The binding domain of p75NGFR contains four "repeats" of a 6-cysteine pattern. To test whether these repeats have any structural or functional independence, each repeat has been separately deleted. In each case, deletion led to the loss of most nerve growth factor (NGF) binding activity. The epitopes of two monoclonal antibodies, MC192 and 271c, could be distinguished, however. Repeat IV was found to be unnecessary for binding MC192, whereas Repeat I was not required for binding 217c. This suggests that either terminal repeat can be removed without loss of native-like structure in the remaining repeats. Trp<sup>555</sup> in the fourth repeat forms an essential part of the 217c epitope but is not required for either MC192 or NGF binding. Deletion of the linker region between the membrane-spanning domain and the cysteine-rich domain does not affect the binding of NGF or MC192, and has only a slight, if any, effect on 217c binding. Cyclic permutation of the four repeats failed to yield protein capable of binding NGF or MC192.

The low affinity receptor, p75NGFR (1), is one of at least two different receptor proteins that bind nerve growth factor (NGF). The function of p75NGFR itself is still unknown but it is able to bind not only NGF, but also three other neurotrophins: brain-derived neurotrophic factor (BDNF) (2,3), neurotrophin-3 (NT-3) (5), and the recently described neurotrophin-4 (NT-4) (4). The fact that these have different effects on different cells suggests that p75NGFR complexes with other protein factors that convey the specificity of signal transduction. The binding domain of this receptor shares homology with several other proteins, including two different tumor necrosis factor receptors (5-7), certain cell surface antigens of unknown function (8-11), and a viral open reading frame (12). Thus an understanding of the structural domains of p75NGFR could provide information on ligand binding, on the cellular role of this receptor and its interactions with other proteins, and on the structure and function of other members of the receptor family.

Based upon the cDNA sequence (1), rat p75NGFR is a 396-amino acid protein with a single membrane-spanning domain. The extracellular portion of the polypeptide chain begins with an N-terminal cysteine-rich domain, 180 amino acid residues in length, which is necessary and sufficient for NGF binding (13). It is linked to the membrane by a 62-residue domain rich in proline, serine, and threonine. By analogy with other proteins (14) this latter domain is believed to be O-glycosylated and to function as a stalk to extend the NGF binding domain beyond the glyocalyx of the cell surface.

The cysteine-rich domain, when secreted from cells as a truncated version of the receptor had previously been shown to bind NGF (13). In order to compare the affinity of NGF for the cysteine-rich domain relative to the wild type receptor when both are membrane-bound, we have specifically deleted most of the linker region, generating a mutant protein in which the cysteine-rich domain is closely connected to the membrane-spanning domain. We show that this protein can bind NGF to an extent comparable to intact receptor when expressed in cos 7 cells.

Binding of either NGF or the MC192 antibody (15) is completely eliminated when the receptor protein is reduced. Thus at least some of the 24 cysteine residues in the domain must be in disulfide linkage and it is reasonable to suppose that all may be, since extracellular proteins are usually found in the oxidized form and more than a single free sulphhydryl group is seldom seen (16). Based upon the nearly identical spacing of 6 cysteine residues in each of four "repeats," as well as a low level of additional internal homology, the domain may be formally divided into four subdomains, which suggests that the overall structure evolved by gene duplication. Whether these subdomains are truly structurally or functionally distinct, however, is unclear. It was the purpose of this study to shed further light on this question.

Previously, we had shown that two N-terminal deletion mutants displayed a small but significant ability to bind NGF (13). In one, most of the first repeat was deleted, and in the other, nearly all of the first repeat and half of the second. In each case, however, 2 or more cysteines were left in an altered environment. The fact that any binding at all was observed we took as evidence that the remaining repeats must have folded independently. If all the disulfides had been scrambled, one would expect the protein to bear no resemblance to the native structure.

In the present report, we describe the separate, precise removal of each of the four repeats. The results show that no repeat can be deleted without loss of most of the binding activity for NGF. Interaction with two different monoclonal antibodies, however, lends support to our conclusion that the
Deletion of 1st repeat: 
- 5' GCCACATTAATTCCG 3'
- 5' GTCAAGGCGTGCACGACCCTGAGG 3'
- 5' GCCACATTAATTCCG 3'
- 5' GCACGGTTCACCTACACTT 3'

Deletion of 2nd repeat (2 versions of junction): 
- 5' GTTACGAACTGCTCCTGACACTCTACGTTCTC 3'
- 5' GTTACGAACTGCTCCTGACACTCTACGTTCTC 3'

Deletion of 2nd repeat (2 versions of junction): 
- 5' ATCTCGCCTCTGACACTCTACGTTCTC 3'
- 5' ATCTCGCCTCTGACACTCTACGTTCTC 3'

Deletion of 4th repeat: 
- 5' GACAGGCGTACCTTACACACTACGTTCTC 3'
- 5' GACAGGCGTACCTTACACACTACGTTCTC 3'

Cyclic permutation, II-III-IV-I (3 versions of junction IV-I):
- 5' ATGCCCAGAGGGCACAT 3'
- 5' ATGCCCAGAGGGCACAT 3'
- 5' ATGCCCAGAGGGCACAT 3'

FIG. 1. Oligonucleotides used to construct mutants by PCR mutagenesis.

Dubbelco's modified Eagle's medium (DMEM) containing 5% horse serum and 5% supplemented calf serum at a CO2 pressure of 7.5%. Transfections were carried out by a modification of the method of Luthman and Magnusson (27). Cells were washed three times with medium minus serum before transfection. To each 100-mm plate, a transfection mixture containing 10 μg of plasmid DNA plus 1 mg of DEAE dextran was added, and the cells were incubated for 30 min at 37°C. Four ml of medium containing 0.08 mM chloroquine was added, and the incubation continued for 2.5 h. The cell supernatant was then removed and the cells were shocked by addition of 4 ml of medium containing 10% dimethyl sulfoxide. After 2.5 min at room temperature, the shock solution was removed and medium containing serum was restored to the cells. Cells were harvested for analysis 48–72 h after transfection.

Cross-linking of [125I]NGF to Transfected Cells and Product Analysis—Cells were pelleted and resuspended at a density of 10^5 cells/ml in binding buffer (Kreb's Henseleit solution) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). 125I was added to give an NGF concentration of 2 nM. The cells were incubated on ice for 1.5–2 h. EDAC, dissolved in water immediately prior to use, was added to give a final concentration of 5 mM. After incubation for 30 min at room temperature, excess EDAC was destroyed by addition of 0.1% Triton-X-100 in phosphate-buffered saline (PBS) containing PMSF. The cells were then collected by centrifugation for 5 min at 2,000 rpm and washed three times with the Triton-X-100 buffer. They were dissolved in an equal volume of 2× SDS treatment buffer containing 0.05 M DTT or 5% 2-mercaptoethanol, and boiled for 5 min before loading on aliquot onto a 6% Laemmli gel (28). The gel was dried and autoradiography was carried out using Kodak XAR film.

Western Analysis—Transfected COS-7 cells were suspended in lysis buffer at a cell density of approximately 10^6 cells/100 μl. Lysis buffer contained 0.5% Nonidet P-40 in Tris-Cl, pH 7.5. Protease inhibitors PMSF (1 mM), leupeptin (20 μM), benzamidine (10 μM), and 1,10-phenanthroline (50 μM) were added just before use. Cells were lysed on ice for 15 min, and the cell debris removed by centrifugation for 15 min at 12,000 rpm. A 15–25-μl aliquot of extract was added to an equal volume of SDS treatment buffer and the sample was boiled for 5 min. For those samples to be stained with the anti-peptide polyclonal antibody, 50 mM DTT or 5% 2-mercaptoethanol was included; in those to be analyzed by 217C or MC192, reducing agent was omitted. After electrophoresis, protein was transferred electrophoretically to Millipore Immobilon P (36) or nitrocellulose membrane, using the transfer buffer system of Towbin et al. (29). The membrane was blocked with phosphate-buffered saline containing 0.1% Tween-20 and 5% non-fat milk. After incubation with primary antibody and horse-radish peroxidase-conjugated secondary antibody, proteins were visualized by diaminobenzidine development.
RESULTS

Deletion of the Linker Domain between the Cysteine-rich Domain and the Membrane-spanning Domain Does Not Diminish the Affinity of the Receptor for NGF, as Judged by Cross-linking—Although we had previously found that a truncated, secreted form of the receptor, immunoprecipitated with MC192, was capable of binding NGF (13), it was difficult to determine whether it did so with wild type affinity. Here, we have constructed another mutant, ΔB/S, in which nearly all of the polypeptide chain between the membrane-spanning domain and the cysteine-rich domain has been deleted. This deletion from Ile286 to Gly298 left only 11 amino acids out of the original 62 between the last cysteine residue and the membrane-spanning domain.

The ΔB/S mutant was expressed in COS 7 cells, and the protein was visualized on a Western blot by either 217c or MC192 staining (Fig. 2A). The position of the band indicated a reduced molecular weight, as expected. An additional band of higher molecular weight, possibly representing a dimer, was observed in our hands, provided the x-ray film is not exposed beyond the linear range of sensitivity of the film. Whereas a change in the equilibrium amount of the NGF-receptor complex might have been compensated by an opposite change in the ease of cross-linking NGF and receptor in the complex, it seems more probable that both reactions are unchanged. If this assumption is valid, then the extracellular linker region does not appear to contribute to the free energy of NGF binding.

No Single Repeat Can Be Deleted Without Loss of Expression or NGF Binding—Next we asked whether the NGF binding site could be localized more closely within the cysteine-rich domain. In particular, we wondered whether it might be possible to delete precisely any one of the 4 cysteine repeats without destroying the binding of NGF. This question also bears upon the structure of the cysteine-rich domain itself.

The 4 cysteine repeats are very closely spaced. Only 2 residues, Glu-Pro, separate the last half-cysteine of the first repeat and the first half-cysteine of the second repeat. The second and third repeats are separated by a single residue, Arg, and the third and fourth repeats by the dipeptide Glu-Glu.

In deleting the first and the fourth repeats, only a single mutant was made in each case, because the deletion fell at an end of the domain and did not result in an artificial junction between repeats. For ΔI, the sequence chosen to precede the first cysteine that was retained was Lys-Glu-Pro. In the wild type protein, the sequence Lys-Glu-Thr precedes the first cysteine repeat. Lysine was retained to ensure cleavage of the signal peptide. Proline rather than threonine was chosen to precede the first half-cysteine in case it was important for the folding of the second repeat. No sequence choice was necessary in deleting the fourth repeat, since the sequence Glu-Glu is found in the wild type protein both between the third and fourth repeats and immediately following the last cysteine of the fourth repeat.

In the case of the internal deletion of either repeat II or repeat III, the situation is more complicated because a new junction between adjacent repeats must be created. We therefore attempted to make two versions of each of the internal deletions. The second repeat was deleted two ways: in one, Glu-Pro, corresponding to the wild type junction between repeats I and II, was retained as the junction between repeats I and III; in the other, Arg, corresponding to the wild type junction between repeats II and III was retained. Likewise, in deleting repeat III, Arg was retained as the junction between repeats II and IV in one construct, and Glu-Glu in a second version.

In practice, we succeeded in isolating only one of the ΔIII constructs (with Glu-Glu as the junction). Our failure to obtain a PCR product of the correct size with Arg as the junction between repeats II and IV was most likely the result of secondary structure in the region of overlap between the two initial PCR fragments.

Deletion of the second repeat presented a different problem. Four ΔI1 clones with the correct sequence, two representing each type of junction, were isolated and used to transfected COS 7 cells. None, however, yielded protein that was recognized on a Western blot by the polyclonal antibody made against a peptide homologous to a hydrophobic region in the cytoplasmic domain (21). This same antibody recognized all other constructs we made, including all of the other deletion mutants (Fig. 2A). Thus deletion of the second repeat prevents stable expression of the protein. We do not know at this stage why this particular deletion prevents stable expression, but we suppose that it must expose some site or sites in the remaining protein to proteolysis.

All the deletion mutants failed to bind NGF to any significant extent (Fig. 3B), although trace amounts of cross-linked products corresponding to the three proteins that were expressed were observed if the film was exposed for a sufficient length of time (data not shown). The ΔI mutant typically showed slightly more cross-linking. The faint band observed could not be eliminated by the presence of a 500-fold excess of cold NGF during the incubation, although the same concentration of cold NGF completely eliminated the band seen when the wild type complex was studied (data not shown).

FIG. 2. Expression and NGF binding properties of p75NGFR ΔB/S. A, Western analysis of Nonidet P-40 extracts from transfected COS 7 cells. Proteins were separated on a 7.5% gel. Lanes 1 and 2 were stained with 217c; lanes 3 and 4 with MC192. B, binding of NGF. Transfected cells were incubated with [125I]NGF, cross-linked with EDC, and analyzed by SDS-polyacrylamide gel electrophoresis on a 6% gel followed by autoradiography as described under "Materials and Methods."
Cysteine-rich Domain of p75NGFR

FIG. 3. Expression and NGF binding properties of p75NGFR deletion mutants. A, Western analysis. Proteins were separated on a 7.5% gel, and the blots were stained with an anti-peptide antibody directed against the intracellular domain. Two separate clones of each ΔII construct were analyzed in lanes 4-7. The upper arrow indicates the position of the wild type protein in lane 1, and the lower arrow indicates the approximate position of mutant protein bands in lanes 3, 8, and 9. Narrower bands seen in all lanes are nonspecific.

B, analysis of cross-linked [3H]NGF-receptor complexes on a 6% gel as described under "Materials and Methods" and Fig. 2. Extremely faint bands corresponding to the mutant complexes can usually be seen on the x-ray film but are lost in the reproduction. The lower band seen in the C(+) lane is presumed due to limited proteolysis sometimes seen in these experiments.

This observation suggests that the $K_d$ for these mutant proteins is higher than $5 \times 10^{-7}$ M, the concentration of NGF in these incubation tubes. It does not rule out the possibility that the structure of the ligand-receptor complex was so altered that the cross-linking agent could not gain access, or that NGF binding occurred without bringing acidic and basic groups into close enough proximity for coupling to occur. For this to be the primary effect, however, each of the separate deletions would have to result in a similar loss of side-chain interactions. Although the extent of binding is too low to allow making a Scatchard analysis with these mutants, it seems probable that the affinity is drastically reduced in each case.

The fact that slightly more binding was seen with the ΔI receptor than with ΔIII or ΔIV might mean that the first repeat contributes less to the binding site than do the other repeats but might alternatively reflect the presence of fewer acidic and basic residues in the first repeat than in subsequent ones. Thus, the probability of cross-linking when EDAC is used as a reagent would be affected less by deletion of the first repeat than by deletion of the third or fourth repeats, if all charged groups (or all acidic residues) represent potential targets for cross-linking.

It was puzzling to us at first that less cross-linking of NGF was seen with the ΔI mutant than we have previously seen with the N-terminal deletion mutants ND1 and ND2 described earlier (13), in which the excised portions of the protein did not correspond to any obvious elements of tertiary structure. This paradox was resolved when we realized that the ND1 and ND2 mutants had been compared with the doubly glycosylated version originally thought to be wild type, whereas later we used the genomic version, with only a single glycosylation site, as the basis of comparison. When all of these mutants were analyzed together, none showed significant cross-linking compared to the genomic wildtype protein G(+) (data not shown). The effect of N-glycosylation on the properties of the receptor will be described more fully elsewhere.

Monoclonal Antibodies MC192 and 217C Do Not Require All Four Repeats—ΔI, ΔIII, and ΔIV were not identical in their interaction with two separate monoclonal antibodies, MC192 (15) and 217c (18-20). As shown by the Western blot in Fig. 4A, ΔI and ΔIII both failed to recognize MC192. In contrast, the ΔIV protein was detected by MC192. Thus MC192 has an absolute requirement for repeats I and III, but is not appreciably affected by the loss of repeat IV.

Conversely, 217c failed to detect the ΔIII or ΔIV proteins, but stained the ΔI protein (Fig. 4B). Since these antibodies require the disulfide bonds to be intact, these results together provide evidence that removal of repeat I or repeat IV does not affect the binding site.

FIG. 4. Selective recognition of p75NGFR deletion mutants by monoclonal antibodies MC192 and 217C. Western blots were analyzed by immunostaining with MC192 (A) or 217c (B) as described. In B, the upper arrow indicates the position of wild type protein in lane 1, and the lower arrow marks the position of the ΔI mutant protein in lane 3.
not prevent the correct folding of the remaining cysteine repeats. This is an important point, since it strongly suggests that the individual repeats, in spite of their close connectivity, must fold independently, and that correct folding can occur when either end of the cysteine-rich domain is defective. Repeat IV is not required for folding of the protein into the conformation seen by MC192, and repeat I is not required for 217c.

Since repeat III was required for interaction with all three proteins tested, namely NGF, MC192, and 217c, and since we were unable to test mutants lacking repeat II, it is not possible to say whether either of these internal repeats contributes directly to the binding sites. Deletion of repeat III may simply destroy the spatial relationship between the remaining repeats, even if they are individually correctly folded. Thus we cannot at present distinguish between an NGF binding site that involves contact residues distributed over at least three (and possibly four) repeats and a domain structure so tightly integrated that loss of any one quarter destroys the correct binding conformation. The separation of antibody epitopes, however, makes the former model seen more probable.

Cyclic Permutation of the Repeats Fails to Generate a Functional Domain—A variety of models for the structure of the cysteine-rich domain can be drawn in which the N-terminal end of the domain repeat I would be physically close to the C-terminal end of repeat IV. We therefore investigated whether binding activity could be detected in mutants in which the repeats had been circularly permuted to place the first repeat after the fourth repeat. This kind of experiment was first successfully carried out by Goldenberg and Creighton (30) on bovine pancreatic trypsin inhibitor (BPTI). More recently, Luger et al. (31) demonstrated activity in cyclically permuted mutants of the βα barrel phosphoribosyl anthranilate isomerase from Escherichia coli (ePRAI), and T4 lysozyme activity has been recovered in a cyclically permuted version constructed by Alber et al.3 Thornton and Sibanda (32) observed that some 30% of single domain globular proteins in the x-ray data base had their N-terminal ends in close spatial proximity to their C-terminal ends.

We chose to place the first repeat after the fourth repeat rather than, for example, the fourth repeat before the first repeat, so that interaction with MC192 as well as with NGF could be used as a criterion for overall conformation. We made three versions of the -II-III-IV-I- permutation with different junctions between repeat IV and I. cp(II-III-IV-I)α joined these two repeats by the sequence Glu-Thr, which precedes repeat I in the wild type protein. cp(II-III-IV-I)β utilized the sequence Glu-Glu, which follows repeat IV in the wild type protein. The third construct, cp(II-III-IV-I)γ, provided for a more extensive peptide linker between repeats IV and I, in case more flexibility was needed in the bridge. The sequence chosen for this linker was Glu-Glu-Gly-Gly-Glu-Thr.

All three of these proteins were stably expressed and detected by the polyclonal antibody to the cytoplasmic domain (Fig. 5A). None, however, could be detected by cross-linking to [125I]NGF (Fig. 5B), or on a Western blot stained with the antipeptide antibody to the intracellular domain. B, autoradiogram of protein from transfected cells cross-linked to [125I]NGF and fractionated on a 6% gel.}

*The authors thank J. J. O'Shea for the T4 lysozyme monoclonal antibody J556.

**Cyclically permuted mutants of the protein were expressed in a bacterial expression system and purified to homogeneity.**

---

![Fig. 5. Expression and NGF binding properties of cyclic permutation mutants, and of point mutant WF155. A, Western analysis of reduced protein separated on a 7.5% gel and stained by the antipeptide antibody to the intracellular domain. B, autoradiogram of protein from transfected cells cross-linked to [125I]NGF and fractionated on a 6% gel.](http://www.jbc.org/)

---

![Fig. 6. Selective recognition of cyclic permutation mutants and WF155 by MC192 and 217c. Western analysis was carried out as described under “Materials and Methods” and Fig. 2.](http://www.jbc.org/)

---

Tryptophan 155 Is Not Required for NGF Binding but Is an Essential Part of the 217c Epitope—Because aromatic residues have been found at the interface between antibodies and antigens (33), and because Trp155 in the fourth repeat seemed to have no counterpart in the other repeats, we changed this residue to phenylalanine. The WF155 protein was expressed and analyzed. As predicted from the deletion experiments,
WF155 did not affect recognition by MC192 (Fig. 6A), but completely eliminated recognition by 217c (Fig. 6B). This is a striking confirmation of the importance of the fourth repeat to the 217c epitope. Equally strikingly, WF155 could bind NGF with affinity comparable to that of wildtype protein as judged by cross-linking (Fig. 5B). Preliminary Scatchard analysis of this mutant is consistent with this conclusion.

**DISCUSSION**

The high proportion of half-cysteine residues found in the low affinity NGF receptor is a general characteristic of the sequence of the binding domains of a number of receptors, such as the low density lipoprotein (LDL) receptor (34) and epidermal growth factor (EGF) receptor (35); of various circulating proteins such as coagulation factors (36); and of cell surface antigens likely to be involved in cell-cell interactions, such as Notch (37). These domains are not subjected to the reducing environment of the cytoplasm. Although these proteins may be large, the extensive disulfide bridging indicates that they are probably composed of individual folding domains that are small, with necessarily limited hydrophobic cores.Domains smaller than about 70 residues in length are predicted not to fold unless stabilized by disulfide bonds or extensive secondary structure (38). In support of the theory, certain small extracellular proteins, such as BPTI (39), EGF itself (40), TGFβ (41), hirudin (42, 43), and various toxins (44–46) are stabilized by disulfide bonds. Many larger proteins seem to have combined small stable motifs. Moreover, in a few cases, the small folding units have been shown to play distinct functional roles. In Notch, mutations specific for different developmental steps are clustered within a small number of the 36 EGF-like repeats (37), and in the LDL receptor, some (but not all) of the 9 six-cysteine repeats are functionally distinguishable (34).

There are, however, several features of the p75NGFR sequence, shared by other members of the family, which indicate a structure fundamentally different from that found in proteins containing the EGF motif as follows.

1. Adjacent repeats are separated by only 1 or 2 amino acid residues, instead of several, suggesting an integrated structure.

2. Whereas the number of EGF-like repeats within a single protein varies from 2 in factor X (47) to 36 in Notch, in the proteins of the p75NGFR family so far known, the number of repeats is nearly always four. Exceptions are the MRC OX40 protein (10), in which repeat III is reduced in size, and hFas (11), which has only three repeats. No known protein has a more extended chain of repeats.

3. Whereas each EGF repeat in proteins compiled of EGF-like domains is generally encoded by a single exon (48), in p75NGFR, the pattern of exons does not correspond to the junction of repeats. The entire 4-repeat domain is encoded by only two exons, with the intervening sequence falling just after the beginning of the second repeat, within codon 42 (50).

4. Certain specific differences among repeats within an individual protein are highly conserved among members of this family. For instance, the second and third cysteines in repeat I are immediately adjacent (except in hFas, which lacks the first disulfide bridge in the first repeat), whereas in the other three repeats they are separated by 2 residues. Clearly, the first repeat is structurally distinct from the subsequent ones. In several of the proteins (but not in p75NGFR), the spacing of cysteines in repeat III is different from that in the other repeats, suggesting that, in these proteins, the fold of this repeat may also be different. Again, this suggests that the different repeats do not play interchangeable roles.

In designing the experiments reported here, we realized that it might not be possible to excise an interior segment of the cysteine-rich domain without disrupting the overall structure. However, the strict conservation of the cysteine pattern (with the variations noted above) from one repeat to another within this protein, as well as the conservation within a family of proteins, led us to believe that the 6-cysteine repeat itself might represent a basic unit of structure. The fact that two different monoclonal antibodies that do not recognize the reduced protein do recognize versions of the protein in which a single repeat has been deleted from one end or the other lends some support to this view. However, both ends of the four-repeat structure, and probably all four repeats, are needed to create the normal NGF binding site.

Obviously, the four repeats are tightly connected, with no more than 2 amino acid residues between successive repeats, but within this structure, they might be arranged in at least three different ways that might be distinguishable experimentally. These three models reflect different kinds of symmetry (Fig. 7).

In model 1, the repeats would be arranged in a line group along an axis of rotation, with or without rotation of the repeats relative to each other. This model is consistent with the observation of Vissarajahala and Ross (51) that a secreted form of the extracellular domain was highly asymmetric. 50 amino acids of the linker region were included in their peptide, however, and the linker region might well be highly extended (14). This model would also be consistent with the close spacing of the repeats. It has against it the fact that the number of repeats in proteins known so far that display this particular cysteine pattern is either exactly four, or sometimes less. This limitation on the number of repeats is in marked contrast to proteins which use EGF as a repeating motif, and suggests a closed structure.

In model 2, the four repeats would be arranged roughly in a ring, with approximate pseudo 4-fold symmetry around an axis of rotation (Fig. 7, Model 2A), or in some distorted version of this plan, such as a horseshoe with pseudo 2-fold symmetry. In drawing 3B the tetrahedral faces are indicated to show why binding of different proteins might be nonoverlapping.
symmetry (Fig. 7, Model 2 B). Wheat germ agglutinin is an example of such a structure (52). It too has four repeats of a globular domain rich in disulfides. The size of each repeat (41 amino acids) is almost identical to the size of each repeat in p75NGFR, although there is a different cystine pattern, with four disulfides per repeat. Recently, a low resolution projection structure of the ligand-binding domain of the EGF receptor has been published (53). Apparently this structure also consists of four domains, arranged in a ring. Each of these globular domains is larger than a single NGFR repeat, and contains more cystines.

Whereas it is also possible to draw a single, continuous structure with rotational symmetry similar to that shown in Fig. 7, Model 2A, such a structure would be likely to involve disulfide bonds between repeats, and this seems unlikely both a priori and in light of our antibody results. Moreover, if the entire set of four repeats constituted a single domain, as in a $\beta$-barrel, the hydrophobic core should be large enough not to require multiple disulfide bonds.

In model 3, the repeats would be clustered in such a way as to display pseudo point group symmetry, as in a tetrahedral array.

These models lead to different predictions about how NGF and the two monoclonal antibodies might bind. With the tetrahedral model, any other protein binding to the receptor would be likely to see three but not four of the receptor repeats, simply because of their arrangement in space. This is consistent with the behavior of the two monoclonal antibodies, MC192 and 217c, each of which requires three but not four repeats, and which have been shown to have nonoverlapping epitopes (20) and not to compete with NGF for binding (15, 20). In fact, MC192 enhances the binding of NGF (15), presumably by stabilizing a confirmation of the receptor favorable to NGF binding. Based upon model 3, we would predict that the binding site for NGF might involve, for example, repeats I, III, and IV, but not II. Unfortunately, the $\Delta$II constructs did not lead to viable protein, although the sequences of four separate clones, representing two different junctions between the first and third repeats, were correct. Without this final piece of information, this model cannot be ruled out or confirmed.

The cyclic permutation mutants were intended to test whether the N and C termini of the 160-amino acid domain were physically close together. This would be predicted to be the case in any variation of model 2, and possibly also in model 3. Such a result would not be unexpected based on known protein structures. A study of globular proteins in the 1983 data base by Thornton and Sibanda (32) revealed that some third of all single domain globular proteins have their termini in close proximity, far more than would be expected on a statistical basis. It is less common for multidomain proteins, but examples can be found (actinidin, yeast hexokinase, phosphoglycerate isomerase, glyceraldehyde-3'-phosphate dehydrogenase). Many more multidomain proteins, which we have no way to guess.

Without this final piece of information, this model cannot be ruled out or confirmed.

The cyclic permutation mutants were intended to test whether the N and C termini of the 160-amino acid domain were physically close together. This would be predicted to be the case in any variation of model 2, and possibly also in model 3. Such a result would not be unexpected based on known protein structures. A study of globular proteins in the 1983 data base by Thornton and Sibanda (32) revealed that some third of all single domain globular proteins have their termini in close proximity, far more than would be expected on a statistical basis. It is less common for multidomain proteins, but examples can be found (actinidin, yeast hexokinase, phosphoglycerate isomerase, glyceraldehyde-3'-phosphate dehydrogenase). Many more multidomain proteins, however, contain individual domains in which the termini are spatially close together. The likelihood of getting a viable cyclic permutation of the p75NGFR binding domain therefore would depend on whether the true structure is the four-repeat structure or the individual repeat. It would also, of course, depend on the exact spacing of the terminal alpha carbons, which we have no way to guess.

Using a value of 3.5 Å for the C$_{\alpha}$-C$_{\alpha}$ distance in a fully extended peptide chain, 2 half-cystines separated by 2 other residues would be about 10–11 Å apart in space, as an upper limit. Thus our first two cyclic permutation mutants would generate active protein only if the N and C termini of the wildtype domain were approximately 10 to 11 Å apart, or less. This is close to the spacing seen in a number of single domain proteins, and in certain multidomain proteins such as glyceraldehyde-3'-phosphate dehydrogenase, phosphoglycerate kinase, and T4 isozyme (32). It is what one might have expected if the four repeats formed a continuous ring or basket-shaped structure, or if the structure fell into one of the classes of recognized super-secondary structures ($\beta$ barrels, parallel and antiparallel $\beta$ barrels, Greek key proteins, 4-helical bundles), all of which display strong termini proximity (39).

Our third version of the cyclic permutation contains a linker of 7 amino acids between the last half-cystine of the fourth repeat and the first half-cystine of the first repeat. This would allow up to 24.5 Å between the two repeats. This is still less than the 30 Å between the terminal $\alpha$ carbons of wheat germ agglutinin, which is not considered to make close terminal contacts. The failure of this mutant protein to bind either NGF or MC192 does not therefore rule out the possibility of a helix-like shaped domain whose ends do not meet. It is also possible that the receptor stem, which connects the binding domain to the membrane, might interfere with the binding of proteins that interact with both repeat I and repeat II. 217c, whose epitope clearly includes residues in the fourth repeat, failed to stain the first two cyclic permutation proteins, in which the first repeat was inserted after the fourth without extra spacing. It did, however, recognize the third mutant, with the flexible linker between the fourth and the first repeats. This suggests that the linker allowed the inserted domain sufficient conformational freedom so that it did not sterically block the antibody from binding to its epitope.

While this manuscript was in preparation, a paper by Yan and Chao (54) appeared, in which it was shown that an insertion following residue 105, which falls in the second cysteine loop of the third repeat, and an insertion after residue 130, in the first loop of the fourth repeat, both completely block NGF binding, whereas an insertion in the second loop of the first repeat after residue 26 only partially does. Insertions outside the cysteine-rich domain had no effect on NGF binding. These results localize the role of repeats three and four, and suggest a lesser role for the first repeat. It is not, however, possible to say whether their insertions permit folding of the repeat in question, or whether these mutants are structurally similar to our deletion mutants.

REFERENCES

1. Radeke, M. J., Misko, T. P., Hau, C., Herzenberg, L. A., and Shooter, E. M. (1987) Nature 325, 593-597
2. Rodrigues-Tebar, A., Dechant, G., and Barde, Y.-A. (1990) Neuron 4, 487-492
3. Squinto, S. P., Stitt, T. N., Aldrich, T. H., Davis, S., Bianco, S. M., Radziejewski, C., Glass, D. J., Masaiakowski, P., Furth, M. E., Valenzuela, D. M., DiStefano, P. S., and Yancopoulos, G. D. (1991) Cell 65, 1-20
4. Hallibo, F., Ibanez, C. F., and Persson, H. (1991) Neuron 6, 845-858
5. Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Grenner, G. A., Lents, R., Raab, H., Kohn, W. J., and Goeddel, D. V. (1990) Cell 61, 361-370
6. Loetscher, H., Pan, Y.-C. E., Lahm, H.-W., Genta, R., Brockhaus, M., Tabuchi, H., and Kessler, W. (1990) Cell 61, 351-359
7. Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. (1990) Science 248, 1019-1022
8. Stamenkovic, I., Clark, E. A., and Seed, B. (1989) EMBO J. 8, 1403-1410
9. Kwon, B. S., and Weissman, S. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1963-1967
10. Mallett, S., Fossum, S., and Barclay, A. N. (1990) EMBO J. 9, 1065-1068
11. Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S.-I., Samegai, M., Hase, A., Seto, Y., and Nagata, S. (1991) Cell 66, 233–243
12. Upton, C., DeLange, A. M., and McFadden, G. (1987) Virology 160, 29–39
13. Welcher, A. A., Bitter, C. M., Radeke, M. J., and Shooter, E. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 159–163
14. Jenoff, N. (1990) Trends Biochem. Sci. 15, 291–294
15. Chandler, C. E., Parsons, L. M., Hosang, M., and Shooter, E. M. (1984) J. Biol. Chem. 259, 6882–6889
16. Thornton, J. M. (1981) J. Mol. Biol. 151, 261–287
17. Ledbetter, J. A., Seaman, W. E., Tsu, T. T., and Herzenberg, L. A. (1981) J. Exp. Med. 153, 1503–1514
18. Peng, W. W., Bressler, J. P., Tiffany-Castiglioni, E., and de Vellis, J. (1982) Science 215, 1102–1104
19. Kumar, S., Huber, J., Pena, L. A., Perez-Polo, J. R., Werrbach-Perez, K., and de Vellis, J. (1990) J. Neurosci. Res. 27, 408–417
20. Ferrari, G., Fabris, M., Polato, P., Skaper, S. D., Fiori, M. G., and Yan, Q. (1991) Exp. Neurol. 112, 183–194
21. Allendoerfer, K. L., Shelton, D. L., Shooter, E. M., and Shatz, C. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 187–190
22. Johnson, D., Lanahan, A. Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., and Chao, M. (1986) Cell 47, 545–554
23. Large, T. H., Weskamp, G., Helder, J. C., Radeke, M. J., Misko, T. P., Shooter, E. M., and Reichardt, L. F. (1989) Neuron 2, 1123–1134
24. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 156, 367–382
25. Ho, S. N., Hunt, H. D., Horton, R. M., Pollen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
26. Elliott, J. F., Albrecht, G. R., Gilladogs, A., Handunneti, S. M., Neequaye, J., Lallinger, G., Minjas, J. N., and Howard, R. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6363–6367
27. Luthman, H., and Magnusson, G. (1983) Nucleic Acids Res. 11, 1295–1305
28. Laemmli, U. K. (1970) Nature 227, 680–685
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
30. Goldenberg, D. P., and Creighton, T. E. (1983) J. Mol. Biol. 165, 407–413
31. Lugier, K. J., Hommel, U., Herold, M., Hofsteenge, J., and Kirschner, K. (1989) Science 243, 206–210
32. Thornton, J. M., and Shibanda, B. L. (1983) J. Mol. Biol. 167, 443–460
33. Padlan, E. A., Silverton, E. W., Sheriff, S., Cohen, G. H., Smith-Gill, S. J., and Davies, D. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5909–5942
34. Russell, D. W., Brown, M. S., and Goldstein, J. L. (1989) J. Biol. Chem. 264, 21682–21688
35. Yarden, Y., and Ullrich, A. (1988) Annu. Rev. Biochem. 57, 443–478
36. Patthy, L. (1985) Cell 41, 657–663
37. Kelley, M. R., Kidd, S., Deutsch, W. A., and Young, M. W. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5894–5898
38. Dill, K. A. (1985) Biochemistry 24, 1501–1509
39. Richardson, J. S. (1981) Adv. Protein Chem. 34, 167–339
40. Montelione, G. T., Wuthrich, K., Nice, E. C., Burgess, A. W., and Scheraga, H. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8594–8598
41. Kohda, D., Shimada, I., Miyake, T., and Inagaki, F. (1989) Biochemistry 28, 953–958
42. Clere, G. M., Sukumaran, D. K., Nilges, M., Zarbock, J., and Gronenborn, A. M. (1987) EMBO J. 6, 529–537
43. Haruyama, H., and Wuthrich, K. (1989) Biochemistry 28, 4301–4312
44. Rees, B., Bitwe, A., Samama, J. P., and Moras, D. (1990) J. Mol. Biol. 214, 281–297
45. Pease, J. H. B., and Wemmer, D. E. (1988) Biochemistry 27, 8491–8498
46. Gray, W. R., Olivera, B. M., and Cruz, L. J. (1988) Annu. Rev. Biochem. 57, 665–700
47. Leytus, S. P., Foster, D. C., Kurachi, K., and Davie, E. W. (1986) Biochemistry 25, 5098–5102
48. Doolittle, R. F. (1985) Trends Biochem. Sci. 10, 233–237
49. Yan, H., and Chao, M. V. (1991) J. Biol. Chem. 266, 12099–12104
50. Sehgal, A., Patil, N., and Chao, M. (1988) Mol. Cell. Biol. 8, 3160–3177
51. Vissavajhala, P., and Ross, A. H. (1990) J. Biol. Chem. 264, 4746–4752
52. Wright, C. S. (1977) J. Mol. Biol. 111, 439–457
53. Lax, I., Mitra, A. K., Ravera, C., Hurwitz, D. R., Rubin, M., Ullrich, A., Stroud, R. M., and Schlessinger, J. (1991) J. Biol. Chem. 265, 13828–13833
Studies on the structure and binding properties of the cysteine-rich domain of rat low affinity nerve growth factor receptor (p75NGFR).

A N Baldwin, C M Bitler, A A Welcher and E M Shooter

*J. Biol. Chem. 1992, 267:8352-8359.*

Access the most updated version of this article at [http://www.jbc.org/content/267/12/8352](http://www.jbc.org/content/267/12/8352)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/267/12/8352.full.html#ref-list-1](http://www.jbc.org/content/267/12/8352.full.html#ref-list-1)