A short segment within the cytoplasmic domain of the neural cell adhesion molecule (N-CAM) is essential for N-CAM-induced NF-κB activity in astrocytes

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The neural cell adhesion molecule (N-CAM) is expressed on the surface of astrocytes, where its homophilic binding leads to the activation of the transcription factor NF-κB. Transfection of astrocytes with a construct encompassing the transmembrane region and the cytoplasmic domain of N-CAM (designated Tm-Cyto, amino acids 685–839 in the full-length molecule) inhibited this activation up to 40%, and inhibited N-CAM-induced translocation of NF-κB to the nucleus. N-CAM also activated NF-κB in astrocytes from N-CAM knockout mice, presumably through binding to a heterophile. This activation, however, was not blocked by Tm-Cyto expression, indicating that the inhibitory effect of the Tm-Cyto construct is specific for cell surface N-CAM. Deletions and point mutations of the cytoplasmic portion of the Tm-Cyto construct indicated that the region between amino acids 780 and 800 were essential for inhibitory activity. This region contains four threonines (788, 793, 794, and 797). Mutation to alanine of T788, T794, or T797, but not T793, abolished inhibitory activity, as did mutation of T788 or T797 to aspartic acid. A Tm-Cyto construct with T794 mutated to aspartic acid retained inhibitory activity but did not itself induce a constitutive NF-κB response. This result suggests that phosphorylation of T794 may be necessary but is not the triggering event. Overall, these findings define a short segment of the N-CAM cytoplasmic domain that is critical for N-CAM-induced activation of NF-κB and may be important in other N-CAM-mediated signaling.

In the past several years, a number of cell adhesion molecules (CAMs) have been described and have been shown to play significant roles in the development and the formation of the three-dimensional structure and normal function of tissues in whole organisms (1, 2). Among the many CAMs are a large variety of Ig-like CAMs that contain several Ig-like domains and typically bind cells to each other in a homophilic fashion, i.e., a CAM on one cell binds the same CAM on another cell. Whereas the adhesive properties of CAMs have been well studied, less is known about their signaling properties, which may be equally, if not more, important.

Neural cell adhesion molecule (N-CAM), the first CAM to be characterized (3), mediates homophilic binding (4–6) and initiates signals that lead to changes in gene expression (7–9). This protein has a number of differentially spliced forms, including two transmembrane forms (sd and ld) and a glycosylphosphatidylinositol-linked form (ssd) (10, 11). After translation, N-CAM molecules can be modified in a number of ways, including N-linked polysialylation (12), phosphorylation on multiple intracellular serine and threonine residues (13), and palmitoylation of cytosolic cysteines (14, 15).

It is now clear that, on binding, CAMs transduce signals across the plasma membrane that regulate cell function (see ref. 2 for review). In particular, N-CAM binding promotes neurite outgrowth (16, 17), inhibits astrocyte proliferation *in vitro* and *in vivo* (18, 19), and activates the NF-κB transcription factor and the glucocorticoid receptor (7, 9, 20). Several models have been proposed describing possible mechanisms of N-CAM signaling. One suggests that cis interactions between the extracellular domains of N-CAM and a CAM homology domain in the fibroblast growth factor (FGF) receptor are responsible for CAM-mediated neurite outgrowth (16, 21, 22). Another model proposes that the cytoplasmic domain of the sd isoform of N-CAM associates with $p59^{fyn}$, a member of the src family of tyrosine kinases, and can associate with the focal adhesion kinase $p125^{fak}$ in a signal-dependent fashion (23). However, neither of these proposed pathways appears to play a major role in N-CAM signaling via NF-κB activation (24).

NF-κB is a heterodimer, typically consisting of p50 and p65 subunits, that was first found to stimulate transcription in B lymphocytes of the Ig κ light chain (25). It is normally present in the cytoplasm bound to a third subunit termed IκB, which keeps the protein in a latent form by masking a nuclear localization signal. NF-κB can be activated by several cytokines, by neurotrophic factors, and after various kinds of cell stress (reviewed in ref. 26). These factors stimulate the phosphorylation and subsequent degradation of IκB, which allows nuclear translocation of NF-κB. Recently, NF-κB has been demonstrated to play a role in the regulation of anti-apoptotic gene expression and the promotion of cell survival (27), and it may be involved in neuronal survival during development (28), as well as in synaptic plasticity (reviewed in ref. 29).

Previous studies in our laboratory have shown that treatment of astrocytes with N-CAM led to IκB degradation, followed by increased NF-κB-mediated transcription. Preventing IκB phosphorylation by overexpression of a non-phosphorylatable IκB blocked the ability of N-CAM to activate NF-κB (7). Other studies have shown that NF-κB can be activated in glial cells by various cytokines, including IL-1β and tumor necrosis factor (TNF)-α (30–34). Kainate-induced toxicity resulted in increased NF-κB expression in reactive astrocytes *in vivo* (35), suggesting that elevated levels of NF-κB are a response of astrocytes to injury. These findings indicate that, in response to injury or disease of the nervous system, N-CAM may share some signaling mechanisms with other ligands that activate NF-κB in astrocytes.

In the present study, we show that expression of an N-CAM cytoplasmic domain construct (Tm-Cyto) in normal astrocytes partially blocks the signal transduction pathway leading to

Abbreviations: N-CAM, neural cell adhesion molecule; CAM, cell adhesion molecule; LPS, lipopolysaccharide; GFP, green fluorescent protein; TNF, tumor necrosis factor.

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www.pnas.org/cgi/doi/10.1073/pnas.041597098

NF-κB activation, presumably through a dominant negative effect on the endogenous N-CAM. Inhibition was not observed in primary astrocytes from N-CAM-null mice nor in cells stimulated with other ligands, such as cytokines, indicating that it was specific for N-CAM-mediated NF-κB activation. By expressing several truncation constructs of the N-CAM cytoplasmic domain, we localized a small region in the cytoplasmic domain that is necessary for N-CAM-induced NF-κB activation. The sequence in and around this region contains four threonines, three of which appear to be critical for the transduction of the N-CAM binding signal across the plasma membrane, and at least one of which may be phosphorylated.

Materials and Methods

DNA Constructs. The plasmid pcDNA3.1$^+$ was purchased from Invitrogen, and the constructs pEGFP-N2, pEGFP-C1, and pCMV-β-gal were purchased from CLONTECH. The NF-κB-luc reporter construct (36) was a generous gift from Mercedes Rincon (Immunology Program, University of Vermont, Burlington). pcDNA-Tm-Cyto and its palmitoylation-deficient mutant were subcloned from rat N-CAM cDNA (15). The cDNA encoding Tm-Cyto was subcloned into the *Bam*HI and *Hin*dIII sites of pEGFP-N2 and pEGFP-C1 by using standard methods. Truncation constructs and point mutants of pcDNA-Tm-Cyto were generated with the QuikChange *in vitro* Mutagenesis kit (Stratagene) by using the mutagenic oligonucleotides indicated in supplemental Table 1, which is published as supplemental data on the PNAS web site, www.pnas.org.

Transcriptional Activation Assays. Primary cultures of astrocytes were obtained from the forebrains of postnatal day 3–4 rats as described (19) and from the forebrains of postnatal day 2–3 N-CAM-null mice (37). Primary astrocytes were transfected via electroporation after 7–10 days *in vitro* as previously described (9) with either 5 μg of pcDNA3.1$^+$ or 5 μg of the cytoplasmic domain construct being tested, as well as 5 μg of NF-κB-luc, and 5 μg of CMV-β-gal by using a Gene Pulser electroporator (Bio-Rad). After electroporation, 10^5 cells per well were plated on 24-well culture plates and incubated overnight. The medium was changed to serum-free DMEM for 24 h before treatment. Cells were treated with a number of different reagents: 100 μg/ml purified N-CAM (19), or 100 μg/ml recombinant IgIII (5), or 1 ng/ml IL-1β, or 1 ng/ml TNF-α, or 20 μg/ml lipopolysaccharide (LPS) for 7 hr. The cells were harvested and assayed for luciferase activity (9). β-galactosidase activity was measured by using a FluoReporter lacZ/galactosidase kit from Molecular Probes in a CytoFluor 2450 fluorescence measurement system (Millipore). The luciferase activity was normalized with respect to the β-galactosidase activity to compensate for differences in transfection efficiency.

Immunoblot Analysis. Separation and detection of specific proteins were performed as described (15), except that an N-CAM cytoplasmic domain-specific polyclonal antibody was used to detect N-CAM and the Tm-Cyto constructs. Green fluorescent protein (GFP)-tagged constructs were detected by using a monoclonal anti-GFP antibody (CLONTECH). Specific bands were visualized by using the Renaissance Western kit (NEN) after incubation with an horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Roche Molecular Biochemicals) or an HRP-sheep anti-mouse secondary antibody (Sigma-Aldrich).

Cell Staining. Primary astrocytes were electroporated with 5 μg of either pEGFP-N2 or the appropriate Tm-Cyto-GFP construct and plated on 8-well Biocoat glass slides precoated with polylysine (Becton Dickinson; 10^4 cells per well). After overnight incubation, the medium was replaced with serum-free DMEM for 24 h. The cells were then either mock-treated with PBS or treated with 1 ng/ml TNF-α or 100 μg/ml IgIII for 2 hr, after which they were fixed and stained as described (24). NF-κB was visualized with rabbit anti-p65-A antibody (Santa Cruz Biotechnology) at a concentration of 1:100, followed by Alexa-Fluor 594 conjugated secondary antibody (Molecular Probes). GFP expression (508 nm) and immunofluorescence (605 nm) were examined in the same field.

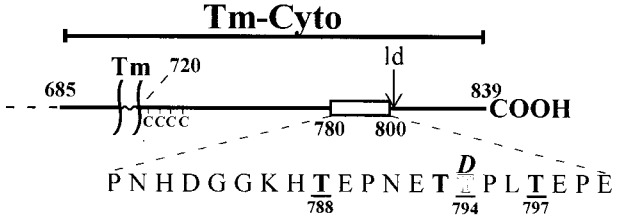

Fig. 1. Schematic drawing of the Tm-Cyto construct. The plasma membrane is represented by the pair of vertical lines. The 21-amino-acid region found to be important in N-CAM signaling is shown as an open box. The four threonines within this region are shown in bold. Underlined residues represent those whose substitution with alanine abolished the inhibitory activity of Tm-Cyto. Substitution of the threonine at position 794 (shown in outline) with aspartate (shown in italics) allows Tm-Cyto-mediated inhibition. The cytoplasmic cysteines (C) and the site of the ld insertion in the cytoplasmic domain are also indicated.

Results

Expression of the Cytoplasmic Domain of the N-CAM Molecule in Astrocytes Partially Inhibits N-CAM-Induced NF-κB Activation. To determine the region of the N-CAM molecule responsible for transducing a signal within the cytoplasm, we generated a construct, termed Tm-Cyto, that we suspected might have a dominant negative effect on N-CAM signaling. This protein includes 17 residues from the extracellular region and all of the cytoplasmic and transmembrane regions of the sd form of rat N-CAM (residues 685–839, GenBank accession number X06564; Fig. 1). Previous experiments had shown that the inclusion of the transmembrane domain and extracellular amino acids gave greater expression of the protein than the cytoplasmic region alone, presumably because the protein was more stable (data not shown). *In vivo*, the four cytoplasmic cysteines (Fig. 1) can be acylated with palmitate, and this modification is important for proper membrane association (15). To test the influence of fatty acid acylation on N-CAM signaling, we also used a palmitoylation-deficient Tm-Cyto molecule (Tm-Cyto/palm$^-$), in which the four cysteines were mutated to serines (15).

Constructs expressing either form of Tm-Cyto were transfected into rat astrocytes, and their expression was confirmed by immunoblotting a protein product that migrates at ≈45 kDa (Fig. 2*A*), a size comparable to those observed for other N-CAM cytoplasmic domain constructs (15). The weaker band at a lower molecular mass is probably a degradation product and was more pronounced with the Tm-Cyto/palm$^-$ construct. The Tm-Cyto construct contains the cytoplasmic region from the sd form of N-CAM and, as demonstrated by the band at 140 kDa (Fig. 2*A*), this form of N-CAM is endogenously expressed in astrocytes. The antibody used for immunoblotting recognizes the sd cytoplasmic domain, and thus the intensity of the immunoblots reflects the level of overexpression of the transfected construct relative to the level of endogenous transmembrane N-CAM.

Transfected astrocytes were treated with purified full-length N-CAM, the recombinant third Ig domain of N-CAM (IgIII), or other known NF-κB-activating reagents, including IL-1β, TNF-α, and LPS. A typical result of luciferase activation in response to these treatments is shown in Fig. 2*B*. The basal level of luciferase activity in untreated cells was similar in all trans-

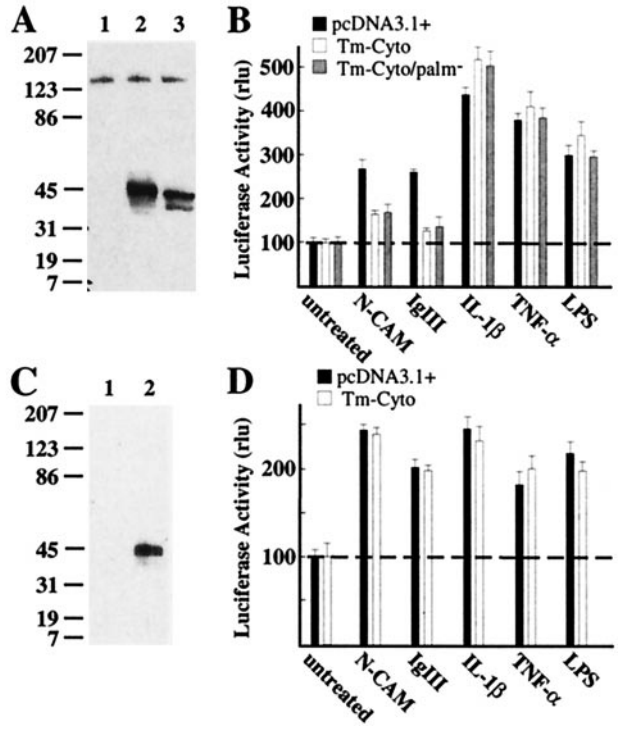

Fig. 2. Expression of Tm-Cyto inhibits the N-CAM-induced NF-κB activation in transfected primary wild-type astrocytes but not in N-CAM-null cells. (*A*) Immunoblot of astrocytes transfected with pcDNA3.1$^+$ (lane 1), pcDNA-Tm-Cyto (lane 2), or pcDNA-Tm-Cyto/palm$^-$ (lane 3). Upper bands (140 kDa) are endogenous sd N-CAM; lower bands are the Tm-Cyto proteins. (*B*) The transfected cells were treated and assayed as described in *Materials and Methods*. The results are shown as the ratio of the luciferase activity normalized to the β-galactosidase activity for each treatment condition. The error bars represent the standard deviation from quadruplicate samples of a representative experiment. Each experiment was replicated a minimum of four times. (*C*) Immunoblot of N-CAM knockout astrocytes transfected with pcDNA3.1$^+$ (lane 1) or pcDNA-Tm-Cyto (lane 2). (*D*) Luciferase assay in transfected knock-out astrocytes. The results are shown as described above.

fected cell lines and was normalized to 100% for comparison of effectiveness of the treatments. N-CAM and IgIII elicited comparable increases in pcDNA3-transfected control cells, and the level of induction of luciferase activity was reduced by approximately 35%–40% in the Tm-Cyto and Tm-Cyto/palm$^-$ expressing cells. In contrast, induction of NF-κB by IL-1β, TNF-α, and LPS gave the same response in control astrocytes and in astrocytes expressing either form of Tm-Cyto. These results demonstrate that the inhibitory activity of Tm-Cyto is specific for N-CAM- and IgIII-induced activation of NF-κB and is independent of palmitoylation.

Astrocytes from N-CAM-null mice (37) were also tested for NF-κB activation by N-CAM and IgIII. Astrocytes from N-CAM-null mice were transfected with either the empty expression vector pcDNA3.1$^+$ or Tm-Cyto. Immunoblot analysis demonstrated the expression of Tm-Cyto in these cells and lack of endogenous N-CAM (Fig. 2*C*). These cells were treated with the NF-κB-activating reagents listed above, and the results are shown in Fig. 2*D*. Despite the absence of N-CAM in these cells, addition of N-CAM or IgIII, as well as IL-1β, TNF-α, and LPS, induced NF-κB activation, but in no case did expression of the Tm-Cyto construct block NF-κB activation. These results are in accord with the hypothesis that a heterophilic receptor specific for N-CAM or IgIII is present on these cells that is capable of stimulating NF-κB activation, but this putative receptor uses a different proximal pathway than cell surface N-CAM. This receptor may also be present on astrocytes from normal mice, which would explain why the inhibition caused by Tm-Cyto is consistently 35%–40%; i.e., the uninhibitable NF-κB activity is presumably due to stimulation via the heterophile.

Expression of Tm-Cyto Blocks the N-CAM Signaling Pathway Before NF-κB Translocation to the Nucleus. Activation of NF-κB target genes requires the translocation of NF-κB from the cytosol to the nucleus, and previous experiments showed that N-CAM reagents stimulated NF-κB translocation to the astrocyte nucleus (25). To determine whether Tm-Cyto expression inhibits N-CAM-mediated NF-κB translocation, astrocytes were transfected with either a construct expressing a GFP-tagged form of Tm-Cyto (Tm-Cyto-GFP) or with a construct expressing GFP (pEGFP-N2) as control. This approach allowed direct visualization of transfected cells via GFP fluorescence (Fig. 3 *a*, *c*, *e*, *g*, *i*, and *k*) and comparison of NF-κB localization in transfected and untransfected cells via immunostaining (Fig. 3 *b*, *d*, *f*, *h*, *j*, and *l*).

Staining for the p65 subunit of NF-κB in untreated astrocytes revealed almost complete cytosolic localization (Fig. 3 *b* and *d*) in cells transfected with pEGFP-N2 or with Tm-Cyto-GFP. After IgIII treatment, astrocytes expressing pEGFP-N2 showed NF-κB nuclear translocation comparable to untransfected cells (Fig. 3 *e* and *f*). In contrast, astrocytes expressing Tm-Cyto-GFP showed strong p65 staining in the cytoplasm, with little p65 localization to the nucleus after treatment with IgIII (Fig. 3 *g* and *h*, straight arrows). An untransfected astrocyte in the same field shows strong p65 staining in the nucleus (Fig. 3*h*, crooked arrow). Treatment with TNF-α of either pEGFP-N2- or Tm-Cyto-GFP-transfected cells resulted in near total nuclear localization of p65 (Fig. 3 *i*–*l*). Similar results were seen when the astrocytes were transfected with the palmitoylation-deficient Tm-Cyto-GFP construct (data not shown). These results indicated that the expression of the Tm-Cyto protein inhibited N-CAM-induced, but not cytokine-induced, signal transduction by blocking NF-κB localization to the nucleus.

A Short Region (Amino Acids 780–790) Is Required for Tm-Cyto To Inhibit N-CAM-Induced NF-κB Activation. To identify the region of Tm-Cyto that is important for its signaling, *in vitro* mutagenesis was used to generate several truncation mutants of Tm-Cyto. These truncation constructs included Tm-Cyto Δ824–839, Tm-Cyto Δ801–839, Tm-Cyto Δ791–839, Tm-Cyto Δ780–839, and Tm-Cyto Δ745–839 (Figs. 1 and 4*A*). All constructs were detected by immunoblot assay of transfected astrocytes, with the exception of Tm-Cyto Δ745–839, which presumably lacked the epitopes recognized by the antibody. To demonstrate expression of this protein, an N-terminal GFP-tagged version (GFP-Δ745–839) was made. The protein was detected in transfected astrocytes by using a monoclonal anti-GFP antibody (Fig. 4*A*, lane 8), and this construct was used in inhibition studies (see below).

The truncated proteins were tested for their ability to inhibit N-CAM signaling in astrocytes in the luciferase assay (Fig. 4*B*). The three largest proteins, Tm-Cyto Δ824–839, Tm-Cyto Δ801–839, and Tm-Cyto Δ791–839, inhibited IgIII-induced NF-κB activation to about the same extent as the full-length Tm-Cyto, and, in each case, the level of induction of luciferase activity was reduced 35%–40%. In contrast, the smaller truncation mutants, Tm-Cyto Δ780–839 and Tm-Cyto Δ745–839, and the GFP-Δ745–839 did not inhibit. None of the Tm-Cyto truncation constructs had any effect on TNF-α–induced NF-κB activation (Fig. 4*B*), nor on IL-1β and LPS-induced NF-κB activation (data not shown). These data indicated that the region between amino acids 780–800 is essential for the inhibitory activity of Tm-Cyto and presumably for N-CAM-mediated NF-κB activation.

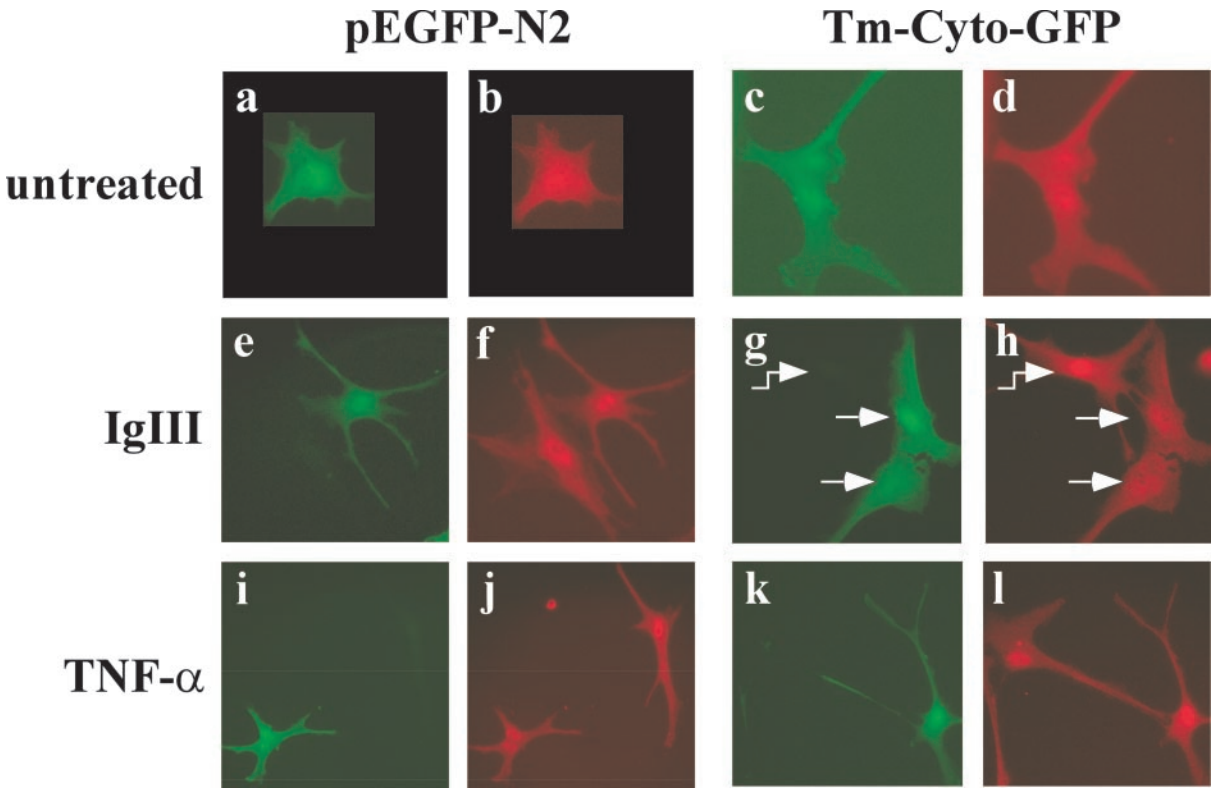

Fig. 3. Expression of GFP-tagged Tm-Cyto inhibits the IgIII-induced translocation of NF-κB to the nucleus. Astrocytes were transfected with pEGFP-N2 (*a*, *b*, *e*, *f*, *i*, and *j*) or Tm-Cyto-GFP (*c*, *d*, *g*, *h*, *k*, and *l*). After transfection, these cells were either untreated (*a–d*), or treated with 100 μg/ml recombinant IgIII (*e–h*) or 1 ng/ml TNF-α (*i–l*). Straight arrows indicate astrocytes expressing Tm-Cyto-GFP and treated with IgIII; the crooked arrow indicates nontransfected, IgIII-treated astrocytes (*g* and *h*).

Three Threonine Residues Are Critical for the Inhibition of N-CAM-Induced NF-κB Activation. The amino acid sequence around the region involved in inhibiting N-CAM-induced NF-κB activation revealed the presence of four threonines (at positions 788, 793, 794, and 797; Fig. 1). Because earlier reports have shown that N-CAM is phosphorylated on threonines and serines *in vitro* (38), we tested whether these potential phosphorylation sites play a role in N-CAM signaling. Point mutations in Tm-Cyto were generated with the individual threonines replaced with an alanine residue. These constructs, termed Tm-Cyto T788A, Tm-Cyto T793A, Tm-Cyto T794A, and Tm-Cyto T797A, were individually expressed in transfected astrocytes as shown by immunoblot analysis (Fig. 5*A*, lanes 3–6). The mutated constructs were then tested for their ability to inhibit N-CAM signaling in astrocytes (Fig. 5*B*). Of these constructs, only Tm-Cyto T793A was able to inhibit the IgIII-induced NF-κB activation, suggesting that the other three threonines (T788, T794, T797) are essential for this activity. The efficiency of inhibition of IgIII-induced NF-κB activation was equal to that of the nonmutated Tm-Cyto construct (Fig. 5*B*). Similar results were seen after treatment with N-CAM (data not shown). Treatment of these transfected cells with TNF-α, IL-1β, and LPS gave equivalent levels of NF-κB activation, but none was reduced by expression of any of the Tm-Cyto constructs (data not shown).

To test further whether phosphorylation of any of these threonines was essential for inhibitory activity, Tm-Cyto constructs were generated in which T788, T794, and T797 were mutated to aspartic acid residues. Mutation of threonine or serine to aspartic acid has been shown in some systems to mimic phosphorylated residues (39). These constructs (Tm-Cyto T788D, Tm-Cyto T794D, and Tm-Cyto T797D) were expressed as shown by immunoblot analysis (Fig. 5*A*, lanes 7–9), and their ability to inhibit N-CAM signaling was tested (Fig. 5*B*). Only Tm-Cyto T794D retained the ability to inhibit IgIII-induced NF-κB activation. The same results were obtained whether IgIII or N-CAM was used to induce NF-κB activation (data not shown). None of the Tm-Cyto mutants inhibited NF-κB activation with TNF-α (Fig. 5*B*), or with IL-1β or LPS (data not shown).

Whereas expression of Tm-Cyto T794D inhibits IgIII- or N-CAM-induced NF-κB activation, it did not itself induce a constitutive NF-κB response, and the basal level of NF-κB activity in astrocytes expressing Tm-Cyto T794D was equal to that in the other transfected cells. Together, the data suggest that the presence of threonines at positions 788, 794, and 797 is required and that phosphorylation of the threonine at position 794 may be required but would not itself be sufficient for normal N-CAM signaling.

Discussion

The results presented here show that the activation of the transcription factor NF-κB in astrocytes by either purified N-CAM or recombinant IgIII is partially blocked by the expression of Tm-Cyto, a construct containing the sd N-CAM cytoplasmic domain, transmembrane segment, and a small region of its extracellular region. Expression of this construct did not inhibit NF-κB activation by other reagents such as IL-1β, TNF-α, or LPS, indicating that the inhibitory effect of Tm-Cyto is specific for activation via cell surface N-CAM. Expression of Tm-Cyto also blocked translocation of NF-κB to the nucleus stimulated by N-CAM reagents but not by cytokines. N-CAM reagents activated NF-κB in N-CAM knockout astrocytes, but this activation was not inhibited by Tm-Cyto. Together, the findings indicate that exogenous N-CAM activates NF-κB

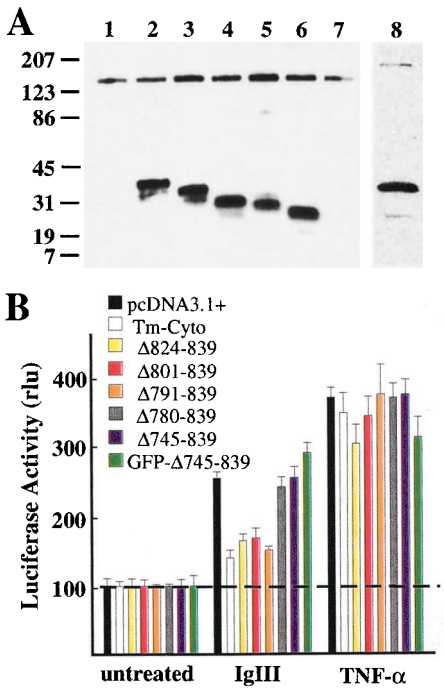

Fig. 4. IgIII-induced NF-κB activation is inhibited by the expression of Tm-Cyto truncation mutants containing the amino acids 780–790. (*A*) Immunoblot of astrocytes transfected with Tm-Cyto constructs. Lane 1, pcDNA3.1$^+$; lane 2, Tm-Cyto; lane 3, Tm-CytoΔ824–839; lane 4, Tm-CytoΔ801–839; lane 5, Tm-CytoΔ791–839; lane 6, Tm-CytoΔ780–839; lane 7, Tm-CytoΔ745–839; and lane 8, GFP-Δ745–839. The band at higher molecular weight in lane 8 is nonspecific and is present in nontransfected astrocytes (data not shown). (*B*) Luciferase activity in transfected astrocytes. The results are shown as described above.

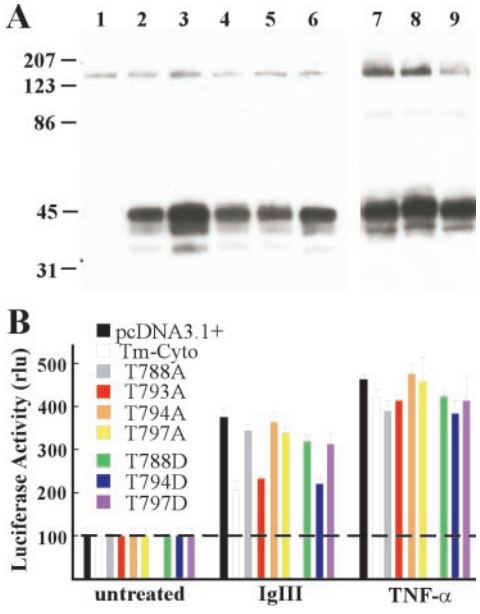

Fig. 5. Specific threonine to alanine or aspartate mutations in the Tm-Cyto construct block IgIII-induced NF-κB activation. (*A*) Immunoblots of transfected astrocytes. Lane 1, pcDNA3.1$^+$; lane 2, Tm-Cyto; lane 3, Tm-Cyto T788A; lane 4, Tm-Cyto T793A; lane 5, Tm-Cyto T794A; lane 6, Tm-Cyto T797A; lane 7, Tm-Cyto T788D; lane 8, Tm-Cyto T794D; and lane 9, Tm-Cyto T797D. (*B*) Luciferase activity of transfected astrocytes. The results are shown as described above.

through cell surface N-CAM, as well as through an as yet unknown heterophile. Activation via cell surface N-CAM is sensitive to inhibition by Tm-Cyto, which appears to exercise a dominant negative effect.

A cytoplasmic segment critical for Tm-Cyto inhibition was localized to the amino acid sequence corresponding to residues 780 and 800 in full-length N-CAM. Three threonines (T788, T794, and T797) in this region are crucial for the inhibitory activity, in that mutation of these residues to alanines eliminated the inhibitory activity of Tm-Cyto. The sequence of ten amino acids containing the three threonines (amino acids 788–797) is conserved in N-CAM from all species except in *Xenopus* and in the N-CAM-related molecule N-CAM2/O-CAM (40, 41), both of which have nine of the ten amino acids but lack the first threonine (T788 in N-CAM). When the threonines at position 788, 794, and 797 were mutated to aspartic acid, a mimetic of phospho-threonine, only the T794D mutant retained inhibitory activity, suggesting that phosphorylation of T794 may be important for Tm-Cyto activity. The loss of inhibitory activity of the T788D and T797D mutants suggests that phosphorylation of these residues may not play a role in N-CAM signaling. These residues are most likely critical for a structural element in the cytoplasmic domain because mutation of either of these threonines to alanine also eliminates the inhibitory activity of Tm-Cyto. The sequence critical for N-CAM signaling described here is distinct from the C-terminal segment reported to be necessary for N-CAM-stimulated neuritogenesis (42).

Our finding that NF-κB can be activated by N-CAM in astrocytes derived from knockout mice lacking all forms of N-CAM indicates that there is a molecule other than N-CAM on the cell surface that on binding N-CAM can transduce a signal leading to NF-κB activation. The proximal pathway, however, appears different from that mediated by cell surface N-CAM, in that expression of the Tm-Cyto construct in cells lacking N-CAM does not inhibit NF-κB activation. N-CAM can activate signaling through heterophilic receptors in at least one other system. For example, studies from our laboratory indicate that treatment of neural progenitor cells from N-CAM-null mice with N-CAM, but not with IgIII, leads to an inhibition of proliferation and a concomitant induction of differentiation to the neuronal lineage (43). These findings suggest that N-CAM, in addition to binding itself, has other receptors that may activate different signal transduction pathways.

In receptors with single transmembrane domains, such as the receptor for the cytokine TNF-α, a common mechanism for signal transduction involves ligand-induced dimerization or oligomerization of the receptor, leading to close association of the cytoplasmic domains and subsequent activation of associated kinases (44). An example of a signaling molecule of the Ig superfamily that spans the plasma membrane only once is the adhesion molecule neurofascin. The cytoplasmic domain of this molecule interacts with ankyrin, and this interaction is modulated by tyrosine phosphorylation of neurofascin. Furthermore, a recombinant form corresponding to the neurofascin cytoplasmic domain forms dimers (45), raising the possibility that neurofascin signaling proceeds through a dimerization and cytoplasmic interaction mechanism. It is possible that a dimerization or oligomerization event is required for N-CAM signal transduction and the dominant negative effect of Tm-Cyto may act by preventing the formation of higher order complexes.

Understanding signaling by N-CAM has been hampered by the inability to identify cytosolic molecules that interact with its cytoplasmic region. The data presented here suggest that key residues in Tm-Cyto may have to be phosphorylated in order for such interactions to occur. Early reports demonstrated that the kinases glycogen synthase kinase 3 (GSK-3) and casein kinase I could phosphorylate N-CAM *in vitro*, although prior phosphor-

ylation of other residues of the N-CAM molecule was required (38). The sd N-CAM isoform has multiple potential phosphorylation sites for casein kinase I and II, as well as a single potential phosphorylation site for GSK-3. However, of the threonines in the region important in the inhibition of N-CAM-induced NF-κB activation, only the one at position 797 fits the consensus sequence (-S/T(P)-X-X-S/T-) for casein kinase I (46). The sole GSK-3 kinase consensus sequence -S/T-X-X-X-S/T(P)- (47) in the sd N-CAM cytoplasmic domain is outside this region (at S761). Because our results have indicated that phosphorylation at T794 may be necessary but not sufficient for induction of NF-κB activation, further studies are required to determine the role of phosphorylation events in the N-CAM cytoplasmic domain.

N-CAM binding can transduce signals in a variety of cell types. Understanding the signaling pathway mediated by N-CAM in different cell systems is made potentially more complex by the fact that N-CAM exists in multiple isoforms. The region shown to be crucial for the inhibitory activity of Tm-Cyto is common to both the sd and ld N-CAM isoforms and, in fact, ends at the ld insertion site. Like the sd N-CAM, ld N-CAM is phosphorylated on multiple threonine and serine residues, although to different extents and at different sites than in the sd N-CAM (38). Because the ld isoform is present only in neurons and not in other adult tissues that express N-CAM, such as skeletal muscle (14), it is possible that the differential phosphorylation of the sd and ld isoforms may be related to specific interactions with proteins in different signaling pathways, whereas the phosphorylation of sites in common to both isoforms may represent overlapping functions. Furthermore, the glycosylphosphatidylinositol-linked ssd N-CAM may be a potential signaling receptor for N-CAM binding. Further study of all isoforms, including experiments in which individual isoforms of the N-CAM molecule are expressed in cells with N-CAM-null backgrounds, will be necessary to clearly delineate their respective roles in N-CAM signal transduction.

We thank Anna Tran, Melanie King, and Lisa Remedios for excellent technical assistance and Dr. Vincent P. Mauro for critical reading of the manuscript. This work was supported by National Institutes of Health Grants HD16550 (to B.A.C.) and HD09635 (to G.M.E.), and a grant from the G. Harold and Leila Mathers Charitable Trust. B.A.C. and K.L.C. are consultants to Becton Dickinson.

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