The neural cell adhesion molecule NCAM binds glial cell line-derived neurotrophic factor (GDNF) through specific determinants located in its third immunoglobulin (Ig) domain. However, high affinity GDNF binding and downstream signaling depend upon NCAM co-expression with the GDNF co-receptor GFRα1. GFRα1 promotes high affinity GDNF binding to NCAM and down-regulates NCAM-mediated homophilic cell adhesion, but the mechanisms underlying these effects are unknown. NCAM and GFRα1 interact at the plasma membrane, but the molecular determinants involved have not been characterized nor is it clear whether their interaction is required for GFRα1 regulation of NCAM function. We have investigated the structure-function relationships underlying GFRα1 binding to NCAM in intact cells. The fourth Ig domain of NCAM was both necessary and sufficient for the interaction of NCAM with GFRα1. Moreover, although the N-terminal domain of GFRα1 had previously been shown to be dispensable for GDNF binding, we found that it was both necessary and sufficient for the efficient interaction of this receptor with NCAM. GFRα1 lacking its N-terminal domain was still able to potentiate GDNF binding to NCAM and assemble into a tripartite receptor complex but showed a reduced capacity to attenuate NCAM-mediated cell adhesion. On its own, the GFRα1 N-terminal domain was sufficient to decrease NCAM-mediated cell adhesion. These results indicate that direct receptor-receptor interactions are not required for high affinity GDNF binding to NCAM but play an important role in the regulation of NCAM-mediated cell adhesion by GFRα1.

Many growth factors exert their effects through binding and activation of multicomponent receptor complexes. Different receptor subunits often play distinct roles in the complex, such as ligand binding and transmembrane signaling. The extent to which direct interactions between individual components in these complexes are required for receptor function is unclear and has been the subject of some debate. For example, neurotrophin high affinity binding has been shown to require both the p75NTR and Trk receptor subunits (4–6), but it is still unclear whether direct interaction between these two receptors underlies the generation of high affinity sites (7–9). In the glial cell line-derived neurotrophic factor (GDNF)2 ligand family, four structurally related glycosylphosphatidylinositol-anchored receptor subunits (GFRα1 to 4) provide ligand-specific binding activity. Structural and functional studies of GFRα molecules have distinguished N-terminal, central, and C-terminal domains of roughly 100, 200, and 100 residues, respectively, herein termed domains I, II, and III (3, 10). The central domain II has been shown to be both necessary and sufficient for GDNF binding (3). GFRα proteins cooperate with alternative transmembrane subunits for downstream signaling, such as the RET receptor tyrosine kinase (11) and the neural cell adhesion molecule NCAM (2). Although RET has on its own no affinity for GDNF, chemical cross-linking studies have shown that it can make direct contact with this ligand in complex with the GFRα1 subunit (11, 12). RET and GFRα1 may interact to some degree even in the absence of GDNF (13–15), but this interaction is not readily detectable by standard co-immunoprecipitation experiments.

Unlike RET, NCAM can interact directly with GDNF (2). The extracellular domain of NCAM comprises five N-terminal Ig domains followed by two fibronectin-like domains, and recent work has delineated the sequences in NCAM involved in GDNF binding (1). The third NCAM Ig domain was found to be necessary and sufficient for GDNF binding, and a combination of molecular modeling and site-directed mutagenesis studies identified 4 amino acid residues in this domain that are required for the interaction of NCAM with GDNF. Interestingly, mutation of these residues abolished the ability of NCAM to bind GDNF but left intact its capacity to mediate cell adhesion, indicating that these two functions are genetically separable (1). Although NCAM can interact with GDNF on its own, high affinity binding and downstream signaling require co-expression of the GFRα1 co-receptor (2). In addition, co-expression of GFRα1 attenuates the ability of NCAM to mediate homophilic cell adhesion in a dose-dependent manner (2), a GFRα1 function that is independent of GDNF. NCAM and GFRα1 interact at the plasma membrane in the absence of GDNF, but the molecular determinants involved have not been

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2 The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; NCAM, neural cell adhesion molecule; PBS, phosphate-buffered saline; HA, hemagglutinin.
characterized and the functional significance of NCAM/GFRα1 interaction is unclear. GFRα1 binding to NCAM could conceivably induce conformational changes in the latter that allow enhanced GDNF binding. Alternatively, GFRα1 may potentiate GDNF binding to NCAM by ligand concentration and presentation, without physical contact with NCAM, a model that has also been proposed to explain the role of p75NTR in neurotrophin high affinity binding (16). Finally, it is not clear whether direct NCAM/GFRα1 interactions are required for the ability of GFRα1 to inhibit NCAM-mediated cell adhesion.

In this study, we have characterized the sequences in NCAM and GFRα1 that mediate their interaction, using a panel of deletion and single domain constructs. A GFRα1 molecule that was markedly impaired in its ability to interact with NCAM but that retained normal GDNF binding was generated. This allowed us to probe the role of NCAM/GFRα1 interactions in NCAM-mediated cell adhesion and high affinity GDNF binding.

EXPERIMENTAL PROCEDURES

NCAM and GFRα1 Deletion Constructs—The NCAM deletion constructs have been described previously (1). Domain boundaries in GFRα1 were assigned based on the structure of domain II of GFRα3 (10). The boundaries used were as follows (numbering excludes the 17-residue signal peptide): Domain I, Ser-1-Lys-133; domain II, Gly-134-Trp-337; domain III, Gln-338-Gly-407; glycosylphosphatidylinositol-anchoring signal, Leu-408-Ser-451. The GFRα1–ΔI construct was made by PCR, using Phusion DNA polymerase (Finnzymes), with a sense primer corresponding to the beginning of domain II and an antisense primer corresponding to the end of the full-length GFRα1 cDNA. The GFRα1–I and GFRα1–II constructs were made by fusing two PCR fragments. One fragment was made using primers corresponding to the beginning and end of the respective domain. The other fragment was made using a sense primer corresponding to the beginning of the sequence responsible for glycosylphosphatidylinositol anchoring the protein and an antisense primer corresponding to the end of the GFRα1 cDNA. The GFRα1–ΔIII construct was made similarly, with the same second fragment containing the glycosylphosphatidylinositol-anchoring site but this time with the first fragment made using the full-length sense primer and the domain II end antisense primer in order to exclude only domain III from the construct. The GFRα1–ΔII construct was made similarly as for the GFRα1–I construct, but with the second fragment made with a sense primer corresponding to the beginning of domain III, thus excluding only the second domain from the construct. The PCR fragments were digested with SfiI and NotI (New England Biolabs) and ligated into a SfiI/NotI-digested pSecTag 2A-Hygro vector (Invitrogen) modified with a Myc tag insertion between the secretion tag and the SfiI site. In the cases where two PCR fragments were ligated, the ligation site between the secretion tag and the SfiI site. In the cases where two PCR fragments were ligated, the ligation site between the secretion tag and the SfiI site. In the cases where two PCR fragments were ligated, the ligation site between the secretion tag and the SfiI site. In the cases where two PCR fragments were ligated, the ligation site between the secretion tag and the SfiI site. In the cases where two PCR fragments were ligated, the ligation site between the secretion tag and the SfiI site. In the cases where two PCR fragments were ligated, the ligation site between the secretion tag and the SfiI site.

Selective Immunoprecipitation of Surface Molecules—In order to study only receptor molecules expressed at the cell surface, we performed selective immunoprecipitation of cell surface molecules in living cells. COS-7 cells grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in 100-mm plates were transfected with 20 μg of the appropriate DNA constructs with 2 μg of polyethyleneimine/μg of DNA. Two days after transfection, cell monolayers were washed with phosphate-buffered saline (PBS) and incubated with 10 μg/ml anti-HA antibodies (clone 12CA5; Roche Applied Science) or anti-Myc antibodies (clone 71D10; Cell Signaling) in binding buffer (PBS, 1 mg/ml bovine serum albumin, 1 mg/ml d-glucose, 0.1 mM CaCl2, 0.1 mM MgCl2) for 1 h at 4 °C. The plates were then washed six times with PBS and lysed with 0.75 ml of lysis buffer (PBS, 60 mM octyl-β-glucoside, 1% Nonidet P-40, 10% glycerol, 2 mM EDTA, and protease inhibitors), and cells were collected with a cell lifter. After a 1-h incubation at 4 °C with shaking, lysates were centrifuged for 10 min at 10,000 × g. GammaBind protein G-Sepharose (Amersham Biosciences) was added to the cleared lysates, and the samples were incubated for 1 h with shaking, centrifuged at 500 × g, and washed four times. In the case of PNGaseF (New England Biolabs) treatment, samples were incubated with PNGaseF (without any addition of buffers or additives) for 2 h at 37 °C, either before or after the protein G-Sepharose pulldown. The samples were run on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Autoradiographs were scanned in a STORM 840 Phosphorimager. The membranes were immunoblotted with anti-HA or anti-Myc antibodies, developed with enhanced chemifluorescence (GE Healthcare), and scanned in a STORM 840.

GDNF Binding and Chemical Cross-linking—COS-7 cells grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in 100-mm plates were transfected with 20 μg of the appropriate DNA constructs with 2 μg of polyethyleneimine/μg of DNA. Two days after transfection, the plates were washed three times with PBS and incubated with 125I-GDNF (labeled by the lactoperoxidase method) at a concentration of 20 ng/ml in binding buffer for 2 h at 4°C with gentle rocking. After cross-linking for 45 min with EDAC/NHS-S (Pierce), plates were quenched with 50 mM glycine and washed three times with PBS.

Cell Adhesion Assay—Jurkat cells were transfected in 12-well plates with NCAM and GFRα constructs together with either green fluorescent protein or Ds-Red-encoding plasmids using FuGENE 6 (Roche Applied Science) in 2 ml of complete medium containing 10% fetal calf serum. On the following day, 100 μl each of green fluorescent protein- and Ds-Red-transfected cells were combined and mixed with 100 μl of serum-free medium in 48-well plates. After 48 h of incubation, green cells, red cells, and cell aggregates were quantified under green and red fluorescence illumination on a motorized Axiovert 200 microscope controlled by OpenLab software (Improvision). Cell adhesion was calculated as the percentage of green cells present in clusters that also contained red cells and normalized to the value obtained with full-length NCAM.

RESULTS

Glycosylation of GFRα1—GFRα1 is a glycoprotein in mammalian cells, subjected to both N- and O-glycosylation. We first set out to determine whether these modifications play any role in the ability of GFRα1 to interact with NCAM. NCAM molecules HA-tagged at their N terminus were selectively immuno-
precipitated from the surface of transfected cells as previously described (1), fractionated by SDS/PAGE, and immunoblotted to detect co-immunoprecipitated GFRα1 molecules carrying an N-terminal Myc tag. Removal of N-glycosylations by digestion with PNGaseF, either prior to or after immunoprecipitation, did not diminish the ability of NCAM to pull down GFRα1 from the plasma membrane (Fig. 1A). An increase in the interaction between the two molecules was detected after PNGaseF digestion (Fig. 1A), indicating a possible negative role of N-glycosylation. O-glycosylation is predicted to be clustered in a threonine-rich segment in the C-terminal domain III of GFRα1. We found that GFRα1-ΔIII, lacking all C-terminal O-glycosylations, interacted with NCAM equally well as the wild type molecule (Fig. 1B). We conclude from these data that neither N- nor O-glycosylation is required for GFRα1 binding to NCAM at the plasma membrane.

**GFRα1 Binding Determinants in NCAM**—To determine sites in NCAM involved in its interaction with GFRα1, we took advantage of a large collection of NCAM deletion constructs previously generated at our laboratory (1). A schematic diagram of the domain structures of NCAM and GFRα1 is shown in Fig. 2, A and B, along with a depiction of the rationale used for the nomenclature of deletion constructs. The C-terminal deletion analysis shown in Fig. 2C indicates the relative importance of domains 4 (Δ4–7) and 2 (Δ2–7), whereas domains 6–7 (Δ6–7), 5 (Δ5–7), and 3 (Δ3–7) appeared to be dispensable for NCAM binding to GFRα1. The fact that deletion of domains 3 to 7 (Δ3–7) or 1 to 4 (Δ1–4) decreased the interaction with GFRα1 confirmed the importance of domain 4 (Fig. 2D). It would, however, appear that domain 2 can be deleted without affecting binding as long as domain 4 is still present in NCAM (as in Δ1–2 in Fig. 2D). The sufficiency of individual NCAM domains for GFRα1 binding was assessed using constructs carrying single domains as the sole extracellular region of the molecule. Domain 4 was found to bind GFRα1 very well on its own, whereas other domains bound only weakly or not at all (Fig. 2E). In a separate analysis, domain 4 was compared against domains GFRα1-ΔII, respectively) or consisted of each single domain on its own (GFRα1-I and GFRα1-II, respectively). The N-terminal domain I of GFRα1 interacted very efficiently with NCAM on its own at levels comparable with those of the full-length molecule, and its deletion markedly reduced the interaction (Fig. 3A). In contrast, the larger GDNF binding region (domain II) of GFRα1 bound weakly on its own to NCAM and could be deleted without affecting the interaction (Fig. 3A). These data indicate that the N-terminal domain of GFRα1 is sufficient on its own and necessary for an efficient interaction with NCAM and therefore contains the main NCAM binding determinant in GFRα1. On the other hand, domain II was not necessary for NCAM binding and although it displayed some binding capacity on its own, this was much weaker. Previous work has shown that domain I of GFRα1 is dispensable for GDNF binding (3) and for ligand-induced cell adhesion, a novel cell-cell interaction mechanism mediated by GDNF-bound GFRα1 (17). NCAM binding therefore represents the first identified function for this domain of the GFRα1 molecule. To test whether the single domains identified in NCAM and GFRα1 were sufficient to interact on their own, we performed co-immunoprecipitation studies between GFRα1 domain I and the first four N-terminal Ig domains of NCAM. In agreement with our previous observations, GFRα1-I interacted most strongly with the fourth Ig domain of NCAM (Fig. 3B), indicating that these two domains comprise the main interaction interface between the two molecules.

**GDNF Binding Potentiation to NCAM and Receptor Complex Formation**—The finding that GDNF and NCAM binding activities resided to a large degree in different domains of the GFRα1 molecule indicated that these two functions may be genetically separable and allowed us to test the functional relevance of the GFRα1/NCAM interaction for the ability of GFRα1 to regulate NCAM function. GFRα1 can potentiate GDNF binding to NCAM (2), and a tripartite 2:2:2 complex between the three proteins has been detected in intact cells by chemical cross-

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**NCAM/GFRα1 Interactions**

**A**

**B**

**FIGURE 1.** *G*~Glycosylation of GFRα1.* A, N-glycosylation does not affect GFRα1 interaction with NCAM. GFRα1 (Myc-tagged) and NCAM (HA-tagged) were co-expressed in COS-7 cells, and NCAM was immunoprecipitated from the plasma membrane using HA antibodies. The upper panel shows co-immunoprecipitation (IP) of Myc-tagged GFRα1. The lower panel shows HA reprobing. PNGaseF treatment was used to test the role of GFRα1 N-glycosylation in its interaction with NCAM. Panels to the right correspond to the total cell lysates. B, O-glycosylation does not affect GFRα1 interaction with NCAM. Co-immunoprecipitation is shown of Myc-tagged wild type GFRα1 (GFRα1^T^) and GFRα1 lacking O-glycosylated domain III (GFRα1-ΔIII) with HA-tagged NCAM. The upper panel shows co-immunoprecipitation of Myc-tagged GFRα1. Bottom panels correspond to the total cell lysates.
The mechanism by which GFRα1 enhances GDNF binding to NCAM is unknown. Interaction between GFRα1 and NCAM could conceivably induce a change in conformation in NCAM that allows high affinity GDNF binding. If this were the case, the N-terminal domain I of GFRα1 should on its own also be able to potentiate GDNF binding to NCAM. Alternatively, GFRα1 may enhance GDNF binding by ligand concentration and presentation. Unlike the previous case, this mechanism only requires that GFRα1 is able to bind GDNF, and therefore GFRα1 lacking domain I should be able to enhance GDNF binding to NCAM to the same extent as the full-length receptor. To distinguish between these possibilities, we compared the abilities of full-length GFRα1, GFRα1-I, and GFRα1-ΔI to potentiate binding of 125I-GDNF to NCAM by chemical cross-linking and immunoprecipitation. As expected, co-expression of full-length GFRα1 significantly increased GDNF binding to NCAM.
binding to NCAM (Fig. 4). However, the N-terminal domain of GFRα1 was on its own unable to potentiate GDNF binding to NCAM (Fig. 5). In contrast, GFRα1 lacking this domain was still able to enhance GDNF binding to NCAM at levels comparable with the full-length molecule (Fig. 4). These results indicate that a preformed complex between GFRα1 and NCAM is not required for high affinity GDNF binding to NCAM. In support of this idea, bands corresponding to the tripartite complexes of NCAM, GDNF, and either full-length GFRα1 or GFRα1-ΔI could be detected after chemical cross-linking and immunoprecipitation (Fig. 4), indicating that GFRα1-ΔI can still be incorporated into a tripartite complex despite being much less efficient at binding NCAM.

**Regulation of NCAM-mediated Cell Adhesion by GFRα1**—A second regulatory function of GFRα1 on NCAM involves its ability to down-regulate NCAM-mediated homophilic cell adhesion (2). We therefore tested whether direct GFRα1/NCAM interactions are required for this activity by comparing the abilities of full-length GFRα1, GFRα1-ΔI, and GFRα1-ΔI to inhibit NCAM-mediated cell adhesion. As expected, full-length GFRα1 inhibited NCAM-mediated cell adhesion in a dose-dependent manner (Fig. 5). Importantly, GFRα1-ΔI was as potent as the full-length molecule (Fig. 5), indicating that the N-terminal domain of GFRα1 is by itself sufficient to negatively regulate NCAM-mediated cell adhesion. In comparison, GFRα1-ΔI displayed a markedly reduced potency (Fig. 5). These results support the notion that direct contact between NCAM and GFRα1 molecules is required for regulation of NCAM-mediated cell adhesion and indicate that the N-terminal domain of GFRα1 plays an important role in the ability of GFRα1 to efficiently inhibit this activity.

**DISCUSSION**

In this study, we have identified the molecular determinants in NCAM and GFRα1 that underlie their interaction at the plasma membrane of mammalian cells. We have shown that neither N- nor O-glycosylation of GFRα1 is required for its binding to GDNF or interaction with NCAM and found that...
N-glycosylation may in fact play a negative role in the interaction between the two receptor molecules. We have also demonstrated that the fourth Ig domain of NCAM comprises the main determinant mediating the association of this molecule with GFRα1, with a smaller contribution from the second domain. Our previous studies had shown that GDNF interacts with a set of 4 positively charged residues in the third Ig domain of NCAM and that this domain is both necessary and sufficient for NCAM binding to GDNF (1). Because neither the second nor fourth Ig domains of NCAM appears to play any role in GDNF binding, together with the results from our present study these data indicate that different regions of the NCAM molecule mediate its interactions with GDNF and GFRα1. We have also found that the N-terminal domain of GFRα1 constitutes a crucial determinant for the efficient interaction between GFRα1 and NCAM. Because this domain is dispensable for GDNF binding to GFRα1 (3), this represents the first function that has been attributed to this region of the GFRα1 molecule. Using single-domain constructs, we could show that the fourth Ig domain of NCAM and the N-terminal domain of GFRα1 can efficiently interact in the absence of any other receptor sequences, suggesting that they represent the main binding interface between the two molecules. Earlier electron microscopy studies have indicated a heavily kinked conformation of the NCAM extracellular domain (18, 19). This would conceivably allow a relatively C-terminal region in this molecule, such as the fourth Ig domain, to interact with the N-terminal domain of GFRα1. Importantly, neither the three-dimensional structure nor the topology of the latter is currently known, and it is likely that the linker sequence connecting this domain to the ligand binding region will afford a great degree of flexibility between these two sections of the GFRα1 molecule.

Despite its markedly reduced binding to NCAM, GFRα1-D1 could still be incorporated into a tripartite complex containing GDNF, GFRα1, and NCAM. This suggests that prior complex formation between GFRα1 and NCAM, and hence allosteric changes in the latter, are not required for GDNF high affinity binding to NCAM. This notion is reminiscent of the role of GFRα1 in RET activation by GDNF, in which significant RET binding to either GDNF or GFRα1 can only be detected after formation of a GDNF/GFRα1 complex. It is, however, still unclear whether GDNF high affinity binding to NCAM requires prior GDNF binding to GFRα1 or whether a tripartite interaction can occur simultaneously without preassembly of a binary complex. Some studies have attributed a ligand concentration/presen-

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