as the possible contribution to a BRET^2 signal of *trans*-dimerization, all constructs were designed to have Rluc or GFP^2 fused to the *C*-terminus of the NCAM isoforms (Figs. 2B and 3C).

2.2. Cell culture and transfection

COS-7 cells were purchased from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamax, 10% fetal bovine serum, penicillin-streptomycin, fungizone, and 1.5 g/l sodium bicarbonate (all purchased from Invitrogen) in 5% CO_2 at 37 °C. For transient transfection, COS-7 cells were seeded at a density of 3×10^6 cells per T-80 flask (Nunc). Transfections were performed the following day using Lipofectamine™ 2000 (Invitrogen) or FuGENE 6 (Roche) according to the manufacturers' instructions. Cells were harvested in 5 mM EDTA 48 h after transfection, washed twice in phosphate-buffered saline (PBS; Invitrogen), and resuspended in Dulbecco's PBS supplemented with 1 g/l glucose (Invitrogen) to a density of 1×10^6 cells/ml.

2.3. BRET^2 assay

The BRET^2 technique is based on resonance energy transfer between a light-emitting donor (Renilla luciferase [Rluc]) and a fluorescent acceptor (Green fluorescence protein^2 [GFP^2]). The technique allows the detection of physical interactions at distances up to 100 Å between two proteins fused to Rluc and GFP^2 moieties.

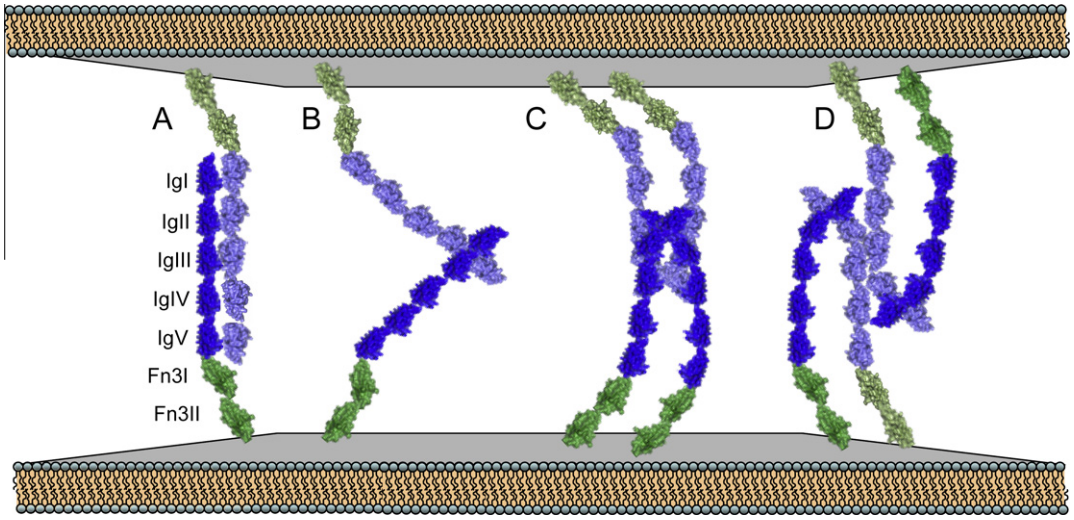

Fig. 1. Models of the NCAM homophilic adhesion complex. NCAM molecules are shown attached to the cell membranes of two opposing cells, with Ig modules in blue and the two membrane-proximal Fn3 modules in green. The models are based on (A) cell and microsphere aggregation experiments, (B) surface plasmon resonance and nuclear magnetic resonance analysis and the crystal structure of the NCAM IgI–II fragment, and (C and D) the crystal structure of the NCAM IgI–III fragment [6].