The Fibroblast Growth Factor Receptor Acid Box Is Essential for Interactions with N-Cadherin and All of the Major Isoforms of Neural Cell Adhesion Molecule*

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Interactions between the neural cell adhesion molecules NCAM and N-cadherin with the fibroblast growth factor receptor (FGFR) are important for a number of developmental events and have also been implicated in tumor progression. The factors regulating these interactions are not known. We have used co-immunoprecipitation and co-clustering paradigms to show that both adhesion molecules can interact with the 3Ig IIIC isoform of the FGFR1 in a number of cell types. Interestingly, whereas the interaction can be seen over most of the cell surface, it is not seen at points of cell-cell contact where the adhesion molecules accumulate at stable junctions. We also demonstrate for the first time that all of the major isoforms of NCAM can interact with the FGFR. Using deletion mutagenesis we have found that the adhesion molecule/FGFR interaction can withstand the removal of most of any one of the FGFR immunoglobulin-like domains (D1–D3). In contrast, the FGFR interaction with N-cadherin and NCAM (but not FGF) is absolutely dependent on the presence of the acid box motif that can be found in the linker region between D1 and D2. As this motif can be spliced out of all four FGFRs, it suggests that this is one mechanism that can regulate the interaction of the receptor with different ligand classes.

Cell adhesion molecules (CAMs) of the immunoglobulin and cadherin superfamilies have a wide range of functions during development. For example, in the nervous system they play roles in cell migration, axonal growth and guidance, and synapse formation (1). Although initially thought to function by modulating adhesion between cells, it is now clear that some CAM functions require the activation of specific second messenger signaling cascades in cells. For example, there is now substantial evidence that the ability of NCAM, N-cadherin, and L1 to stimulate axonal growth is dependent on the tyrosine kinase activity of the fibroblast growth factor receptor (FGFR) in neurons (2–7). Furthermore, evidence from studies on Drosophila suggests that a functional interaction between CAMs and the FGFR evolved perhaps 1 billion years ago (8, 9). Indeed, there now appears to be an emerging consensus that CAM interactions with the FGFR are not only important for neuronal function but also for contact dependent survival of some cell types (10, 11) and the development or progression of some cancers (12–14).

Divergent evolution has generated four functional gene products that constitute the FGFR family of tyrosine kinase receptors, and these can interact with up to 23 fibroblast growth factors (FGFs) (15). Responsiveness to these conventional ligands is determined in part by alternative splicing of the exons that encode the extracellular domains of the receptors (15). However, little is known about the interplay between CAMs and FGFs in the context of FGFR signaling nor the factors that influence CAM interactions with the FGFRs. In some cancer cells, an interaction between N-cadherin and the FGFR1 appears to stabilize receptors on the cell surface and may thereby potentiate signaling by endogenous FGFs (12). In other cancer cells an interaction between NCAM and the FGFR4 appears to orchestrate the formation of a higher order-signaling complex that can signal independently of the conventional ligands (13). Likewise, in neurons CAMs appear to be able to act as surrogate ligands for the FGFR and signal via the receptors independently of FGFs (3, 16). A better understanding of this relationship clearly requires more insights into the factors that influence the ability of CAMs to interact with the FGFRs.

The extracellular domain of the FGFR can be composed of up to three distinct Ig-like domains (D1, D2, and D3) with an unusual stretch of acidic amino acids (the “acid box”) being present in the linker region between D1 and D2. Alternative splicing generates full-length FGFRs that differ in the second half of the membrane proximal D3 and/or shorter versions of the receptor that lack D1 and/or the acid box (17). The structures of FGFR1/2 with the membrane proximal D2 and D3 domains of the FGFR1/2 have been solved, and this has allowed for a very detailed understanding of the nature of this class of ligand-receptor interface (18, 19) and rational design of novel peptide antagonists (16). FGFs bind to an interface that is formed by the membrane proximal D2/D3 domains, as well as the linker region between the domains, in a manner that can explain why alternative splicing of the exons that encode D3 can

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The abbreviations used are: CAM, cell adhesion molecules; CHD, CAM-homology domain; NCAM, neural cell adhesion molecule; FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; GPl, glycosylphosphatidylinositol; VASE, variable alternatively spliced exon.
determine ligand binding specificity. The structures of two distinct higher order receptor-ligand structures that also accommodate heparin have also been solved (20, 21), with recent evidence suggesting that both might be biologically relevant (22).

Whereas there is a large body of evidence relating to FGF/FGFR interactions, very little is known about how CAMs interact with the FGFR. FGFRs contain an ~20 amino acid motif within the D2 domain that shares sequence homology with functional motifs present in NCAM and N-cadherin (23). This “CAM homology domain” (CHD) forms a contiguous sequence with the acid box and antibodies raised against the acid box or the CAM homology domain inhibit CAM stimulated neurite outgrowth (3). Importantly, synthetic peptide mimetics of motifs from this region of the FGFR also inhibit the CAM responses (3). This formed the basis for the suggestion that the CAMs might directly interact in cis with this region of the FGFR (23).

The membrane proximal D3 domain of the FGFR is essential for FGF binding but not required for N-cadherin binding (12). The interaction of N-cadherin and the FGFR appears to involve a direct cis interaction between D1 and D2 domains of the receptor with an N-cadherin region that includes the fourth of the five cadherin domains (12, 16, 24). Likewise, surface plasmon resonance and NMR data have shown direct interactions between the FGFR1 D1 and D2 domains with the fibronectin type III domains of NCAM (25). In the present study we have used co-immunoprecipitation and co-clustering assays to map sites on the 3Ig IIIc isoform of the FGFR1 that are required for an interaction with N-cadherin and all of the major isoforms of NCAM. Our results show that the FGFR1 will robustly interact with N-cadherin and all five of the major isoforms of NCAM, and this provides the first evidence that alternative splicing of NCAM does not directly influence its interaction with the FGFR1. In the context of the FGFR, our results demonstrate that whereas the CAM homology domain is dispensable for the CAM/FGFR interactions, the proximal acid box region is absolutely required. Interestingly, the acid box can be spliced out of the FGFR and is not required for FGF function (26). These results show for the first time a positive requirement of the acid box for an FGFR function and substantiate the emerging view that CAMs can interact with regions of the receptor that are not involved in binding FGFs.

**EXPERIMENTAL PROCEDURES**

**Structures**—The structure of the membrane proximal D2/D3 Ig like domains of the FGFR1/2 has been solved (Protein Data Bank accession numbers 1cvs and 1fq9). The structure of the D1 Ig-like domain was defined by homology modeling with the Swiss-Model server using the telokin structure (1tlk) as a template. The structure of the linker region between D1 and D2 has not been solved, and there are no structures for similar sequences in the public domain. However, a secondary structure prediction (with the 3D-psmb server) is that of a random coil. We modeled the linker region as a random coil and joined the three structures for the complete model. We make no inference on the relationship between D1 and D2/D3; this aspect of the model is for illustrative purposes only.

**Antibodies**—The NCD2 monoclonal antibody (27) specifically reacts with the ECD1 (EGFR extracellular domain 1) of chicken N-cadherin and was obtained from R&D. 3B9 (28) is an N-cadherin monoclonal antibody obtained from Zymed Laboratories Inc.. ERIC 1 is a monoclonal antibody targeted against the second fibronectin domain of human NCAM (29) and was obtained from Santa Cruz Biotechnology. A rabbit antibody (06-177) raised against the acid box containing region of FGFR (30) was obtained from Upstate Biotechnology. A rabbit antibody raised against the catalytic domain of the FGFR1 (FlgC-15) (31) was obtained from Santa Cruz Biotechnology. A polyclonal rabbit serum raised against a highly conserved peptide sequence from the cytoplasmic domain of the epidermal growth factor receptor (EGFR) was a kind gift from Prof. W. J. Gullick (University of Kent, Canterbury, UK). Goat anti-rat, anti-mouse and anti-rabbit secondary antibodies coupled to Alexa Fluor 488 or 594 were obtained from Molecular Probes. Anti-active and total MAPK antibodies were obtained from Promega and used according to manufacturer’s instructions. Horseradish peroxidase-labeled horse anti-mouse or anti-rabbit antibodies were obtained from Vector.

**Plasmids and Mutagenesis**—Expression constructs for the various isoforms of human NCAM and full-length chicken N-cadherin have been described previously (32, 33). An epitope-tagged FGFR1 expression construct was obtained by cloning the 2.3-kb HindIII-XbaI cDNA fragment containing the entire coding sequence of the 3Ig IIIc isoform of the mouse FGFR1 into the pcDNA3.1/Myc-His vector using standard protocols (Invitrogen). A construct lacking the cytoplasmic domain of the FGFR (DN-FGFR1) was created by PCR from the FGFR1 plasmid, again using standard procedures. Likewise, constructs with substantial deletions within D1, D2, and D3 of the FGFR1 and the linker region between D1 and D2 were assembled with PCR-generated fragments and standard molecular cloning techniques. In brief, for each deletion construct, FGFR1 sequences were generated by PCR separately incorporating specific deletions, and then, using these PCR products as templates, a second-step PCR was performed to piece together individual fragments. The final PCR products were cloned into the pcDNA3.1/Myc-His plasmid.

**Cell Lines and Cell Transfection**—BT-549 breast carcinoma cells and L6 myoblasts were obtained from the American Tissue Culture Collection. Transfected NIH 3T3 cell lines expressing all of the major isoforms of human NCAM (34) were routinely maintained at 37 °C in a humidified atmosphere of 8% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfected L6 cells that stably express the 3Ig IIIc isoform of the human FGFR1 (L6-FGFR; a kind gift from Dr Claesson-Welsh) (35) and NIH 3T3 cells that stably express full-length chicken N-cadherin (the LK8 line) (33) were maintained in selection medium (720 ng/ml G148 in 10% FCS-DMEM). Importantly, NIH 3T3 cells predominantly express full-length 3Ig forms of the FGFR1 and FGFR2 (36). L6 cells were transfected with 1 μg of the plasmid encoding the control or mutated versions of the 3Ig IIIc isoform of the mouse FGFR1 construct and/or 0.5–1 μg of the plasmid encoding the full-length chicken N-cadherin using a Lipofectamine-Plus transfection reagent (Invitrogen). In some
experiments the N-cadherin construct was transfected into the L6 cell line that stably expresses the 3lg IIIC isoform of the human FGFR1. Transfected cells were allowed to recover in serum-containing medium overnight before being subcultured onto poly-l-lysine-coated glass cover slips for microscopic analysis or standard tissue culture plates for co-immunoprecipitation studies. Post-natal day 3 mouse cerebellar neurons were isolated as previously described (36) and plated at a density of $8 \times 10^6$ cells per well in individual wells of a 6-well culture dish and allowed to attach overnight at 37°C.

**Immunoprecipitations and Western Blotting**—In general, transfected cells and control cells were washed with cold PBS and lysed in buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 10 mM NaF, and 1 mM sodium orthovanadate in the presence of a mixture of protease inhibitors (Complete, Roche Applied Science). Extracts were clarified by centrifugation and protein concentrations were determined by the BCA protein assay. Cell lysates (0.5–1 mg) were incubated with FGFR antibodies (generally flg at 1 μg/ml, but in some experiments a combination of flg and 177) or N-cadherin antibody (NCD2 at 1 μg/ml) for 3–12 h at 4°C with rotation. The immunocomplexes were captured with 10 mg of prewashed protein A-Sepharose (from Sigma) for 2 h at 4°C. Precipitates were then washed three times with cold lysis buffer and re-suspended in 2 × Laemmli sample buffer. The proteins were separated on 7.5% SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with the indicated primary antibody. The primary antibodies were subsequently detected with horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG conjugates (Vector) at a 1:3000 dilution. Bound antibodies were detected by adding chemiluminescence substrate solution (ECL, Amersham Biosciences) followed by exposure to Kodak Bio-Max light film.

**Membrane Preparation for Western Blotting**—Transfected L6 cells from a 10-cm tissue culture plate were washed three times with ice-cold PBS and re-suspended in 500 μl of Buffer STM (10 mM Tris-HCl (pH 8), 0.25 mM sucrose, 10 mM MgCl2, 0.1 mM dithiothreitol, protease, and phosphatase inhibitors). All the procedures were carried out on ice or in a 4°C centrifuge. The cells were homogenized (25 times) on ice and centrifuged at 600 × g for 15 min to pellet the nuclei and attached membrane. The supernatant was kept (Smt1). The pellet was re-suspended in STM again and centrifuged at 600 × g for 15 min, and this second supernatant was kept (Smt2). The pellet from Smt2 was re-suspended in STM Buffer containing 0.5% Nonidet P-40 and centrifuged at 600 × g for 15 min to release the proteins from the membrane. The nuclei are kept intact in the pellet and are lysed in RIPA Buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) to obtain the nuclear fraction.

**Clustering Experiment**—Cells were taken from semi-confluent dishes and grown overnight on poly-l-lysine-coated glass coverslips at a density of 25,000 cells/well in 10% FCS/DMEM. For clustering of N-cadherin or NCAM, the cultures were incubated with the NCD2 or ERIC monoclonal antibodies in 10% FCS/DMEM (dilution 1:400) for 30–60 min at 37°C in tissue culture incubator, washed three times with culture media, and then incubated for 1 h with goat anti-rat Alexa Fluor 488 (for NCD2) or goat anti-mouse Alexa Fluor 488 (for ERIC), both at a 1:1000 dilution in 10% FCS/DMEM for a further 60 min at 37°C. Cultures were fixed by initially adding an equal volume of 4% paraformaldehyde (in PBS with 4% sucrose) to the culture medium for 5 min; this was then gently removed and replaced with fresh 4% paraformaldehyde solution and incubated for further 10 min. In some experiments cells were fixed prior to incubation with the N-cadherin and NCAM antibodies (non-clustered). Non-clustered cells were also incubated with NCD2 or ERIC. The cultures were washed three times with PBS and then blocked with 10% FCS/PBS for 30–60 min at room temperature. Non-clustered and clustered cells were then incubated with the rabbit anti-FGFR antibody (177) or the rabbit anti-EGFR antibody at a 1:400 dilution in 10% FCS/PBS overnight at 4°C. After washing, the cultures were finally incubated with goat anti-rabbit Alexa Fluor 594 in 10% FCS/PBS (to detect the FGFR) for 1 h at room temperature. Following washing in PBS, the coverslips were mounted in Mowiol and viewed on an inverted Zeiss microscope (Axiovert 135) with a 63× objective lens (Plan-Apochromat 63×/1.40). We would emphasize that we only detected positive immunoreactivity for the FGFR when both the primary and secondary antibodies were included, and we did not in any circumstance see “bleeding” from the green N-cadherin channel into the red FGFR channel. For example, evidence in support can be seen in Fig. 4 where saturable staining for N-cadherin is seen in the green channel in the absence of any corresponding staining in the red channel.

**Detection of Active MAPK (ERK1/2)**—Parental or transfected L6 cells were starved in serum free media for 24 h. Cells were then treated with FGF2 (1 μM) for 5 min and aliquots of the cell lysates fractionated on a 12% SDS-PAGE gel. Active MAPK was detected by immunoblotting using an anti-active MAPK antibody (Promega) according to manufacturer’s instructions (1:5000 dilution) and a horseradish peroxidase-labeled anti-rabbit secondary antibody (1:10,000 dilution) followed by chemiluminescent detection.

**RESULTS**

**N-Cadherin Co-immunoprecipitates with the FGFR from a Variety of Cell Types**—Our initial objective was to determine whether N-cadherin interacts with the FGFR in a variety of cell types. As functional interactions between N-cadherin and the FGFR have been reported in breast cancer cells and neurons (see Introduction), we determined whether N-cadherin was present in the FGFR immunoprecipitate obtained from lysates from samples of both cell types. When lysates from the BT549 breast cancer cell line were subjected to a standard immunoprecipitation protocol with two independent FGFR antibodies, N-cadherin was found in both precipitates, with a more robust response being seen with these cells when the antibodies were used in combination (Fig. 1a). We observed a similar co-immunoprecipitation from post-natal day 3 cerebellar neurons that had been cultured overnight (Fig. 1b) and from a number of other cells and intact tissues (e.g. the chick retina; data not shown). To confirm the specificity of the interaction, and to be able to map interaction sites, we wanted to identify a cell type that did not express endogenous N-cadherin or the FGFR. We could not detect endogenous N-cadherin or FGFR in Western
Cells but is excluded from sites of cell-cell contact and N-cadherin antibodies. (Data not shown). As expression of both molecules is required the mouse FGFR1 were transiently co-transfected into L6 cells. Results were seen when N-cadherin and the 3Ig IIIc isoform of the same LK8 line used here is associated with a co-clustering of transfected N-cadherin in NIH 3T3 fibroblasts for N-cadherin in LK8 cells (see Fig. 2, a–c). Similar results were found in parental L6 cells (control) L6 cells stably transfected with the human FGFR1 (FGFR1), L6 cells transiently transfected with N-cadherin (N-cad), or L6 cells stably transfected with the FGFR1 and transiently transfected with N-cadherin (FGFR/N-cad) were obtained and subjected to immunoprecipitation with control rabbit IgG (lane 1) or a combination of two anti-FGFR antibodies (lane 2). In c lyses obtained from parental L6 cells (control), L6 cells stably transfected with the human FGFR1 (FGFR1), L6 cells transiently transfected with N-cadherin (N-cad), or L6 cells stably transfected with the FGFR1 and transiently transfected with N-cadherin (FGFR/N-cad) were obtained and subjected to immunoprecipitation with control rabbit IgG (−) or immunoprecipitation with the combination of the two anti-FGFR antibodies (+). In all cases the lyses were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the 3B9 anti-N-cadherin monoclonal antibody. Bars indicate relative positions of molecular mass markers of −200 and −125 kD. Blots of lyses of the LN6 muscle cell line that has previously been reported to be negative for FGFR expression (35). In accord, we did not detect N-cadherin by Western blotting in FGFR immunoprecipitates from control L6 cell lyses or from L6 cells transfected with the FGFR or N-cadherin constructs on their own (Fig. 1c). However, when N-cadherin was transfected into an L6 cell line that stably expresses the transfected 3Ig IIIc isoform of the human FGFR1, N-cadherin was now found in the FGFR immunoprecipitate (Fig. 1c, final two lanes). Similar results were seen when N-cadherin and the 3Ig IIIc isoform of the mouse FGFR1 were transiently co-transfected into L6 cells (data not shown). As expression of both molecules is required to obtain the result, this controls for the specificity of the FGFR and N-cadherin antibodies. The FGFR co-localizes with a sub-pool of N-Cadherin in cells but is excluded from sites of cell-cell contact—The FGFR and N-cadherin will co-immunoprecipitate from lysates from a variety of cell types; we wished to determine to what extent these molecules co-localize with each other in cells. We have previously reported low level “diffuse” staining of endogenous FGFR at the cell membrane, and this precludes meaningful co-localization studies (37). However, antibody-induced clustering of transfected N-cadherin in NIH 3T3 fibroblasts (the same LK8 line used here) is associated with a co-clustering of the endogenous FGFR (37). In the present study we first asked if the 3Ig IIIc isoform of the human FGFR1 co-localizes with N-cadherin clusters in the L6 cell line that stably expresses this receptor. Following transient transfection of the N-cadherin construct, we observed extensive (but not absolute) co-localization of the FGFR1 and the clustered N-cadherin protein (e.g. Fig. 2, a–c). Similar results were found in parental L6 cells following transient co-transfection of the mouse 3Ig IIIc isoform of the FGFR1 with N-cadherin (Fig. 2, d–f). Similar findings were made with other cell types; for example in NIH 3T3 cells that stably express transfected N-cadherin (the LK8 cell line) the endogenous FGFR co-localizes with N-cadherin over most of the cell membrane (Fig. 2, g–i). However, we noted that the FGFR is excluded from the cell-cell junction that is enriched for N-cadherin in LK8 cells (see arrowhead in Fig. 2, g–i) and in

**FIGURE 1. Co-immunoprecipitation of N-cadherin with the FGFR1.** In a BT-549 cells lyses were subjected to immunoprecipitation with control rabbit IgG (lane 1), the flg rabbit antibody that recognizes the intracellular domain of the FGFR1 (lane 2), the “177” rabbit antibody that recognizes the CHD/AB region of the FGFR1 (lane 3) or a combination of both antibodies (lane 4). In b lyses from cultured post-natal day 3 rat cerebellar granule cells were subjected to immunoprecipitation with control rabbit IgG (lane 1) or a combination of the two anti-FGFR antibodies (lane 2). In c lyses obtained from parental L6 cells (control), L6 cells stably transfected with the human FGFR1 (FGFR1), L6 cells transiently transfected with N-cadherin (N-cad), or L6 cells stably transfected with the FGFR1 and transiently transfected with N-cadherin (FGFR/N-cad) were obtained and subjected to immunoprecipitation with control rabbit IgG (−) or immunoprecipitation with the combination of the two anti-FGFR antibodies (+). In all cases the lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the 3B9 anti-N-cadherin monoclonal antibody. Bars indicate relative positions of molecular mass markers of −200 and −125 kD.

**FIGURE 2. Clustering of N-cadherin in transfected cells.** a–c show L6 cells that express the 3Ig IIIc isoform of the human FGFR1 (stable transfection) together with chicken N-cadherin (transient transfection). d and e show L6 cells that express the 3Ig IIIc isoform of the mouse FGFR1 together with chicken N-cadherin (transient co-transfection). g–l show NIH 3T3 fibroblasts that express chicken N-cadherin (stable transfection, the LK8 cell line). All cell types were treated with the NCD2 anti-chick N-cadherin monoclonal antibody for 30–60 min at 37 °C, washed three times, and then treated with anti-mouse Alexa Fluor 488 for a further 60 min at 37 °C. The cells were then washed and fixed with paraformaldehyde (see “Experimental Procedures”). The fixed cells were then immunostained with rabbit polyclonal antibodies to the FGFR (antiserum 177) or the EGFR as indicated (note for EGFR staining the cells were permeabilized by treatment with methanol). Specifically bound rabbit antibodies were visualized with goat anti-rabbit Alexa Fluor 594. Micrographs show pseudo-colored images of N-cadherin (green), the FGFR (red), and the merged image as indicated. In “H” Hoechst staining highlights the location of the cell nuclei. The scale bar is 20 μm.
transfected L6 cells (data not shown). This is an interesting observation that also acts as an important internal control as it demonstrates the specificity of the antibodies used for the staining. Additional control experiments for the specificity of the interaction were carried out. For example, LK8 cells express low levels of the EGFR that can be detected with a rabbit antiserum raised against the cytoplasmic domain of the receptor (Fig. 2k). Importantly, the EGFR does not co-localize with clustered N-cadherin (Fig. 2, j–l). These data extend the co-immunoprecipitation data by showing that N-cadherin is associated with the FGFR in the cell membrane and by showing that this interaction can be regulated as it is excluded from points of cell-cell contact where N-cadherin accumulates.

Deletions within FGFR1 D1–D3 Identify Sequences That Are Not Required for the N-Cadherin Interaction—The intracellular domain of the FGFR is not required for interactions between the FGFR1 and N-cadherin (12), and we have confirmed this observation (data not shown). In contrast, deletion of the entire D1 or D2 (but not D3) Ig-like domains from the FGFR1 results in the loss of N-cadherin association as determined by co-immunoprecipitation (12). In this study, rather than deleting the entire Ig-like domain, we deleted the regions spanning the conserved cysteines (removing the coding sequence for amino acids 48–108, 171–237, and 270–348 to generate the ΔD1, ΔD2, and ΔD3 constructs). Our deletions are shown on a modeled structure of the FGFR in Fig. 3 (see “Experimental Procedures” for details on the modeling). Interestingly, in contrast to the results seen when the entire globular domain is deleted, we still observed co-precipitation of the ΔD1 FGFR with N-cadherin (Fig. 3). Likewise, co-precipitation was seen with the FGFR1 that lacked most of D2, and as expected from previous results (12), most of D3 (Fig. 3). Taken together with the previous results of Suyama et al. (12), our new data narrows down the N-cadherin/FGFR interaction site from the first two Ig-like domains, to an ~60-amino acid sequence that was not deleted in our constructs, but was deleted in the Suyama constructs. This sequence (residues 108–171) lies on the face of D2 and also serves to link D2 to D1 (Fig. 3).

Identification of the FGFR Acid Box as a Motif Required for the N-Cadherin Interaction—There are three conspicuous motifs within the ~60-amino acid sequence that appears to be required for the N-cadherin/FGFR interaction, and these are shown schematically in Fig. 4a. These are the acidic box 

### FIGURE 3. Ig domain deletions of FGFR1 do not affect association with N-cadherin. L6 cells were co-transfected with chicken N-cadherin and the ΔD1, ΔD2, and ΔD3 FGFR1 constructs. Regions deleted are highlighted on a modeled structure of the FGFR as indicated. Cell lysates obtained from the transfected cells were immunoprecipitated with the NCD2 anti-N-cadherin antibody. Precipitates were run on standard gels, transferred to nitrocellulose membranes, and immunoblotted with the pig rabbit polyclonal antiserum that is reactive with the cytoplasmic domain of the FGFR (top panel). The presence of transfected FGFR and N-cadherin in the cell lysates was confirmed by Western blotting (bottom two panels as indicated).

### FIGURE 4. The acid box is required for the FGFR1/N-cadherin interaction. L6 cells were co-transfected with chicken N-cadherin and the ΔCHD, ΔHAV, and ΔAB (acid box) FGFR1 constructs. Regions deleted are highlighted on a modeled structure of the FGFR as indicated. Cell lysates obtained from the transfected cells were immunoprecipitated with the NCD2 anti-N-cadherin antibody and lysates analyzed for the presence of the FGFR as described in the legend to Fig. 3. The FGFR lacking the HAV motif (lane 1) or CHD (lane 2) were found in the N-cadherin immunoprecipitate. In contrast, the FGFR lacking the acid box motif did not co-immunoprecipitate with N-cadherin (lane 3). The presence of both the FGFR and N-cadherin in the original lysates was confirmed by Western blotting (Fig. 4b as indicated). In Fig. 4c, the presence of the FGFR was determined by Western blotting total cell lysates obtained from L6 cells transfected with full-length (lane 4) or the ΔAB FGFR (lane 5). The presence of the FGFR was also determined by Western blotting in cytosolic (lanes 6 and 8) and membrane fractions (lanes 7 and 9) obtained from L6 cells transfected with full-length (lanes 6 and 7) or the ΔAB FGFR (lanes 8 and 9). In d L6 cells expressing transfected N-cadherin and the ΔAB FGFR were treated with a specific anti-chick N-cadherin monoclonal antibody (NCD2) to induce clustering of N-cadherin as described in the legend to Fig. 3. The cells were then washed and fixed with paraformaldehyde and immunostained with the rabbit polyclonal 177 antibody targeted against the extracellular acid box region of the FGFR as described in Fig. 2. Staining for N-cadherin is shown in micrograph i, with staining for the FGFR shown in micrograph ii. The merged image is shown in micrograph iii with the cell nuclei highlighted by Hoechst staining. The scale bar is 20 μm.
Forms part of the face of D2 in the FGFR, with the acid box forming part of the linker sequence between D1 and D2 (illustrated in the model in Fig. 4a). As the presence of the HAV motif within the CAM homology domain was the driving force for the hypothesis that N-cadherin might functionally interact with the FGFR (3), we deleted both to determine whether this region is required for the N-cadherin/FGFR interaction. We also deleted the seven aspartic acids that constitute the most conspicuous aspect of the acid box region. All three constructs were co-transfected with N-cadherin into the L6 cell line and lysates subjected to immunoprecipitation with the NCD2 N-cadherin antibody (Fig. 4b). The results clearly show that FGFRs that lack the HAV motif (lane 1) or the entire CHD sequence (lane 2) can still co-immunoprecipitate with N-cadherin. In contrast, the FGFR1 construct that lacked the acid box failed to co-immunoprecipitate with N-cadherin (lane 3). The FGFR lacking the acid box showed similar levels of expression as the other FGFR constructs in total cell lysates (Fig. 4b, lane 3). Furthermore, additional experiments confirmed that like the full-length FGFR, the FGFR lacking the acid box was present in the membrane and not the cytosol (Fig. 4c, lanes 4–9). As with the full-length receptor, there was no evidence for clustering of the acid box-deleted receptor on the surface of transfected cells (Fig. 4d, lane II). However, in contrast to the full-length receptor, we were unable to detect the acid box-deleted receptor in N-cadherin clusters. Nonetheless, the acid box-deleted receptor was readily detected in clusters at the cell surface of transfected cells following direct clustering with an FGFR antibody confirming that it is indeed expressed in the cell membrane (Fig. 5a). This conclusion is reinforced by that fact that FGF2-induced phosphorylation of ERK1/2 was readily detectable in cells that express the acid box-deleted receptor (Fig. 5b, lane labeled ΔAB) confirming that the receptor is not only at the cell surface, it is also clearly capable of binding and responding to FGF2. However, in accord with previous studies we found that the HAV motif is absolutely required for FGF function (Fig. 5b, ΔHAV lane). As an additional control we found that phosphorylation of ERK was dependent on the presence of the FGFR1 catalytic domain (Fig. 5b, DN lane).

All of the Major NCAM Isoforms Interact with the FGFR1—NCAM is another major molecule that has been proposed to interact with the FGFR (see Introduction). Alternative splicing generates three major classes of NCAM isoforms that share a common five Ig-like domains in tandem with two membrane proximal fibronectin domain III-like domains (1). These NCAM isoforms can be GPI-anchored to the cell membrane (NCAM120) or be class 1 transmembrane glycoproteins with a small (NCAM140) or large (NCAM180) cytoplasmic domain. In addition, a 10-amino acid variable alternatively spliced exon (VASE) changes the structure of the fourth Ig domain of NCAM to a variable Ig-like domain, with alternative usage of a number of small exons introducing an additional stretch of amino acids between the two fibronectin domain III domains (NCAM125 when present in the GPI-anchored isoform of NCAM). We have previously generated stably transfected lines of NIH 3T3 fibroblasts that express similar levels of each of these NCAM isoforms (36, 38). NIH 3T3 cells predominately express full-length 3lg versions of the FGFR1 and FGFR2 (39).

The endogenous FGFR1 was immunoprecipitated from lysates of these cells using the “flg” antibody that reacts specifically with the cytoplasmic domain of the FGFR1. All of the NCAM isoforms were found to co-precipitate with the FGFR1 (Fig. 6a). We also looked for co-localization of the endogenous FGFR and transfected NCAM in these cells. Similar results were found with all the NCAM isoforms and are shown for the 125-kDa GPI-linked isoform. When fixed cells were stained for NCAM, a relatively diffuse staining pattern is seen with some concentration at cell-cell junctions (Fig. 6b, micrographs I). In contrast, the FGFR1 is barely detectable due to low expression levels, but it is evident that it does not accumulate with NCAM at sites of cell-cell contact (Fig. 6b, micrographs II and III). In contrast, when NCAM is clustered on the surface of live cells, and the cells are then fixed and stained for the FGFR1, the FGFR can be seen to show extensive co-localization with the clustered NCAM (Fig. 6b, micrographs V–VI). Please note the presence of two cells that do not express NCAM in this set of micrographs (revealed by the nuclear stain in Fig. 6b, micrograph VI). The FGFR remains barely detectable in these cells and does not appear clustered after treatment with the antibodies to cluster NCAM. This demonstrates that clustering of the
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FGFR1 is absolutely dependent on NCAM expression in the cells.

Identification of the FGFR Acid Box as a Motif Required for NCAM Interactions—We next co-transfected L6 cells with the 125-kDa GPI-anchored NCAM and the various FGFR1 deletion constructs described above to identify motifs required for the interaction. NCAM showed a very robust co-immunoprecipitation with the 3Ig IIIc isoform of the mouse FGFR1 lacking either the HAV motif or the entire CHD (Fig. 6c). However, as was the case for N-cadherin, NCAM failed to interact with the FGFR1 construct lacking the acid box motif (Fig. 6c). Again, robust expression of the acid box-deleted construct is apparent from Western blotting of total cell lysates.

DISCUSSION

FGFRs are critically important for a wide range of fundamental cellular responses including neurogenesis, cell migration, and cell differentiation. In mammals, 23 FGFs have evolved to serve as the conventional ligands for the receptors (15, 40); however, recent evidence has pointed to the possibility that a number of CAMs might be able to serve as surrogate ligands and/or receptor-binding partners that modulate signaling by the conventional ligands. In this context, the ability of NCAM, N-cadherin, and L1 to stimulate neurite outgrowth appears to be dependent on their ability to activate neuronal FGFRs, and the formation of an N-cadherin/FGFR1 and NCAM/FGFR4 signaling complexes can drive biochemical and cellular responses in tumor cells (see Introduction).

There are currently several models, which are not mutually exclusive, that aspire to explain the nature of the CAM/FGFR interaction. In one model, CAMs will cluster upon homophilic binding with the resultant co-clustering of the FGFR leading to receptor activation and biological responses (41). In another model, the association of N-cadherin with the FGFR1 regulates the dynamics of ligand induced receptor internalization and thereby sensitizes the receptor to low levels of conventional ligands by increasing receptor availability at the cell surface (12). In a third model, NCAM orchestrates the assembly of an FGFR4 signaling complex that contains other components (e.g. N-cadherin, phospholipase-Cγ, growth-associated protein-43) within the complex that can signal independently of the conventional ligands (13). A common feature of all three models is the direct cis interaction between the CAM and the FGFR in the same cell membrane.

The original hypothesis that at least three CAMs can directly signal via the FGFR was developed based on two independent sets of observations. First, pharmacological studies suggested that CAM stimulated neurite outgrowth required activation of a second messenger cascade in neurons and pointed to a receptor tyrosine kinase as an early step in the cascade (42). Second, unexpected results from studies on the function of the VASE motif in NCAM led to the observation that FGFRs carry an ~20-amino acid sequence that shares sequence homology with functional motifs present in both NCAM and N-cadherin. In this context, an exon called VASE encodes for 10 amino acids that when spliced into the fourth Ig like domain of NCAM inhibits NCAM-stimulated neurite outgrowth (36, 43, 44). The observation that a peptide mimetic of the VASE sequence could inhibit NCAM-stimulated neurite outgrowth in circumstances where NCAM itself did not use the exon led to a data base search for other molecules with a VASE-like sequence (3, 45). The FGFR came out at the top of the list, and intriguingly the FGFR VASE-like sequence was within a sequence that also shows evolutionary conservation with the HAV containing FGFR VASE-like sequence that is present in N-cadherin (46).

Structure-function studies have shown that deletion of the entire FGFR D1 or D2, but not D3, prevents the interaction between N-cadherin and the FGFR1 suggesting that the first two Ig-like domains of the receptor mediate the interaction (12). Importantly, the authors showed that an interaction was still seen after mutation of the HAV motif to HEV, providing the first evidence that this motif is not essential for the interaction. We have extended these findings by showing that the N-cadherin/FGFR interaction can withstand the removal of most of D1 and D2, and this narrows down the interaction site...
to an ~60-amino acid sequence that was not deleted in our constructs but was deleted in the Suyama constructs. This sequence contains three conspicuous motifs, and these are the acid box, the CAM-homology domain, and the HAV motif within the CAM homology domain. Surprisingly, the N-cadherin/FGFR interaction was still seen when the full CAM-homology domain was deleted. Thus it can be concluded that not only is the HAV motif not required for the N-cadherin/FGFR interaction, the whole of the CAM-homology domain can be dispensed with. However, the HAV motif is required for FGF2 binding (12) and for FGF2 signaling (this study).

The region that links the D1/D2 domains of the FGFR is relatively long, with the presence of an unusual stretch of acidic amino acids called the acid box (47). Interestingly, deletion of a contiguous stretch of seven aspartic acid residues from the acid box prevented the FGFR from interacting with N-cadherin but did not inhibit FGF2-induced activation of the MAPK cascade. The latter observation was to be expected given that the acid box is not required for FGF binding, and if anything, receptors lacking the acid box show an increased affinity for FGFs (48). The former observation is, however, surprising as it suggests that the acid box is absolutely required for N-cadherin binding.

At least three CAMs can interact with the FGFR. In the present study we have shown that in addition to N-cadherin, all of the major isoforms of NCAM can co-precipitate with the FGFR1. A cis interaction between the extracellular domains appears to mediate the interaction as it is seen with the natural GPI-anchored isoforms of NCAM and with the FGFR1 construct that has its cytoplasmic domain deleted. Interestingly, whereas the use of the VASE exon in NCAM compromises its ability to stimulate neurite outgrowth (36), it has no obvious effect on the NCAM/FGFR interaction. As was the case with N-cadherin, the NCAM/FGFR interaction was seen with FGFR constructs that have most of D1, D2, or D3 deleted, and with constructs that have the CAM-homology domain and HAV motif deleted (this study). However, as was the case with N-cadherin, the interaction was lost when the acid-box was deleted from the FGFR. This suggests a similar interaction mechanism governs the interaction of all of the major NCAM isoforms and N-cadherin with the receptor.

To substantiate the results with the co-immunoprecipitation, we also looked at the co-localization of the CAMs and FGFR in the plasma membrane. In general, the FGFR is expressed at low levels in a very diffuse manner and this is not particularly amenable to co-localization studies. However, antibodies can readily cluster CAMs into very discrete patches on the cell surface, and under these conditions we can determine whether the FGFR co-localizes with the clustered CAMs. In brief, there was a complete correlation with the immunoprecipitation data; all of the FGFR1 constructs that co-precipitate with N-cadherin and the various NCAM isoforms were found to co-localize with the clustered CAMs. However, co-clustering was not seen when the acid-box was removed from the FGFR.

The clustering experiments were done with both the mouse and human 3ig IIIC isoforms of the FGFR1 and with the endoge-

3 E. Sanchez-Heras, F. V. Howell, G. Williams, and P. Doherty, unpublished observation.

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Visualization of the FGFR in control 3T3 cells and NCAM clustered 3T3 cells gives the clear impression that there is more FGFR in the NCAM clustered cells (e.g. see Fig. 6b), and this would be in keeping with the suggestion that CAMs can stabilize FGFRs on the cell surface (12). However, it should be noted that we never found any evidence for increased levels of FGFRs in CAM expressing cells by Western blot analysis of lysates, and although the FGFR is normally barely detectable on cells, it is readily visualized following clustering with an antibody. Furthermore, we have not seen any obvious differences in FGF2 responsiveness in N-cadherin expressing NIH 3T3 cells as compared with parental NIH 3T3 cells. Nonetheless, the possibility that CAMs might concentrate FGFRs in discrete membrane compartments might well have implications for FGF signaling in cells.

To date there have only been crystal structures for the D2/D3 forms of the FGFRs; however, modeling studies have allowed for some predictions concerning the position of D1 relative to D2/D3 (18). In this context it has been shown that the acid box has the potential to interact with the CHD on D2, enabling D1 to fold over onto D2 and D3. The results of the present study suggest an additional function for the acid box, which is to allow for CAM interactions with the FGFR. We do not know if the acid box binds directly to the CAMs or mediates an indirect interaction. However, it is perhaps worth speculating that a direct binding of the acid box region to the CAM might in turn open up the D2/D3 interface and thereby facilitates additional CAM interactions or indeed FGF interactions with the CAM homology domain. This in turn would explain why peptides derived from the CAM-homology domain (3) or peptides designed to bind to the CAM homology domain (16) are able to inhibit functional responses to CAMs and FGFs (3, 16). Nonetheless, it is absolutely clear that the FGFR CAM-homology domain is not absolutely required for an initial or basal CAM/FGFR interaction, and it remains possible that CAMs do not interact with this domain under any circumstances. If the latter were the case, it would leave us with the paradoxical situation that the originally contentious hypothesis that CAMs might signal via the FGFR that was formulated based on the presence of the CAM-homology domain in the FGFR has largely been substantiated in the absence of any role for that domain in the interaction. It would also fail to explain why peptides derived from this region of the FGFR, and/or peptides designed to bind to this region of the FGFR, inhibit CAM responses (see above).

FGF1 and FGF2 bind primarily to D3, the linker region between D3 and D2, and to a small region of D2 that contains the HAV motif (165EKRHLHAVPAN173) (16). Our studies and others (12) have shown that these regions are not required for NCAM or N-cadherin binding. In contrast, CAM but not FGF2 binding requires the acid box motif within the D2 and D3 linker region. The observation that CAMs can interact with regions of the receptor that are not involved in binding FGFs clearly suggests that both “ligands” might be able to simultaneously engage the same FGFR, and in support we have been unable to
compete out CAM/FGFR interactions with micromolar concentrations of FGFR2.\textsuperscript{4} The anti-FGFR antibodies used in our clustering and immunoprecipitation experiments specifically recognize the acid box region of the FGFR1 (177) or the cytoplasmic domain of the FGFR1 (flg). Thus we can conclude that NCAM and N-cadherin can interact with the 3 lg domain acid box containing FGFR1 but can make no comment on the other FGFRs as we did not test antibodies that specifically recognize these distinct gene products. However, given that NCAM can interact with the FGFR4 (13), and that the acid box can be found in all four FGFRs (17, 26), the above CAMs are likely to be able to interact with all four FGFRs provided that the acid box is present. Importantly, emerging studies are providing good evidence for the CAM/FGFR interactions being direct (49).

In addition to alternative splicing of the FGFRs, other factors probably regulate CAM/FGFR interactions. In this context, the fact that FGFR immunoprecipitation pulls down CAMs from control cell lysates suggests that the interaction is to some extent constitutive. However, we never found the TGFR co-localized with N-cadherin or NCAM at points of cell-cell contact where the CAMs can accumulate based on their homophilic binding activity and participate in stabilizing cell-cell contacts. To our knowledge, there has been no suggestion that the FGFR would co-operate in this “adhesive” function of the CAMs and therefore no reason for CAMs and FGFRs to co-localize at these sites. This raises the possibility that the FGFR only interacts with the more dynamic pool of CAMs that function in processes such as cell migration and axonal growth and suggests that there must be a mechanism to exclude the FGFRs from stable clusters of CAMs that accumulate at cell-cell junctions.

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