SynCAMs 1/2 Comprise a Novel Adhesion Complex at CNS Synapses

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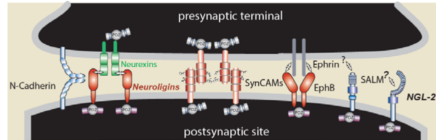
Introduction

Synapses are asymmetric sites of cell-cell adhesion at which neurons communicate chemically to propagate electrical signals. Tight adhesion between the pre-synaptic and post-synaptic neuron is a critical biochemical and morphological feature of synapses in the central nervous system. Synaptic adhesion molecules have been identified, and play roles in synaptic stabilization, formation, development and potentiation. Here we describe our efforts to characterize a novel adhesion complex at central synapses comprised of SynCAMs 1 and 2. SynCAMs 1 and 2 prefer to bind heterophilically *in vitro* and form a stable complex *in vivo* which can be isolated from synaptic fractions. Additionally, SynCAMs 1 and 2 can recruit each other to sites of cell-cell contact, suggesting that this complex forms actively during neuronal development.

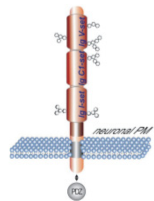
The material in the synaptic cleft. Cryo-EM tomography studies have revealed the first molecular details of the structures in the synaptic cleft (From Lucic, 2005, Structure). What are these complexes and what roles do they play in the organization and stabilization of synapses?

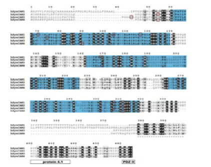


Background

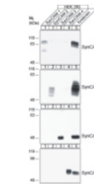
In recent years, our understanding of the roles of adhesion receptors in the formation and development of mammalian central synapses has expanded tremendously (see model above). Adhesion receptor systems identified as important for synaptic development include the neurexin-neuroligin and EphB-Ephrin asymmetric linkages, the symmetric SynCAM linkage, and also the orphan receptors SALM and NGL. All of these proteins influence synapse formation *in vitro* but likely also play important roles in other stages of neuronal and synaptic development.

The SynCAM family is a four-protein family conserved throughout the vertebrate phylum. SynCAMs have three extracellular Ig-like domains which mediate adhesion and a short cytoplasmic tail with protein-interaction motifs for synaptic adaptor proteins and the actin cytoskeleton (Biederer, 2005). In the CNS, it has been shown that SynCAM 1 is a synaptic protein with the capacity to induce functional synapses in cultured neurons (Biederer et al, 2002). However, it remains unclear what protein interactions are critical in this process. We have begun to characterize the protein interactions of the SynCAM family and their roles in neuronal development. Interestingly, the sequences of the four SynCAM cytoplasmic domains is highly conserved while the extracellular domains diverge. This suggests that the intracellular signalling partners of the SynCAMs might converge, but that each SynCAM might be sensitive to specific adhesive cues. Accordingly, we focused first on the extracellular domains of SynCAMs to characterize their unique biochemical and cell biological properties.

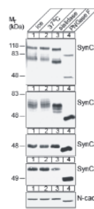




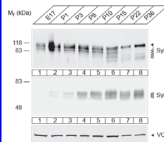
Results



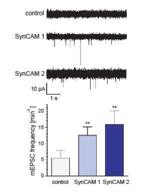
Isoform specific SynCAM antibodies. Antibodies were generated against non-conserved regions of each SynCAM, and purified against the corresponding antigen. Immunoblots of HEK lysate overexpressing each of the SynCAMs, control lysate, or brain lysate shows that each specific antibody recognizes a similar pattern of bands for overexpressed and endogenous SynCAM.



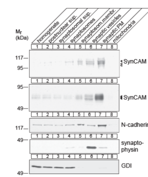
SynCAMs are modified by N-Glycosylation. Equal fractions of brain lysate treated under control conditions, with sialidase, or with PNGase F were analyzed by immunoblotting for each of the SynCAMs. SynCAMs 1 and 2 are both modified by sialic acid, whereas SynCAMs 3 and 4 are not (lane 3). Digestion with PNGase F removes all N-linked carbohydrate and reduces SynCAMs to the predicted molecular weight of 48 kDa, with the exception of SynCAM 1, which has an additional splice variant predicted to be O-glycosylated (lane 4).



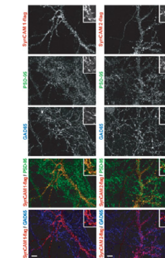
SynCAMs are expressed early in brain development. Equal total protein from brain lysates at the indicated developmental time points (Embryonic, Postnatal) were analyzed by immunoblotting for SynCAMs 1 and 2. SynCAM 2 expression rises in the first two weeks after birth, whereas SynCAM 1 levels are constant but appear to change in glycosylation.



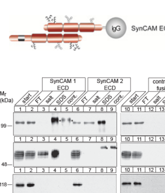
SynCAMs potentiate excitatory neurotransmission. mEPSCs were measured from hippocampal neurons overexpressing SynCAM 1, SynCAM 2, or untransfected control neurons. Overexpression of both SynCAMs 1 and 2 leads to a greater mini-frequency but not mini-amplitude (not shown), suggesting that the patched neurons have a greater number of active synapses. SynCAM 2 has a trend towards higher mini-frequency than SynCAM 1 (A. Krupp and V. Stein).



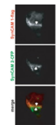
SynCAMs fractionate in synaptic plasma membranes. Equal total protein from the indicated fractions of a subcellular fractionation of rat brain were loaded and analyzed by immunoblotting. Both SynCAMs 1 and 2 enrich strongly in synaptic plasma membranes with the control marker protein N-Cadherin, while synaptophysin serves as a marker for synaptic vesicles and GDI as a marker for soluble fractions.



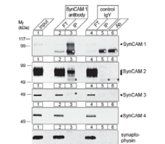
SynCAMs sort to synaptic sites in mature cultured neurons. Hippocampal neurons were transfected with flag-SynCAM constructs and stained at 21 D.I.V. for the synaptic markers PSD-95 and GAD 65. In mature neurons, overexpressed SynCAMs 1 and 2 are both punctate and co-localize significantly with excitatory synaptic markers and to a lesser extent with inhibitory synaptic markers. Overexpressed SynCAM 2 is somewhat more punctate/synaptic than SynCAM 1 (Quantitation not shown).



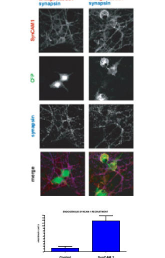
SynCAMs 1 and 2 engage in and prefer a heterophilic interaction. Affinity chromatography experiments were performed using the recombinant extracellular domains of SynCAMs 1 and 2. Total brain membranes were solubilized and passed over the extracellular domains of SynCAMs 1 and 2, and the bound proteins eluted sequentially with high salt (800 mM KAc) and sample buffer (2% SDS), and the fractions subjected to immunoblotting. We observed strong binding of SynCAMs 1 and 2 but no binding of homophilic interactions or control proteins. Asterisks mark background bands.



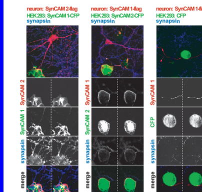
SynCAMs 1 and 2 colocalize at sites of cell-cell contact. HEK 293 cells expressing tagged SynCAM 1 or tagged SynCAM 2 were mixed and analyzed by fluorescence microscopy. SynCAMs 1 and 2 accumulate at sites of cell-cell contact in long adhesive structures.



SynCAMs 1 and 2 form a stable complex *in vivo*. Synaptic fractions were purified from rat brain, solubilized, and incubated with SynCAM 1 antibody. The resulting immunoprecipitate specifically contains SynCAM 2, but not other SynCAM family members or control proteins, suggesting a stable complex of SynCAMs 1 and 2 is formed *in vivo*. Asterisks mark cross-reactivity from IP antibody.

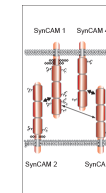


SynCAM 2 recruits endogenous SynCAM 1 in contacting neurons . HEK 293 cells expressing SynCAM 2-CFP or CFP alone were co-cultured with hippocampal neurons, and then stained for SynCAM 1 and synapsin. A significant ($p = 0.0043$) enrichment of SynCAM 1 is seen on the surface of SynCAM 2 cells but not control cells, suggesting that SynCAM 2 is able to actively recruit neuronal SynCAM 1.



Protein recruitment in the co-culture assay. Tagged SynCAMs 1 and 2 were overexpressed in neurons before co-culture with HEK expressing the cognate heterophilic partner or control. When a transfected neuron makes contact with a SynCAM expressing cell, we observed membrane expansion at the sites of contact with enrichment of the heterophilic partner at these sites, as well as synapsin. Thus, it appears heterophilic interaction of SynCAMs is sufficient to initiate contact with neuronal membranes to mobilize the cytoskeleton and synaptic machinery.

Discussion



SynCAMs are a family of four adhesion molecules expressed strongly during the major period of brain circuit development. Previous work has shown that SynCAM 1 can play an active role in synapse development, similar to activities described for neuroligins, EphB receptors, and Netrin G-Ligand. We have now developed tools to study each of the SynCAM isoforms. Our studies focus on SynCAMs 1 and 2, which we hypothesize form a synaptic adhesion complex with roles in synapse development and stabilization. We also observed an interaction between SynCAMs 3 and 4 (not shown here) which plays an important role in PNS myelination (Spiegel et al, 2007; Maurel et al, 2007).

In vitro, SynCAMs were first described as homophilic, but we now show that they prefer heterophilic interactions. SynCAMs 1 and 2 both fractionate with synaptic membranes and co-localize in culture with synaptic markers, suggesting a synaptic localization. SynCAM 1/2 complexes can be co-immunoprecipitated from synaptic fractions, suggesting the formation of a stable complex *in vivo*. Additionally, SynCAMs 1 and 2 are able to reciprocally recruit each other at sites of cell-cell contact between neurons and non-neuronal cells, which is likely the interaction necessary for effects in the co-culture assay. From this we conclude that SynCAMs 1 and 2 form an adhesive complex at central synapses, and that the arrival of one isoform might actively recruit the other to carry out synaptic function. The heterophilic interaction of SynCAMs is "pseudo-asymmetric", in that the extracellular domains have different ligands but the cytoplasmic domains likely share the same effector molecules. Therefore, it seems more likely that that asymmetry of a synapse is encoded by other adhesion receptors, such as neurexin-neuroligin or Ephrin-EphB interactions. Instead, the preference for heterophilic interactions by SynCAMs might help neurons avoid making unwanted long-lasting self-contacts, or could mediate specific contacts between distinct populations of neurons.

References

Akins, M.R., and Biederer, T. (2006). Cell-cell interactions in synaptogenesis. Curr. Opin. Neurobiol. 16, 83-89.

Biederer, T. (2006). Bioinformatic characterization of the SynCAM family of immunoglobulin-like domain-containing adhesion molecules. Genomics 87, 139-150.

Biederer, T., Sara, Y., Mozhayeva, M., Atasoy, D., Liu, X., Kavalali, E.T., and Sudhof, T.C. (2002). SynCAM, a synaptic adhesion molecule that drives synapse assembly. Science 297, 1525-1531.

Fogel, A., Akins, M. R., Krupp, A., Stagi, M., Stein, V. and Biederer, T. SynCAMs organize synapses through heterophilic adhesion. J. Neuroscience, October 31, 2007 27(44).

Lucic V, Yang T, Schweikert G, Forster F, Baumeister W (2005) Morphological characterization of molecular complexes present in the synaptic cleft. Structure (Camb) 13:423– 434.

Maurel, P, et al. Nectin-like proteins mediate axon Schwann cell interactions along the internode and are essential for myelination. J Cell Biol. 2007 Aug 27;178(5):861-74.

Spiegel, I, et al. A central role for Necl4 (SynCAM4) in Schwann cell-axon interaction and myelination. Nat Neurosci. 2007 Jul;10(7):86