Neurotrophins regulate differentiation and survival of vertebrate neurons through binding to members of the Trk family of receptor tyrosine kinases and to a common low affinity receptor, p75\(^{LNGFR}\). The specificity of neurotrophin action is determined by their selective interaction with the different members of the Trk family; TrkA, TrkB, and TrkC serve as cognate receptors for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 (NT-3), respectively. Unlike nerve growth factor and brain-derived neurotrophic factor, NT-3 can to some extent also bind and activate non-cognate TrkA and B receptors, although the physiological relevance of these interactions is unclear. Previous studies established that neurotrophins use an extended surface for binding to cognate Trk receptors, while binding to p75\(^{LNGFR}\) is mediated by a localized cluster of positively charged residues. Here we show that the binding site of NT-3 to its non-preferred receptors TrkA and TrkB is dominated by two positively charged residues, Arg-31 and His-33, previously shown to constitute a main determinant of binding to p75\(^{LNGFR}\). Simultaneous mutation of these two residues into Ala completely abolished NT-3 binding and signaling through TrkA and greatly diminished binding and activation of TrkB. However, NT-3 binding and signaling through its cognate receptor TrkC was unaffected by the mutation. These results show that binding of NT-3 to p75\(^{LNGFR}\), TrkA, and TrkB is mediated by a common determinant, which is distinct from that recognized by TrkC and also different and more localized than the one recognized by TrkA and TrkB in their cognate ligands. Thus, although homologous regions in all neurotrophins are used for binding to Trk receptors, a given Trk may actually contact different residues in different neurotrophins. The mutant NT-3 described here may be of greater advantage than native NT-3 when a trophic activity needs to be specifically targeted to TrkC-expressing neurons and provides a monospecific neurotrophin for future therapeutic development.

Vertebrate neurons are dependent on the continuous supply of a group of proteins generally called neurotrophic factors for their development, differentiation, and survival. The neurotrophins are structurally and functionally related neurotrophic factors that function in the developing and adult nervous system (1-3). In mammals, the neurotrophin family presently includes four members, nerve growth factor (NGF)\(^1\) (4), brain-derived neurotrophic factor (BDNF) (5, 6), neurotrophin-3 (NT-3) (7-12), and neurotrophin-4 (NT-4) (13, 14), also called NGF-3 (15), which share approximately 50% amino acid sequence identity. An additional neurotrophin molecule with structural and functional similarities to NGF has recently been isolated from the platy fish and named NT-6 (16), despite being only the third neurotrophin identified in this species. The neurotrophins are non-covalently linked homodimers of two highly basic 120-residue long polypeptide chains. Each protomer contains three disulfide bridges forming a cysteine knot at the bottom of the structure (17) and three pairs of antiparallel \(\beta\)-strands connected by \(\beta\)-hairpin loops (18). The latter contain most of the variable residues found among all the neurotrophins (19).

Neurotrophins mediate their action by binding to two classes of specific neurotrophin receptors. The low affinity neurotrophin receptor (p75\(^{LNGFR}\)) is a transmembrane glycoprotein that binds all neurotrophins with equal affinity (20, 21). p75\(^{LNGFR}\) is structurally related to the tumor necrosis factor receptor and to CD40, and it contains a short cytoplasmic domain of unknown biochemical function. In contrast to p75\(^{LNGFR}\), members of the Trk family of tyrosine kinase receptors exhibit ligand-dependent activation of endogenous tyrosine kinase activity and can thus clearly function as signaling receptors (22, 23). Specificity of neurotrophin action is achieved by their selective interaction with cognate members of the Trk family of receptors. Thus, p140\(^{Trk}\) (TrkA) is the cognate receptor for NGF, p145\(^{TrkB}\) (TrkB) serves as receptor for BDNF and NT-4, while p145\(^{TrkC}\) (TrkC) is the cognate receptor for NT-3 (22). Unlike the other neurotrophins, NT-3 can also interact with non-cognate Trk receptors, although the physiological importance of these interactions is still unclear (24, 25).

Studies of structure-function relationships in the neurotrophins have identified amino acid residues important for their interaction with cell surface receptors (26). The binding site of neurotrophins to p75\(^{LNGFR}\) is dominated by positively charged residues located in two spatially close variable loop regions (27, 28). Although the precise nature and spatial arrangement of the side chains involved vary among the different proteins, all neurotrophins utilize a similar cluster of positive charges for binding to p75\(^{LNGFR}\) (28). Disruption of p75\(^{LNGFR}\) binding does not interfere with binding and activation of cognate Trk receptors, although it does appear to affect responsiveness to some neurotrophins in cells co-expressing p75\(^{LNGFR}\) and Trks (28).\(^2\)
In contrast to p75NGFR, the interaction of neurotrophins with cognate Trk receptors involves multiple contacts distributed along the side of the neurotrophin dimer, parallel to the 2-fold symmetry axis (19, 29). Taken together, data from different studies on neurotrophin-Trk interactions support a model in which conserved residues in \( \beta \)-strands provide the contacts with the highest binding energy, while variable residues in turns and loop regions along this surface determine biological specificity, either by contributing directly with contacts of lower energy to cognate receptors or indirectly, by preventing interaction with inappropriate receptors. Loss-of-function studies have shown that the binding epitope of NT-3 to its cognate receptor TrkC appears dominated by the conserved residue Arg-103, together with Glu-10, Tyr-51, Glu-54, and Arg-56 (29).

In addition, gain-of-function experiments have established the role of variable residues 39-48 in determining the specificity of binding of NT-3 to TrkC (30).

Although NT-3 can also interact with TrkA and TrkB, this neurotrophin lacks some of the major determinants of binding found in the preferred ligands of these receptors, NGF and BDNF (19, 30), suggesting that the interaction of NT-3 with its non-preferred receptors may be mediated by residues conserved in all three neurotrophins. We show here that, surprisingly, the binding site of NT-3 to TrkA and TrkB is dominated by two positively charged variable residues, Arg-31 and His-33, previously shown to constitute a main determinant of binding to p75NGFR. Mutation of these residues does not affect NT-3 binding or activation of TrkC. Thus, binding of NT-3 to p75NGFR, TrkA, and TrkB is mediated by a common epitope distinct from that recognized by its cognate receptor TrkC.

**EXPERIMENTAL PROCEDURES**

Cell Lines, Antisera, Proteins, and Plasmids—The generation of mouse M87–NIH3T3 fibroblasts expressing Trk receptors, kindly provided by George Yancopoulos, Regeneron Pharmaceuticals Inc., has previously been described (31, 32). Anti-pan Trk rabbit polyclonal antiserum 203, which recognizes TrkA, TrkB, and TrkC, was kindly provided by David Kaplan, National Cancer Institute, Frederick, MD. Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc. Purified mouse NGF was from Promega, and purified recombinant native NT-3 was obtained from Regeneron Pharmaceuticals Inc. Mutant R31A+H33A NT-3 was produced and purified from recombinant baculovirus-infected insect cells as described previously (28). For Trk riboprobe synthesis, cDNA fragments derived from chicken TrkB (kindly provided by Tom Large, Case Western University, Cleveland, OH) and chicken NGF (kindly provided by Finn Hallbo¨o¨k, Biomedical Center, Uppsala, Sweden) were subcloned into pBluescript KS+ (Stratagene).

Binding and Phosphorylation Assays—Purified neurotrophins were labeled with \( ^{125}\text{I} \) by the lactoperoxidase method as described previously (19). Steady state binding was measured in competition assays at 4°C essentially as described previously (28), except that binding mixtures were incubated on 96-well filter plates (Millipore) using \( 1 \times 10^5 \) cells/well. After equilibrium was reached (90–120 min of incubation), vacuum was applied and cells were washed briefly with ice-cold binding buffer. Filters were then collected and counted in a 

\( ^{125}\text{I} \) counter. Nonspecific binding was measured in a parallel incubation to which 300-1000-fold molar excess of unlabeled purified factor was added. All results were corrected for this nonspecific binding. The concentration of the mutant and wild type molecule that gave 50% binding (IC\(_{50}\)) was determined, and relative binding was calculated using the relationship: (mutant \( IC_{50} \)/wild type \( IC_{50} \)) \( \times 100 \). Assays of ligand-induced receptor tyrosine autophosphorylation were performed as described previously (19).

Bioassays—Biological activity of wild type and mutated neurotrophins in Trk-expressing M87–NIH3T3 fibroblasts was assayed as described previously (31, 32). Briefly, cells (\( 2 \times 10^4 \)well) were incubated with serial dilutions of neurotrophins in serum-free Dulbecco’s modified Eagle’s medium in 96-well plates. After a 4-day incubation, cellular acid proteinase activity was analyzed for TrkA, TrkB, and TrkC mRNAs by RNase protection analysis.
When embryonic day 8 chick nodose ganglion and dorsal root ganglion sensory neurons are cultured in the presence of 100 ng/ml NT-3, a prominent TrkC mRNA signal is recovered, indicating specific rescue of TrkC mRNA containing neurons by NT-3 (Fig. 4). No TrkC mRNA is recovered if the ganglia are cultured in medium without NT-3 (Fig. 4). In addition to TrkC mRNA, low but significant amounts of TrkB mRNA are also recovered from these cells after NT-3 treatment (Fig. 4; see also Ref. 33). The effects of NT-3 on TrkB mRNA containing sensory neurons could be due to TrkB and TrkC receptor mRNA coexpression, TrkB mRNA up-regulation, or genuine action of NT-3 through neuronal TrkB receptors. The possibility that NT-3 may in certain circumstances act through TrkB has previously been suggested (34). However, when chick dorsal root ganglion and nodose ganglion neurons were treated with 100 ng/ml of the R31A+H33A NT-3 mutant deficient in TrkB binding, both TrkC and TrkB mRNA signals were recovered at relative levels, which were comparable to those seen with wild type NT-3 (Fig. 4). Identical results were obtained when mutant and wild type NT-3 were tested at 20 ng/ml (not shown). Given that the mutant was previously found to be as potent as wild type NT-3 on sensory neurons (28), these results suggest that recovery of TrkB mRNA after NT-3 treatment is likely due to receptor coexpression and not to direct action of NT-3 on TrkB receptors.

**DISCUSSION**

In this study, a mutant NT-3 previously characterized with respect to its interactions with TrkC and p75LNGFR was used to investigate the role of positively charged amino acid residues in the interaction of NT-3 with its non-preferred receptors TrkA and TrkB. Unexpectedly, the same mutation that abolished binding of NT-3 to the low affinity receptor p75LNGFR (28) also impaired binding to and activation of TrkA and TrkB. The fact that NT-3 binding and signaling through its cognate receptor TrkC was unaffected by the mutation indicates that NT-3 interacts with p75LNGFR, TrkA, and TrkB through a common and localized determinant, which is distinct from that recognized by TrkC. Previous studies of TrkC binding determinants in NT-3 established the importance of conserved residues, especially Arg-103 (29), as well as variable residues, particularly positions 39–48 in variable region II (30). Thus, like NGF and BDNF (19), binding of NT-3 to its cognate Trk receptor appears to be mediated by an extended surface containing variable and conserved residues from different regions of the molecule. Although our results show that Arg-31 and His-33 do not appear to be essential functional epitopes for TrkC binding, their location in the TrkC binding surface suggests they could nevertheless form part of structural binding epitopes, involving multiple Van der Waal and hydrogen bonding contacts. Unlike functional epitopes, such determinants, taken individually, may not contribute sufficient binding energy to be easily identified from mutagenesis experiments.

Despite their importance for NT-3 binding to TrkA and TrkB, positively charged residues at the two positions studied here are not essential for binding of NGF or BDNF to these receptors. Mutation of positively charged residues Lys-32 and Lys-34 in NGF, corresponding to Arg-31 and His-33 in NT-3, affected only marginally the binding of NGF to TrkA (27). This
indicates that TrkA interacts with NGF and NT-3 in different ways, the latter being recognized through a more localized epitope, which only has marginal importance for the binding of the cognate ligand. Interestingly, BDNF lacks positively charged residues at these positions, indicating that TrkB may actually contact different residues in BDNF and NT-3. Despite the lack of positive charges in this region, three positively charged residues (Lys-95, Lys-96, Arg-97) in a spatially close loop in BDNF have been shown to play a role in binding to and activation of TrkB (19, 28). A recently reported crystal structure of a BDNF/NT-3 heterodimer (35) suggests that the side chains of Arg-31 and His-33 in NT-3 could occupy positions equivalent to those of the positively charged residues in BDNF and therefore may be able to provide similar contacts with the TrkB receptor. In any case, while mutation of Arg-31 and His-33 in NT-3 resulted in a 20-fold decrease in the affinity of binding to TrkB, only a 3–5-fold reduction was observed after simultaneous mutation of Lys-95, Lys-96, and Arg-97 in BDNF (19), indicating that the TrkB binding site of this neurotrophin includes additional determinants.

Although NT-3 can interact with TrkA and TrkB and induce receptor activation and survival in fibroblasts ectopically expressing these two receptors (36–38), NT-3 does not appear to be an efficient agonist of TrkA and TrkB in primary neurons and neuronal cell lines (24, 25, 32, 39). It has been proposed that the efficiency of the interaction of NT-3 with non-cognate receptors may depend on the cellular context (32) and on the presence of accessory receptor molecules such as p75<sup>LNGFR</sup> (24, 25). Efficient interaction of NT-3 with TrkA appears also to depend on the specific isoform of TrkA expressed. In particular, a TrkA splice variant containing a 6-amino acid insert in the proximal part of the extracellular domain (40), but not TrkA lacking the insert, has been shown to mediate NT-3-dependent neurite outgrowth when overexpressed in PC12 cells (25). A recent study reported TrkB-dependent neurotrophic activities of NT-3 on developing sensory neurons isolated from mouse embryos homozygous for a null mutation in the <i>trk</i><sub>C</sub> gene (41).

Our analysis of the survival of subpopulations of embryonic chick sensory neurons suggests that in normal cells NT-3 acts predominantly through its cognate receptor TrkC. Comparison of the effects of wild type and mutant NT-3 did not reveal specific actions of NT-3 through TrkB; the TrkB-expressing neurons that responded to NT-3 appeared to co-express TrkC. The differences between our study and the one with neurons from <i>trk</i><sub>C</sub><sup>2</sup> mice may be due to the high doses of NT-3 (over 20-fold higher or up to 100 nM) used in the latter and suggest that, in the absence of TrkC, NT-3 may function through TrkA and TrkB albeit with low efficiency. A more localized binding site of NT-3 to TrkA and TrkB may be responsible for the lower affinity and more limited biological actions of NT-3 on these two receptors.

Neurotrophins are likely to have diverged from a common ancestor. Present time neurotrophins may still share common determinants of binding to different Trk family members, while their variable regions have probably evolved through the acquisition of specific binding determinants that allow specificity. Why have the interactions of NT-3 with TrkA and TrkB been preserved during neurotrophin evolution? Are these molecular relics of interactions between ancestral neurotrophins and Trks? The fact that the R31A+H33A mutation did not affect
binding to TrkC suggests that these residues could not have been maintained because of their importance in TrkC binding as they are dispensable for binding to this receptor. On the other hand, the complete inability of the R31A+H33A mutant to interact with p75NGFR suggests that a need to maintain binding to the low affinity receptor could have imposed evolutionary constraints to the rate of divergence of these residues. Thus, NT-3 binding to TrkA and TrkB may be an indirect consequence of the overlap with the p75NGFR binding site, suggesting that non-cognate interactions of NT-3 with TrkA and TrkB may be physiologically irrelevant. Alternatively, NT-3 could have yet unknown functions, required for normal development and survival, mediated via TrkA and TrkB. Because neurotrophins are dispensable for binding to this receptor. Tom Large for the chicken sera, George Yancopoulos for Trk-expressing fibroblasts and recombinant native neurotrophins, Finn Hallböö for the chicken ilial help, and Mike Fainzilber for comments to the manuscript. Nilsson for excellent technical assistance, Lotta Johansson for secretarial help, and Mike Fainzilber for comments to the manuscript.

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