Journal of Cellular Biochemistry 61:502–513 (1996)

Elucidation of the Molecular Actions of NCAM and Structurally Related Cell Adhesion Molecules

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Abstract The Neural Cell Adhesion Molecule (NCAM) is a founder member of a large family of cell surface glycoproteins that share structural motifs related to immunoglobulin and fibronectin type III (FN III) domains [Walsh and Doherty (1991) (Fig. 1). These glycoproteins have been grouped based on the respective number of each type of domain. In vertebrates members of this family of glycoproteins include L1/NILE, NgCAM, axonin-1/TAG-1, and Thy-1 as well as NCAM. In addition structural homologs of NCAM and L1 have been identified in *Drosophila* and Grasshoppers [*Walsh and Doherty* (1991)]. These insect homologs are called fasciclins and a series of mutants corresponding to these genes have been isolated. A homologue of NCAM has been identified in *Aplysia* where it may play a role in regulating aspects of synaptic plasticity [Mayford et al. (1992) Science 256:638–644]. In vertebrates all of these glycoproteins are expressed in the developing nervous system where they have been identified as candidate molecules for mediating axon outgrowth, fasciculation, regeneration, and target recognition. In addition, NCAM is expressed in a number of different tissues and cell types. For example, NCAM is expressed in a dynamic pattern in developing and regenerating adult muscle. In this review we aim to describe important aspects of the role of these CAMs in development of the nervous system, including the neuromuscular junction. Furthermore, we will explore the prospective use of molecular biology, cell biology, and molecular genetic techniques, such as transgenic mice, to understand the role and molecular action of this family of cell adhesion molecules *in vivo*.

Key words: NCAM, glycoproteins, nervous system, adhesion molecules

Actions of CAMs in the Nervous System

The functioning of the nervous system depends on the appropriate actions of distinct neural circuits [Purves and Lichtman, 1985]. These neural circuits function because the neurones comprising them are connected appropriately to each other. One of the central themes in Neurobiology has always been understanding the molecular mechanisms that regulate the establishment of this intricate pattern of appropriate neuronal connections during development because of its fundamental importance to the operation of the nervous system [Goodman and Shatz, 1993]. A key event in establishing neuronal connectivity is the guidance of the axon to its target via the action and orientation of the growth cone. Initial outgrowth of axons early in development is stereospecific and depends on receptor molecules in the growth cone recognising and transducing extrinsic cues in their environment that define a pathway. Axonal pathfinding probably requires the integration of a number of different cues stimulating various signal transduction pathways that can stimulate or inhibit axonal outgrowth [Dodd and Jessell, 1988; Doherty and Walsh, 1989; Lumsden and Cohen, 1991; Bixby and Harris, 1991; Doherty and Walsh, 1992; Rutishauser, 1993; Dodd and Schuchardt, 1995]. These pathway determining cues have been demonstrated to include neurotrophic and survival factors secreted by intermediate and final targets, components of the extracellular matrix e.g., laminin, and cell adhesion molecules of the immunoglobulin superfamily and cadherin family [Bixby and Harris, 1991]. In our studies we have focused largely on elucidating the mechanisms of axonal growth promoted by members of the immunoglobulin superfamily [Edelman, 1985; Bixby and Harris, 1991]. Direct evidence that these CAMs can act as a neurite outgrowth promoting ligand in a cellular substrata comes from studies where neu-

Received December 1, 1995; accepted December 7, 1995.

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rones were cultures on monolayers of control and transfected fibroblasts which express physiological levels of CAMs [Doherty and Walsh, 1992]. In addition, indirect evidence implicating these molecules in regulating neural development comes from in vivo studies where the function of the molecule has been perturbed by injection of a blocking antibody into developing embryos resulting in abnormal neuronal development [Rutishauser, 1993; Fisher et al., 1986; Fraser et al. 1988; Wood et al., 1990]. In addition to stimulating axon outgrowth CAMs have been implicated in regulating or promoting neuronal migration. Thus, CAMs of the immunoglobulin superfamily would appear to perform vital roles in the establishment of the architecture and connectivity of the nervous system.

Cell Adhesion Molecules Can Stimulate Neurite Outgrowth

The observations that different cellular substrates exhibit distinct differences in their ability to support neuronal survival and axon outgrowth suggested that sets of cell surface molecules could regulate aspects of axonal pathfinding [Bixby et al., 1987; Neugebauer et al., 1988]. The isolation and characterisation of nervous system expression of cell adhesion molecules of the immunoglobulin superfamily suggested that these molecules regulate axonal outgrowth during development. In general these proteins function via transhomophilic interactions between substrate cells and neurones [Doherty and Walsh, 1992; Rutishauser, 1993]. Direct evidence that these CAMs can act as a neurite outgrowth promoting ligand in a cellular substrata comes from studies where neurones were cultured on monolayers of control and transfected fibroblasts which express physiological levels of these CAMs [Doherty et al., 1989; Doherty et al., 1990a; Doherty et al., 1991a; Doherty et al., 1991b; Doherty et al., 1992; Gennarini et al., 1991; Williams et al., 1992; Liu et al., 1993]. As these molecules have been characterised biochemically and functionally as adhesive molecules these original studies interpreted the action of these molecules as mediating differential adhesion of the growth cone to the substrate. Although, this remains a possible mode of action of these molecules in the development of the nervous system subsequent studies implicated functions for these molecules other than simple cell adhesion. It was suggested from studies from neuronal cell cultures [Schuch et al., 1989; Doherty et al., 1991] and from *Drosophila* neurobiology [Elkins et al., 1990] that these cell adhesion molecules could promote development of axonal pathways by the activation of intracellular signal transduction cascades and second messengers.

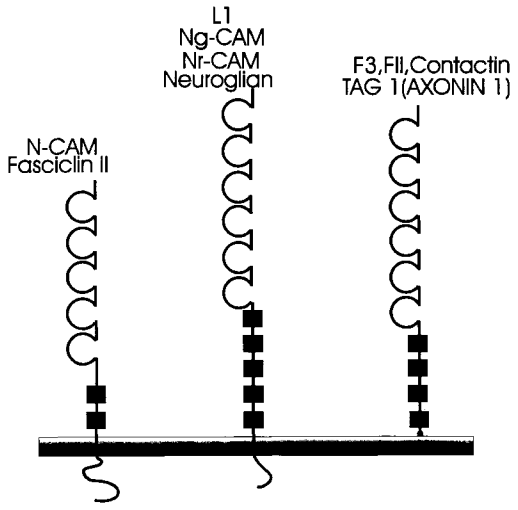

Fig. 1. Proposed structure of immunoglobulin superfamily members of cell adhesion molecules. A representation of some of the immunoglobulin-like proteins expressed in the nervous system and musculature. The molecules contain both immunoglobulin-like domains illustrated with the loops and FNIII like domains indicated as rectangles. [See Goodman and Shatz, 1993; Doherty et al., 1994 for details].

Second Messengers Mediate CAM Stimulated Neurite Outgrowth

Evidence from a series of experiments with bioassays of cultured neurones on CAM transfected 3T3 monolayers support the notion that CAMs may promote axonal development via activation of intracellular second messengers. We have found previously that removal of polysialic acid (PSA), which has been reported to increase adhesion [Hoffman and Edelman, 1983], from NCAM substantially inhibits NCAM dependent neurite outgrowth [Doherty et al., 1990b]. These data cannot be explained simply by an inverse relationship of neurite outgrowth to adhesiveness because we have observed positive cooperative and saturable dose response curves for NCAM level and neurite outgrowth [Doherty et al., 1990b; Doherty et al., 1992]. Furthermore, these data are not obviously consistent with PSA operating by inhibiting the trans-binding of NCAM, as would be expected to inhibit NCAM dependent neurite outgrowth. These data, however, do suggest that NCAM dependent neurite

outgrowth and adhesion can operate separately [Doherty and Walsh, 1992]. This can be explained by NCAM-dependent activation of a second messenger pathway rather than adhesion per se underlying neurite outgrowth response i.e., there is no direct correlation between the ability of NCAM to support adhesion and its ability to support neurite outgrowth. Therefore target recognition and signal transduction may be more important than adhesion in promoting axonal development stimulated by CAMs.

To test this notion a variety of agents were screened for their ability to selectively block CAM dependent neurite outgrowth. In summary, antagonists of L- and N-type calcium channels can inhibit CAM (NCAM, L1, and N-Cadherin 1) dependent neurite outgrowth in primary neurones and PC12 cells [Doherty et al., 1991a; Williams et al., 1992; Doherty et al., 1993]. This implicates a role for extracellular calcium in these processes and that a signal transduction cascade operates to open calcium channels. Further evidence for the involvement of calcium channels arises from the observations that potassium depolarisation of PC12 cells can fully mimic CAM-dependent neurite outgrowth which can be inhibited by N- and L-type calcium channel antagonists [Saffell et al., 1992]. In addition, a similar response could also be induced by the calcium channel agonist BAY K 8644. The major conclusion from these original studies implicated an essential role for calcium influx via activation of voltage sensitive channels into the growth cone as a result of the binding of CAMs. Such observations are consistent with the evidence implicating changes in the levels of intracellular calcium in regulating growth cone navigation [Kater and Mills, 1991; Silver et al., 1990].

Evidence for the Involvement of Protein Tyrosine Kinases in Neurite Outgrowth Promoted by CAMs

A number of observations have demonstrated that receptor and non-receptor tyrosine kinases appear to play a vital role in axonal growth and guidance. Soluble trophic factors such as NGF, BDNF, FGF, and other neurotrophins function by binding and activating receptor tyrosine kinases [Chao, 1992; Barbacid, 1994]. In addition transfection of neurones with constitutively active and inactivating (dominant negative) forms of cytoplasmic non-receptor tyrosine kinases have implicated these molecules in activating neurite outgrowth [Maness, 1992]. Pharmacological manipulation of neuronal cultures with inhibitors of protein kinases [Doherty et al., 1991a] and studies in *Drosophila* genetics [Elkins et al., 1990] were first in identifying a link between the binding of CAMs to neurones with the requirement of protein kinase activity in the responding neurone. This was intriguing since these CAMs do not have defined kinase domains in their sequence [Walsh and Doherty, 1991] and thus implicated an interaction with an effector molecule. Further investigation of the mechanisms mediating axonal outgrowth on fibroblasts expressing physiological levels of CAMs involved screening inhibitors of protein tyrosine kinases for their ability to selectively block neurite outgrowth promoted by CAMs expressed on the surface of fibroblasts. These studies demonstrated that neurite outgrowth stimulated by CAMs require the activity of a protein tyrosine kinase sensitive to inhibition by an analogue of erbstatin [Williams et al., 1994a]. In contrast, the kinase inhibitors lavendustin A and genistein failed to produce inhibition of neurite outgrowth promoted by CAMs at concentrations demonstrated to inhibit the activity of non-receptor protein tyrosine kinases. The experiments of Maness and collaborators [Ignelzi et al., 1994; Beggs et al., 1994] suggest that cell adhesion molecules such as L1 and NCAM activate neurite outgrowth by activation of non-receptor protein tyrosine kinases. In this case, each CAM has a corresponding specific tyrosine kinase e.g., NCAM specific for *fyn* and L1 specific for *src*. In these experiments, cerebellar granule cell neurones from mice in which the tyrosine kinases had been inactivated by use of homologous recombination were CAM expressing substrates. Although, some of the experimental paradigms used in measuring stimulation of neurite outgrowth are distinct from ours, i.e., purified L1 was coated onto a plastic substrate and NCAM was expressed in fibroblasts, it represents an intriguing finding. In experiments of this nature we are still measuring a requirement for the expression of these proteins. A loss of expression of these tyrosine kinases may result in the loss of expression or alteration of activity of other molecules that may be required for CAM mediated neurite outgrowth. In itself it does not represent evidence for activation of these tyrosine kinases by the CAMs. Furthermore, in vivo "knockouts" of these tyrosine kinases do not result in major alterations in the development of axonal projections. In the case of

fyn-mice there are alterations in development of the hippocampus and defects in LTP [Grant et al., 1992]. It is of interest, however, that null-mutants of NCAM also demonstrate defects in spatial learning and hippocampal anatomy [Cremer et al., 1994; Tomasiewicz et al., 1993]. In light of the latter observations it is possible that *fyn* may have a role in mediating aspects of plasticity requiring CAM mediated axonal growth, nevertheless, mutations in *src* do not exhibit a similar phenotype despite its apparent requirement for substrate bound L1 stimulated neurite outgrowth.

In searching for the putative effector tyrosine kinase that mediates CAM stimulated neurite outgrowth a clue to the potential nature of this tyrosine kinase came from experiments of Saffell et al. [1994]. In these experiments an isoform of NCAM containing the VASE sequence [Walsh and Doherty, 1992] was expressed in PC12 cells grown on fibroblasts expressing the non-VASE isoform. In these experiments the PC12 cells lost their ability to respond to NCAM in the cellular substrate. From previous studies it had been demonstrated that NCAM-VASE expressed as a substrate for neurite outgrowth was less supportive of outgrowth than non-VASE NCAM [Doherty et al., 1992a]. An interpretation of the loss of responsiveness to NCAM stimulated neurite outgrowth in PC12 cells expressing the NCAM-VASE was that the VASE sequence may inhibit interaction with the effector molecule and thus block its activation in cis. In screening a database of protein sequences it was discovered that the VASE sequence contained 10 amino acids in common with the FGFR family of receptor protein tyrosine kinases [Saffell et al., 1994] (Fig. 2). In addition, adjacent to the VASE homology sequence in the FGF receptor was the motif HAV [Blaschuk et al., 1990; Byers et al., 1992]. The HAV sequence is an amino acid motif contained within the extracellular domain of many cadherins and is thought to play a role in the homophilic binding of cadherins expressed in different cells. Nevertheless, it appears that the amino acids adjacent to the HAV sequence determine the specificity binding between the extracellular domains of cadherins. On further searches for homology a sequence between the third and fourth immunoglobulin-like domains of the cell adhesion molecule L1 was detected in the FGFR1 sequence containing the homologies to NCAM and N-cadherin (Fig. 2). This region in the FGF receptor has been called the CAM homology domain (CHD) [Williams et al., 1994b]. This sequence lies adjacent to the acid box common to FGF receptors and is well conserved between FGFR1 and FGFR2. The identification of this sequence led to the speculation that this may represent a site of interaction between neuronal FGF receptors and cell adhesion molecules which could lead to the activation of this tyrosine kinase.

This postulate was examined in several ways [Williams et al., 1994b]. Firstly, an antiserum to the acid box of the FGF receptor was tested for its ability to disrupt neurite outgrowth supported by NCAM, L1, and N-cadherin expressed in 3T3 cells. This antiserum demonstrated no detectable effect on basal, integrin mediated, neurite outgrowth on parental 3T3 monolayers. By contrast, on 3T3s expressing CAMs the anti FGF receptor acid box antibody completely inhibited the response to L1 and partially inhibited ($\sim$60%) the response to NCAM and N-cadherin. Secondly, an antiserum raised against the CAM homology domain peptide sequence from the FGF receptor fully and selectively inhibited the neurite outgrowth response stimulated by NCAM, L1, and N-cadherin. Furthermore, pretreatment of the neurones with the anti-CAM homology domain antibody prior to plating on the 3T3 monolayers expressing CAMs suggests that the inhibition observed resulted from inactivation of the FGF receptor in the responding neurone. Thirdly, peptides derived from the

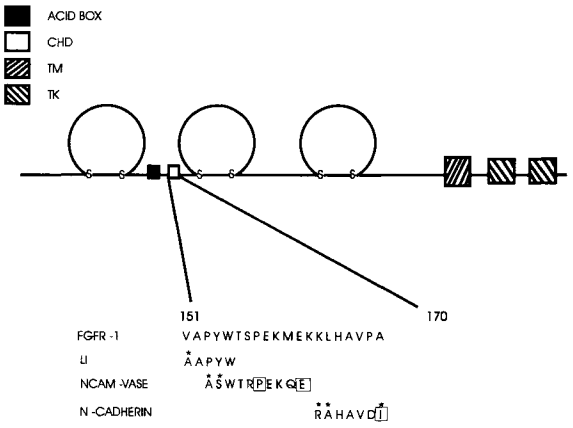

Fig. 2. The CAM homology domain (CHD) in FGF receptors. A schematic representation of the mouse FGFR1 showing the CAM homology domain (CHD) and regions of identity and similarity detected in L1, NCAM, and N-cadherin. An * indicates a conservative amino acid substitution, whereas a box indicates a non-conservative change. TM, transmembrane domain, TK, tyrosine kinase domains.

FGFR1 CAM homology domain selectively inhibit neurite outgrowth responses stimulated by CAMs. Addition of a 31-mer peptide containing the CHD blocked neurite outgrowth responses to NCAM, L1, and N-cadherin in a dose dependent manner. The basal outgrowth on 3T3 cells was unaffected by addition of this peptide and addition of a scrambled version of this sequence to cultures where 3T3 expressed CAMs had no effect on CAM stimulated neurite outgrowth. In addition, peptides corresponding to overlapping subdomains of this sequence selectively blocked CAM mediated neurite outgrowth. In this case, however, the peptides selectively inhibited the response corresponding to the cell adhesion molecule with which they exhibit most identity (Fig. 2). Similarly, peptides derived from the equivalent domains of FGF receptor 2 (FGFR2), which is also expressed in the nervous system, demonstrated equivalent responses in terms of their effectiveness and specificity for blockade of neurite outgrowth responses to CAMs. In addition, FGF was demonstrated to stimulate neurite outgrowth from cerebellar granule cells by utilising a second messenger pathway that was pharmacologically indistinguishable from that activated by CAMs expressed in 3T3s [Williams et al., 1994c] (see below). As with the CAMs antibodies to the acid box sequence and CAM homology domain, as well as peptides from the CAM homology domain, can inhibit neurite outgrowth stimulated by FGF. In addition, a peptide derived from basic FGF (KRTGQYKL), which has been shown previously to block FGF binding to its receptor, blocked neurite outgrowth promoted by FGF but had no effect on neurite outgrowth stimulated by CAMs. This suggests that there is some specificity in the apparent response of the FGF receptor to the CAMs and that neurite outgrowth stimulated by CAMs does not require addition of FGF. The effect of all of these blockade experiments can be alleviated by use of mimetics that activate downstream components of the pathway activated by CAMs, e.g., depolarisation by K^+, which suggests that the FGF receptor is important for a very early component in the signal transduction pathway used for CAMs in promoting neurite outgrowth.

Evidence to support the activation of FGF receptors by binding CAMs is provided by the use of recombinant CAM-Fc chimeras. These molecules represent fusions of the extracellular domain of cell adhesion molecules to the Fc portion of human immunoglobulin. When such fusions are transfected into fibroblastic cells large amounts of soluble dimeric proteins are secreted into the tissue culture supernatant which can be affinity purified by binding to and elution from columns bearing protein A [Simmons, 1993]. Addition of FGF or L1-Fc chimera for 1 h to cultures of cerebellar granule cells plated on polylysine resulted in the increase in tyrosine phosphorylation of a number of common cellular proteins when assayed by immunoblotting using anti-phosphotyrosine antibodies. This increase in apparent tyrosine phosphorylation of a common set of proteins as a result of FGF or L1 treatment could be inhibited by pretreatment of neurones with the antibodies to the CAM homology domain. Unlike previous data which suggest a requirement for tyrosine kinase activity for CAM stimulated neurite outgrowth this data is the first to provide evidence for actual activation of tyrosine kinases in neurones in response to addition of a cell adhesion molecule. Although no direct characterisation of the proteins phosphorylated was achieved these data are suggestive of activation of the FGF receptor by L1 because of the commonality of proteins phosphorylated. The results of subsequent experiments suggest that the soluble L1-Fc chimera could stimulate neurite outgrowth in cerebellar granule cells as effectively as FGF or L1 expressed in 3T3 cells [Doherty et al., 1995]. Furthermore, use of pharmacological tools that block components of the signal transduction pathway downstream of the FGF receptor (see below) also block neurite outgrowth promoted by soluble L1.

In summary, the above results suggest that CAMs may stimulate neurite outgrowth in neurones by activating FGF receptor tyrosine kinases. These results also establish that FGF receptors contain a possible recognition sequence, the CAM homology domain (CHD), which is important for the function of CAMs. One model suggested by these data for the mechanism of activation is illustrated in Figure 3. In this model the trans-homophilic binding of the CAM would lead to recruitment of CAMs at a point of cell-cell contact. This clustering of CAMs could lead to a coclustering of an effector molecule such as the FGF receptor. This could be aided by a direct or indirect binding of the CAM to the FGF receptor (cis/trans or both) [Doherty and Walsh, 1994]. In such models the peptides could function to block sites present on the

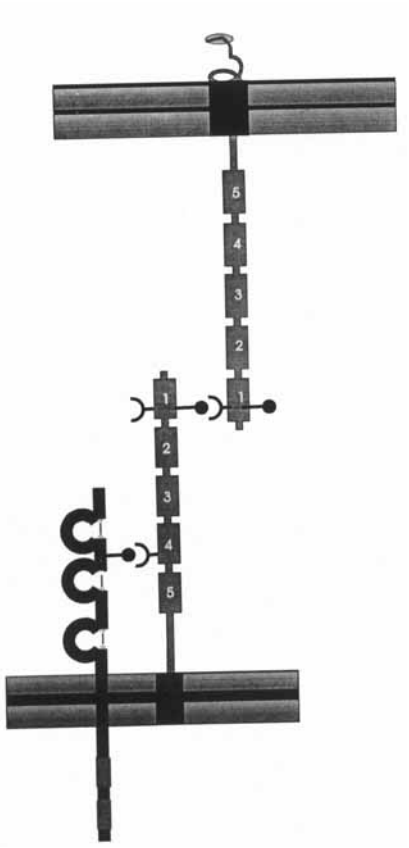

Fig. 3. Model proposed to explain activation of the FGF receptor by CAMs. A postulated model proposing the activation of the FGF receptor as a result of cis-interaction with a CAM on a neurone in response to trans-binding of the CAM with its counterpart in a substrate cell. Such an interaction has been proposed to produce a "cluster" of CAMs resulting in the "capture" of the FGF receptor, leading to its activation and signalling [See Doherty and Walsh, 1994].

CAMs rather than the FGF receptor. Nevertheless, the fact that these peptides also block FGF activation of the FGF receptor suggest that they do not merely function to block homophilic binding.

Relationship of Tyrosine Kinases to Activation of Downstream Pathway Events Involved in Transducing Signals From Cell Adhesion Molecules

The experiments outlined above demonstrate two components of the signal transduction pathway characterised in neurones as a response to stimulation by binding CAMs. Initial steps require FGFR activity and stimulation culminates in calcium influx into neurones [Doherty and Walsh, 1992]. A similar influx of calcium via voltage dependent calcium channels appears a requirement for neurite outgrowth promoted by FGF in cerebellar granule cells. A clue to the pathway of activation of these molecules has been derived from the use of the DAG lipase inhibitor RHC-80267. Addition of the DAG lipase inhibitor to neuronal cultures specifically inhibits neurite outgrowth stimulated by CAMs expressed in 3T3s, FGF, and L1-Fc chimeras. DAG lipase catalyses the conversion of diacylglycerols to fatty acids such as arachidonic and oleic acid. Furthermore, arachidonic and oleic acids have been identified as agents that can activate the opening of voltage dependent, calcium channels [Divecha and Divine, 1995]. Thus, mobilisation of these fatty acids in the neurone could provide a means for activation of calcium channels in response to CAM stimulation. In support of this, application of arachidonic or oleic acid to neuronal cultures can produce neurite outgrowth characteristic of that stimulated by CAMs [Williams et al., 1994d]. This observation is supported by experiments where addition of mellitin, which mobilises arachidonic acid in cells, promotes neurite outgrowth [Williams et al., 1994d]. An interesting observation was made in the study of FGF stimulation of neurite outgrowth in cerebellar granule cells. A biphasic dose response of neurite outgrowth to FGF application was observed. Neurite outgrowth was stimulated maximally by application of 5 ng/ml FGF to the culture, and the response declined at higher doses [Williams et al., 1994c]. This biphasic response, however, did not appear to result from a classical desensitisation of the FGF receptor as tyrosine phosphorylation of cellular proteins was apparent at all doses of FGF. A possible resolution of this apparent conundrum is provided by the observation that a biphasic neurite outgrowth response curve was also observed in application of arachidonic acid [Williams et al., 1994c]. The maximal outgrowth was observed at 10 μM arachidonic acid and a decline in responsiveness was observed at higher dose. This suggests that the signal transduction mechanism downstream of the FGF receptor becomes "desensitised" in response to CAM stimulation thereby restricting responsiveness in terms of neurite outgrowth.

The involvement of DAG lipase in the signal transduction pathway implicates phospholipase c γ (PLCγ) as an earlier event in the signal transduction pathway as arachidonic acid containing diacylglycerol (DAG) is one product of activated PLCγ hydrolysis of phospholipids with the other product of PLCγ activity being inositol

phosphates. One potential signal for the activation of PLCγ is the activation of FGF receptor, which when activated becomes autophosphorylated and can bind PLCγ via its SH2 domain [Schlessinger, 1994] and subsequently activate it by phosphorylation. A summary of these observations is illustrated in the pathway outlined in Figure 4. Thus the observations relating to identification of second messenger components mediating CAM binding to calcium mobilisation can fit the model for FGF receptor activation.

Role of NCAM in Development and Plasticity of the Neuromuscular Junction

It has been suggested that initial events of nerve muscle contact in culture require the participation of NCAM [Edelman 1983; Grumet et al., 1982; Rutishauser et al., 1983]. Muscle cells in culture have been found to bind vesicles reconstituted from purified NCAM and lipid [Grumet et al., 1982]. Furthermore, adhesion between chick embryo spinal cord cells and muscle cells in culture can be inhibited by monovalent Fab fragments of anti-NCAM antibodies [Rutishauser et al., 1983]. This led to the proposal that NCAM might mediate initial adhesive contacts between nerve and muscle during synapse development.

In the chick at stages 17–22 of embryogenesis approximately 20,000 motor neurones send their intermingled axons to different target muscles through eight separate spinal nerves. On reaching the cranial and spinal plexus, these axons defasciculate and wait for a period of approximately 24 h before entering the limb bud. Subsequently, these axons diverge from the main nerve trunk as tightly fasciculated muscle nerves which defasciculate upon reaching their target muscles. This highly organised behaviour indicates the presence of a number of guidance cues. A series

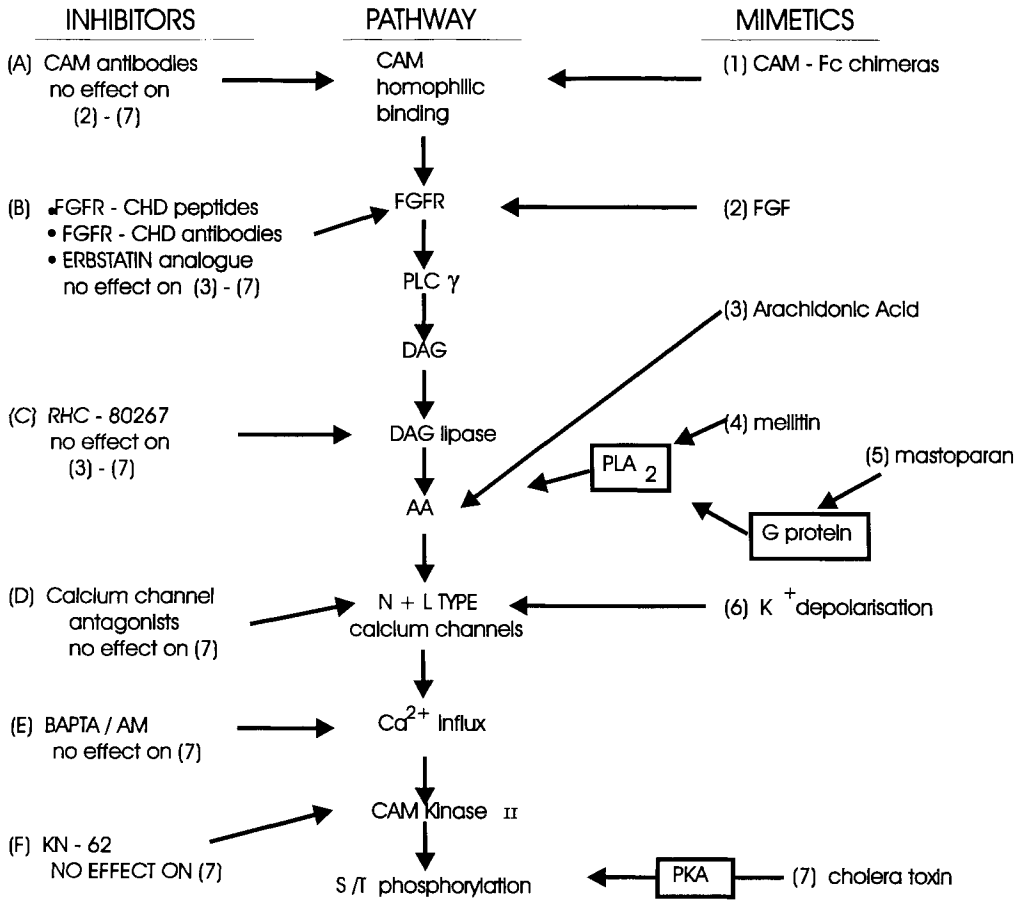

Fig. 4. Summary of signal transduction pathway activated by CAMs to stimulate neurite outgrowth. A linear representation of the steps proposed to be involved in the CAM second messenger pathway. The central column lists the steps involved in this pathway as determined by specific inhibitors and mimetics. Points A–F denote the specific inhibitors, agents able to activate the pathway are denoted as 1–7.

of experimental perturbations have led to the hypothesis that in the chick limb a dual system of axon guidance operates [Lance-Jones and Landmesser, 1980]. A relatively non-specific set of 'public' pathways which support the stereotyped nerve pattern determined in the plexus region and a second set of cues allowing subpopulations of axons to select their individual target. As axons can make correct and specific decisions in the plexus region in the absence of their final target muscles, it appears that these do not play a major role in the initial guidance. Intercellular interactions involving CAM mediate adhesion between axons, and between axons and muscles play a prominent role in the growth and ramification of axons within the muscle masses [Tosney and Landmesser, 1984; Landmesser et al., 1988].

Over 50% of motor neurones projecting to muscle die within a short time, in the chick between stages 28–36. Competition for soluble trophic factors supplied by the muscle is thought to be the basis for this cell death. The enmasse cell death occurring at this stage is thought to act as a mechanism by which the number of motor neurones is matched to the size of the target muscle. Whereas the ability of muscle to promote the survival of motor neurones is thought to depend on soluble trophic factors, CAMs are thought to play an important part in the interactions leading to axonal branching and synaptogenesis. Due to the close apposition of the nerve and muscle membranes (<10 nm) CAMs could provide the first signal for the establishment of a synapse. This notion is in agreement with the speed with which growth cones show changes in their morphology and function, with a rapid increase in presynaptic calcium levels and transmitter release [Xie and Poo, 1986; Sun and Poo, 1987]. Since increased transmitter release from growth cones can be observed in response to contact with an isolated patch of muscle membrane [Xie and Poo, 1986] it is possible that CAMs participate in the establishment of rudimentary synapses.

A further role for CAMs in the process of innervation and synaptogenesis is indicated by denervation studies in adult muscle. When a foreign nerve is implanted into an innervated muscle, although some degree of axonal growth does occur from the cut end of the nerve, no new synapses form. Once the host muscle is denervated, however, massive axonal growth occurs and synapses are formed at the original junctions. New synapses can also form, however there is a spatial constraint on their position and they appear to only formed more that 1mm away from the original sites. NCAM is re-expressed on the surface of muscles in response to denervation and is therefore a candidate for the increased growth of axons from the cut end of the foreign nerve. Intact motor axons often show a sprouting response following reinnervation or pharmacologically induced paralysis. This sprouting is assumed to be part of the process of reinnervation. As well as re-expression following axotomy, NCAM is also found on myofibres following paralysis. A few days after paralysis induced by the injection of Botulinum toxin into muscle terminal sprouting is observed in parallel with the increase in NCAM expression. This sprouting has been shown to be completely inhibited in the presence of antibodies to NCAM, thus indicating a direct role for NCAM in this process [Booth et al., 1990]. Nevertheless, a paradox does exist in implicating CAMs in neuromuscular interactions. The basal lamina which envelops myotubes also separates nerve membranes from muscle membranes. Furthermore, the basal lamina itself has been implicated in regulating neuromuscular interactions during development and regeneration.

The Function of CAMs in Vertebrates Investigated by use of Homologous Recombination and Transgenic Mice

In addition to transfection of cells and manipulation of cultured cells with blocking antibodies and inhibitory molecules, such as peptides, the evolution of transgenic mouse technology is providing new avenues of inspection for the function of cell adhesion molecules during vertebrate development. In particular, the generation of null mutants by gene specific targeting using homologous recombination in ES cells has generated mice deficient in the expression of the NCAM gene. In the case of NCAM, two approaches were adopted to inactivate its expression within the animal. These were to generate a complete null mutant [Cremer et al., 1994] and to generate the selective loss of the 180kd polysialylated isoform [Tomasiewicz et al., 1993], which is predominantly expressed in the nervous system. The most obvious phenotype was observed in the brain, which in both cases was a marked reduction of size of the olfactory bulb. In the olfactory bulb the granule cells were disorganised and reduced in number. The precur-

sors of these cells were detected at their origin in the subependymal zone at the lateral vesicle, which is consistent for a role for NCAM in certain forms of neuronal migration. This is supported by data where the enzymatic removal of polysialic acid from NCAM phenocopied the effect of the knockout [Ono et al., 1995]. In addition, there was an overall reduction in brain size and some minor defects such as in organisation of neurones in the retina, hippocampus, and cerebellum of the brain. In addition, the mice generated as a result of introducing the null mutation exhibited deficits in spatial learning [Cremer et al., 1994]. This observation is made even more intriguing as a result of the observations made in *Aplysia* where changes in the formation of synaptic connectivity is correlated with neurotransmitter mediated down regulation of apCAM on the presynaptic sensory neurone [Mayford et al., 1992]. This suggests that CAMs may play a subtle role in regulating aspects of synaptic plasticity and regeneration involved in remodelling the nervous system. Interestingly, despite the complex expression pattern of NCAM the initial analyses did not describe other phenotypes associated with other tissues such as muscle or the peripheral nervous system. These observations suggest that either CAMs have a very discrete function revealed only by fine analysis or that their is some redundancy or "rescue" of function by other molecules. There are several avenues where such observations can be pursued. Firstly, by examining the phenotypes generated by knockouts of such molecules as L1, Thy-1, or Axonin 1/TAG-1. It is possible that knockouts of these molecules on their own produce only very subtle or mild phenotypes. It is possible that other cell adhesion molecules become upregulated to replace them functionally. Some controversial evidence supporting this notion already exists for knockouts of myelin associated glycoprotein (MAG) [Li et al., 1994; Montag et al., 1994]. In one report of a MAG knockout, NCAM levels become upregulated and postulated to replace MAG functionally in stimulating axonal development. It is possible that the true functions of these CAMs, which appear functionally similar, may only be revealed in double knockout combinations. In addition, since the expression patterns of these molecules are highly dynamic during development tissue specific dominant expression of inappropriate isoforms or mutants incapable of providing CAM function (dominant inhibitory) may be more appropriate to understand the role of these molecules in development. This may be particularly true in the case of understanding the actions of CAMs in muscle development. In addition, generating mutants in signal transduction pathways associated with CAM function may yield information associated with their role in vivo.

We have proposed that cell adhesion molecules such as NCAM, L1, and N-cadherin can stimulate neurite outgrowth by cis-activation of a FGF receptor protein tyrosine kinase in a neurone in response to transhomophilic binding or binding an appropriate ligand on a substrate cell [Williams et al., 1994b]. In contrast to the work of Maness et al. [Ignelizi et al., 1994; Beggs et al., 1994] this represents activation of a common signalling mechanism by quite distinct ligands. The experiments involving "gene specific" inhibitions does demonstrate a clear approach to test the postulate that FGF receptors are required to mediate CAM stimulated axon outgrowth. In contrast, to non-receptor tyrosine kinases germ-line null mutations in FGFR1 results in early embryonic lethality [Deng et al., 1994; Yamaguchi et al., 1994] therefore alternative approaches must be developed to examine its role in axonal development. The clearest approach is to use the strategy of expressing "dominant negative" mutants of the FGF receptor [Amaya et al., 1991] in cultured neurones and also specifically in the nervous system of transgenic mice to specifically inactivate FGF receptor function. This approach will test the postulate of requirement of the FGF receptor for CAM mediated outgrowth in cell culture and its importance as a molecular mechanism determining axonal outgrowth during development.

Recent experiments suggest that some types of human mental retardation and brain abnormalities may be due to mutations that affect L1 [Wong et al., 1995]. The gene for both humans and mice is located on the X-chromosome and has been mapped to band Xq28 in humans. It is well established that L1 exhibits complex interactions with other cell surface molecules in addition to its predominant trans-homophilic mechanism of adhesion and plays multiple roles in stimulating axon outgrowth, neuronal migration, and axon fasciculation. A series of recessive X-chromosome linked mutations have been mapped to Xq28 which result in the absence or diminution of the corticospinal tract as well as

absence of the corpus callosum and fusions of various structures in the brain. Three syndromes are characteristic of mutations associated with Xq28, these being X-linked hydrocephalus (HSAS), MASA syndrome, and X-linked paraplegia. Isolation of human cDNAs encoding L1 from brains of affected individuals led to the identification of a series of mutations in L1 which would lead to the expression of mutant L1 proteins. Clearly an impediment to establishing the direct influences of the L1 mutations in these syndromes is the difficulty of obtaining brain samples. A key step to characterise the actions of these L1 mutants in vivo is to phenocopy these L1 mutations in transgenic mice. This would not only establish these mutations as the basis of these syndromes, but may provide systems in which to investigate possible therapies including gene therapy.

Further Future Prospects

Further tests postulate that CAMs stimulate neurite outgrowth by activation of the FGF receptor involve demonstrating that CAMs as well as FGF can bind directly to the FGF receptor. Such an analysis must include a number of CAMs, in particular N-cadherin since other effector molecules, the catenins, have been identified as transducers of extracellular signals to the cellular cytoskeleton [Ranscht, 1994] and there is some evidence implicating an interaction of L1 with ankyrin [Davis and Bennett, 1994]. Also, as a consequence of this that the FGF receptor itself becomes phosphorylated and that enzymes such as PLCγ become phosphorylated and activated to produce second messenger molecules such as arachidonic acid and inositol phosphates. It is clear that the production of pure soluble chimeric CAMs that can mimic stimulation of neurite outgrowth promoted by CAMs expressed in 3T3s are invaluable tools making these experiments feasible. To date, our evidence implicates the activation of PLCγ by the FGF receptor as sufficient and necessary to transduce signals promoting neurite outgrowth as a result of binding CAMs. These observations raise intriguing questions concerning the potential nature of signalling from the FGF receptor by CAMs and FGF in neurones. Firstly, is the mechanism for activation of the FGF receptor the same in each case? Is there a requirement for dimerisation or can the tyrosine kinase domain be activated via another mechanism? Secondly, there is a great deal of evidence suggesting that p21 ras and MAP kinases are activated by receptor tyrosine kinases, including FGF receptors, in neurones to promote neuronal differentiation [Marshall, 1995]. It will be of great interest to determine whether such a pathway is activated by or required for CAM mediated axonal outgrowth. It is possible that this may vary between different neuronal types and mechanisms of activation of the FGF receptor.

Finally, the use of transgenic mouse technologies may be used to unravel the actions of CAMs in development and regeneration of the neuromuscular system by use of targeted expression of mutant proteins to the muscle.

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