Purification and Characterization of the Recombinant Extracellular Domain of Human Nerve Growth Factor Receptor Expressed in a Baculovirus System*

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To obtain the large quantities of the extracellular domain of the nerve growth factor receptor (NGF-R) necessary for structural analyses, we produced this protein in the baculovirus expression system. A cDNA coding for the extracellular domain of the human NGF-R was first introduced into transfer vector pVL941. Recombinant baculovirus was produced by cotransfecting Spodoptera frugiperda cells with the transfer vector and DNA of Autographa californica nuclear polyhedrosis virus. Recombinant viral plaques were selected by morphology and dot hybridization. The expression of recombinant extracellular domain (RED) was analyzed by Western blot analysis using anti-NGF-R monoclonal antibody. Insect cells infected with recombinant virus synthesized RED and secreted it into the culture supernatant. RED was isolated by ammonium sulfate precipitation, immunoaffinity chromatography, and anion exchange chromatography yielding 4 mg of RED/liter of suspension culture. Since there was no apparent effect of endoglycosidase H or F on RED electrophoretic mobility, and since RED did not bind concanavalin A or soybean lectin, there appears to be little or no glycosylation of RED. Purified RED bound [125I]-NGF as well as anti-NGF-R monoclonal antibodies. Sedimentation analysis and gel exclusion chromatography revealed that RED is an asymmetric molecule and may be a dimer.

Nerve growth factor (NGF) is a neurotrophic factor which plays a critical role in development, growth, and differentiation of sympathetic and sensory neurons. It mediates many biological responses including axonal outgrowth, survival of the developing and adult nervous system, and neural regeneration following nerve injury. Binding studies with 125I-NGF reveal specific nerve growth factor receptors (NGF-R) on the plasma membranes of a variety of cell types. Even though the biochemical events underlying NGF action are unclear, it is believed that binding of NGF to its receptor is the first event in eliciting the biological responses of NGF.

Murine monoclonal antibodies (mAbs) directed against human and rat NGF-R identify an acidic glycoprotein with an apparent molecular weight of 75,000, which partially exists as a disulfide-linked dimer. No kinase activity is associated with the receptor. The genes coding for the human and rat NGF-R were cloned by the method of gene transfer. The human NGF-R gene is about 23 kb long encoding a 3.8-kb mRNA with an unusually long 3'-noncoding region (8-10). This mRNA encodes a protein of 427 amino acids containing a 28-amino acid signal peptide, an extracellular domain of 211 amino acids, a single transmembrane region of 33 amino acids, and a 155-amino acid cytoplasmic domain.

A complete understanding of NGF signal transduction requires a detailed knowledge of NGF-R structure including x-ray crystal structure. Production of the large amounts of protein required for such an analysis is possible only in a recombinant expression system. Since NGF-R produced in bacteria does not fold properly, we have chosen the baculovirus eukaryotic expression system (11). Using this system, large amounts of other mammalian proteins have been produced, including epidermal growth factor receptor (12, 13), the intracellular domain of insulin receptor (14), and human immunodeficiency virus coat protein (15). We have prepared a recombinant baculovirus encoding the extracellular domain of human NGF-R and have expressed large quantities of the recombinant extracellular domain (RED). The resulting protein appears to be properly folded since it binds NGF and conformation-dependent anti-NGF-R mAbs. Since the RED lacks carbohydrate, glycosylation is not required for NGF binding.

EXPERIMENTAL PROCEDURES

Materials—Grace’s insect cell culture medium was the product of Difco Laboratories. Low-melting agarose was supplied by FMC Bio-products. Routine molecular biology tools including linkers, restriction enzymes, and other enzymes were obtained from Boehringer Mannheim or New England Biolabs. NGF was purchased from Bio-products for Science. The bicinchoninic acid protein assay kit was supplied by Pierce Chemical Co., and the silver staining kit was from Bio-Rad. [α-32P]dCTP (3000 Ci/mmol) and Na235S (carrier-free) were purchased from Amersham Corp. Concanavalin A, ethyldimethylpropylaminocarbodiimide (EDAC), cyanogen bromide-activated Sepharose 4B, gentamycin, and endoglycosidase F were from Sigma. Biotinylated soybean lectin and the anti-mouse IgG peroxidase ABC Elite Vectastain kit were from Vector Laboratories. Endoglycosidase

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1 The abbreviations used are: NGF, nerve growth factor; NGF-R, nerve growth factor receptor; mAb, monoclonal antibody; RED, recombinant extracellular domain; EDAC, ethyldimethylpropylaminocarbodiimide; AcNPV, A. californica nuclear polyhedrosis virus; ABC, avidin biotin complex; PBS, phosphate-buffered saline; ConA, concanavalin A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobase pair.

This DNA fragment was inserted into vector pBClB/CMV at the Hind111 and BamHI sites.

Western blotting was carried out with ascites diluted 1:500 followed by biotinylated anti-mouse IgG and avidin biotin complex peroxidase reagent following the manufacturer’s instructions. Immunoreactive protein bands were visualized with diaminobenzidine. Extracts of crude membranes from A875 cells (20) were used as a positive control for NGF-R.

For immunoaffinity chromatography, NGF-R was purified from ascites by chromatography on Protein A-Sepharose. The resulting purified immunoglobulin was coupled for 16 h at 4 °C with cyanogen bromide-activated Sepharose 4B in 0.15 m NaCl, 5 mM sodium phosphate, pH 7.4 (PBS).

Production of Recombinant Baculoviruses—Dr. V. Luckow and M. Summers generously provided vector pVL941 (21). Transfer vector pVL H1-3 coding for RED of human NGF-R (Fig. 1) was constructed as follows. A 0.5-kb cDNA encoding the extracellular domain of human NGF-R (initiation methionine to Ser-211) was excised from a COS cell expression vector, phURH-3, with HindIII and BamHI, and was filled in with WldH1 linkers and was inserted into the unique BamHI site of pVL941 downstream to the polyhedrin promoter. The orientation of the RED insert was determined by double digestion with Sphi and SalI. The resulting plasmid was cleaved with SmaI, and Xbal linkers with a stop codon in each reading frame (CGCGTTAAGACTTNG) were inserted at the 3’ end of the cDNA insert.

Recombinant baculovirus was produced by cotransfecting SF9 cells (2 × 10⁶ cells in a 25-cm² flask) with AcNPV DNA (1 µg) and pVL H1-3 (2 µg), and the resulting culture supernatant was harvested 4–6 days later. Fresh monolayers of SF9 cells in Lux (60 mm) culture dishes were infected with 10-fold serial dilutions of the cotransfection culture supernatant and subsequently overlaid with 0.3% fetal bovine serum, 2

The recombinant plaques were suspended in 0.5 ml of TNM-FH medium, and 0.4 ml was used to infect St9 cells in a 24-well plate (3 plaque-forming units/ml). At 24 h post-infection, the cells were harvested 96 h after infection. Protein from the culture supernatant was precipitated with 60% saturated (NH₄)₂SO₄ and was dissolved in 50 ml of PBS. The sample was passed over a 4-m1 bovine serum albumin-Sepharose column (5 mg of bovine serum albumin/ml) and then over a 4-m1 NGF-R-Sepharose column (5 mg of IgG/ml). The NGF-R column was washed with 20 ml of PBS and then with 20 ml of 0.1 m glycine, pH 4.0, 0.2 mM phenylmethylsulfonyl fluoride. RED was eluted with 0.1 m glycine, pH 2.5, 0.2 mM phenylmethylsulfonyl fluoride in 2-m1 fractions including 0.4 ml of 0.5 M Tris, pH 8.3, in each collecting tube for immediate neutralization.

The eluted fraction from the immunoadfinity column was dialyzed against Solution A (20 mM histidine, pH 6.0, 2 × 500 ml) and applied to a Mono-Q column (0.5 × 5 cm, 4 °C) pre-equilibrated with the same buffer. The column was eluted at 0.5 ml/min over 1 h with a linear salt gradient from 0 to 100% Solution B (1 M NaCl, 20 mM histidine, pH 6.0).

Protein Chemistry—Purified RED was estimated by the bichinonic acid protein assay (22). Automated Edman degradations were performed on an Applied Biosystems 477A sequenator and analyzed with a Model 120A In-Line PTM Analyzer.

Detection of Glycosylation—Samples of RED (4 µg each) were denatured by boiling in a 1.2-fold excess (by weight) of SDS. The samples were reduced with 50 mM sodium citrate, pH 6.0, to give a final concentration of SDS less than 0.2%. Endoglycosidase H (500 units/ml) was added and incubated at 37 °C overnight. Incubation with endoglycosidase F (500 units/ml) was carried out at 31 °C for 1 h. The samples then were analyzed by SDS-PAGE and silver staining or Western blotting.

For lectin-binding studies, 2 µg of RED/lane was applied to an SDS gel and then electrotransferred to an Immobilon membrane. Oligosaccharides were detected with 125I-concanavalin A (ConA, 200,000 cpm/ml), or with biotinylated soybean lectin (10 µg/ml) followed by peroxidase ABC reagent. Glycoprotein C was used as a control protein, which was purified according to Marchesi and Andrews (23).

Affinity Labeling of Purified RED with 125I-NGF—NGF (2.5 S) was iodinated according to Sutter et al. (24). RED (4 µg/ml) was incubated for 45 min at 37 °C with 125I-NGF (1.0 nm) in Dulbecco’s PBS supplemented with 0.1% bovine serum albumin. The chemical cross-linker EDAC (30 µm) was added and incubated for 2 min at room temperature. The samples were reduced and denatured, subjected to electrophoresis, and visualized by autoradiography.

Analytical Gel Filtration and Sucrose Density Gradient Ultracentrifugation of RED—RED (20 µg, 50 µl) was chromatographed on a column (1.0 × 30 cm) of Superose 6 equilibrated with PBS at a flow rate of 0.4 ml/min (4 °C). The column was calibrated using dextran blue dye, thyroglobulin (660,000), cyanocobalamin (1350), human transferrin (92,000), bovine hemoglobin (68,000), bovine serum albumin (66,500), ovalbumin (44,000), and vitamin B12 (1.350). A 125-µl sample including 40 µg of RED, 31 µg of IgG, 25 µg of transferrin, 30 µg of bovine serum albumin, and 25 µg of bovine cytochrome c was layered onto a 5-m1 5–20% (w/v) sucrose density gradient in PBS. Samples were ultracentrifuged for 2 h (4 °C) at 45,000 rpm in a Beckman SW50.1 rotor. Aliquots (0.25 ml) were analyzed by SDS-PAGE and silver staining.

RESULTS

Construction of Recombinant Baculovirus—Transfer vector pVL H1-3 (Fig. 1) was constructed by ligation of a cDNA encoding the RED of the NGF-R into vector pVL941 followed by the insertion of a stop codon at the 3’ end of the insert (“Experimental Procedures”). pVL941 is a baculovirus-derived vector including the polyhedrin promoter and parts of the polyhedrin coding region. The 5’ end of the polyhedrin gene was deleted from earlier vectors to avoid the production of fusion proteins, but recombinant viruses derived from such vectors yielded less recombinant protein. pVL941 includes the 5’ end of the polyhedrin gene with the initiation codon modified by site-directed mutagenesis to ATT so that translation begins at the NGF-R initiation codon.

Following the cotransfection of SF9 cells with pVL H1-3...
and AcNPV DNA, putative recombinant plaques were identified by visual discrimination under a dissection microscope. The wild-type plaques were refractile and crystal-like, and the recombinant plaques were less refractile and occlusion-negative. In the first round of identification, 7 out of 38 putative recombinant plaques were positive by dot hybridization probing with NGF-R cDNA. Although the recombinant plaques appeared to lack occlusions during the initial screening, close examination by phase-contrast microscopy invariably detected a few occlusions. The contaminating wild-type virus was eliminated by 3 rounds of plaque purification, and the resulting pure recombinant virus lacked occlusions even after extensive amplification.

**Secretion of RED into the Culture Supernatant—**Sf9 cells infected with Ac H1-3 and RED production was analyzed by Western blot analysis (10% SDS gel) of nonreduced samples with anti-NGF-R mAb ME20.4. Extracts of cell membranes from about 1 x 10^6 A675 human melanoma cells were used as a positive control. The cell extracts (panel A) and culture supernatants (panel B) represent the equivalent of about 2 x 10^6 Sf9 cells/lane.

**Purification of RED—**Sf9 cells (5.8 x 10^6) grown in suspension culture were infected with Ac H1-3, and the culture supernatant was harvested 96 h post-infection. The culture supernatant protein was concentrated by ammonium sulfate precipitation and then dissolved in PBS. After preadsorption on a bovine serum albumin column, the sample was applied to an anti-NGF-R-mAb immunoaffinity column. After thorough washing, the protein bound to the column was eluted with mild acid, and fractions positive by Western blot analysis with anti-NGF-R-mAb were pooled and dialyzed against 20 mM histidine, pH 6.0. This material was chromatographed on a Mono-Q anion exchange column (Fig. 3) and analyzed by SDS-PAGE (Fig. 4) and Western blot analysis (not shown). Fractions b and c contained the bulk of the RED (M_r = 46,000 under reducing conditions). Silver staining of a heavily loaded gel (Fig. 4A, lane c) revealed a less abundant M_r = 35,000 protein, and, in some preparations, a minor band migrating just behind the tracking dye. No other bands were evident in the silver-stained SDS gels. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product. The RED and the M_r = 35,000 protein were detected in Coomassie Blue-stained SDS gels (Fig. 4B). As judged by scanning densitometry of a typical Coomassie Blue-stained gel, 90% of total staining is associated with the RED breakdown product.
Recombinant Nerve Growth Factor Receptor

ION EXCHANGE CHROMATOGRAPHY OF RED

Fig. 3. Ion exchange chromatography of RED. Proteins eluted from an anti-NGF-R mAb immunoaffinity column were separated on a Mono-Q anion exchange column by a salt gradient as described under “Experimental Procedures.” Fractions (1.0 ml) were analyzed by SDS-PAGE and Western blotting. SDS-PAGE data for fractions a, b, and c which were reactive with anti-NGF-R mAb are presented in Fig. 4.

2-mercaptoethanol

A B

a b c

C C

-116
-97
-66
-45
-29

Fig. 4. SDS-PAGE analysis of RED. Fractions from ion exchange chromatography of RED (Fig. 3) were analyzed by electrophoresis on a 10% SDS gel. A, fractions a, b, and c were reduced (+) or not reduced (-) prior to electrophoresis and then detected by silver staining. B, fraction c was reduced (+) or not reduced (-) prior to electrophoresis and then electroblotted. Soybean lectin binds to terminal N-acetylgalactosamine which, in mammalian cells but not insect cells, is frequently masked by sialic acid. Sf9 extracts and neuraminidase-treated glycoprotein A, and RED were applied to an SDS gel and then electrophoresed. The predicted position of the RED band is marked with an arrow.

Fig. 5. Detection of N-linked oligosaccharides with 125I-ConA. RED (lanes 1-2) or ovalbumin (lanes 3-4) was treated (lanes 2 and 4) or not treated (lanes 1 and 3) with endoglycosidase H and then reduced and denatured. The samples (2 μg/lane) were subjected to electrophoresis on a 10% SDS gel, transferred to an Immobilon membrane, and probed with 125I-ConA. Glycoprotein bands were visualized by autoradiography. The predicted position of the RED band is marked with an arrow.

Insect cells (25). Ovalbumin bound the 125I-ConA, but not RED. This binding was specific since endoglycosidase H-treated ovalbumin did not bind 125I-ConA. Hence, it appears that RED has little or no N-linked carbohydrate. Third, total Sf9 extracts, untreated glycoprotein A, neuraminidase-treated glycoprotein A, and RED were applied to an SDS gel and then electroblotted. Soybean lectin binds to terminal N-acetylgalactosamine which, in mammalian cells but not insect cells, is frequently masked by sialic acid. Sf9 extracts and neuraminidase-treated glycoprotein A reacted strongly, but the untreated glycoprotein A reacted only weakly (not shown). There was no detectable reactivity with RED which suggests that there is little or no O-linked glycosylation. Since both of these lectins bind to many Sf9 glycoproteins, the lack of any apparent reactivity with the RED sample verifies the high purity of the RED.

Affinity Labeling of RED with 125I-NGF—The purified RED was cross-linked to 125I-NGF with EDAC (Fig. 6) resulting in a Mₗ = 56,000 complex. The RED cross-linking was found to be specific since the cross-linking reaction was carried out in the presence of 1 mg/ml BSA and since the addition of nonradioactive NGF inhibited the reaction. Fig. 6 also shows a faint Mₗ = 88,000 radioactive band which is not dependent on the presence of RED or cross-linker. This material is probably 125I-lactoperoxidase from the NGF iodination reaction.

The cross-linking reaction was inhibited 50% by about 1.1 μg/ml NGF (42 nM). If [RED] << Kᵦ, then the Kᵦ would equal 42 nM. However, a relatively high concentration of RED (90 nM assuming Mₗ = 44,700, see below) was necessary to obtain the levels of cross-linking necessary for quantification. Hence,
with 0.15 M NaCl, 5 mM sodium phosphate, pH 7.5, with a flow rate of 0.4 ml/min. Fractions (0.8 ml) were collected and analyzed by SDS-gel electrophoresis and silver staining. The RED was present in fractions 20-22 in the absence of NGF and in fractions 15-16 in the presence of NGF. Details of the ultracentrifugation are given under "Experimental Procedures.”

The RED was analyzed by sucrose density gradient ultracentrifugation and found to yield a sedimentation coefficient of 2.5 ± 0.3 (three determinations) consistent with a protein of about 25,000 (Fig. 8). We used the equations of Siegel and Monty (28) to calculate from the Stokes radius and the sedimentation coefficient an apparent molecular weight of 44,000. The RED Stokes radius (a = 4.3 nm) was calculated from these data by the method of Laurent and Killander (27).

RED was preincubated with NGF (7.7 μM) and then subjected to ultracentrifugation through a sucrose gradient containing NGF (0.20 or 0.46 μM). Under these conditions, the sedimentation coefficient was 3.8 ± 0.2 (three determinations).

DISCUSSION

Utilizing the baculovirus system, RED was prepared in large quantities and compared with native NGF-R. Under the same conditions reported for isolated NGF-R (19), the RED specifically binds NGF. Also, the RED binds anti-NGF-R mAbs directed against disulfide bond-dependent epitopes, and there is a shift in the electrophoretic mobility upon reduction of the RED disulfide bonds. The signal sequence is cleaved at the same amino acid as NGF-R (19) allowing the secretion of the RED into the culture medium. These studies demonstrate that the RED prepared in the baculovirus system is properly folded in a manner closely resembling the extracellular domain of the native NGF-R.

Even though several other mammalian proteins expressed in the baculovirus system are glycosylated (12, 29), the RED was synthesized in the apparent absence of glycosylation. Digestion of RED with endoglycosidases F or H had no effect on RED electrophoretic mobility even though these enzymes are known to be effective against other glycoproteins synthesized in insect cells (12, 29). ConA and soybean lectins did not bind RED. These lectins bind insect glycoproteins particularly effectively, since insect cells lack the enzymes for conversion of high mannose oligosaccharides to complex oligosaccharides and for the addition of sialic acid (30). However, proper folding of the RED in the absence of glycosylation is

Fig. 7. Gel filtration chromatography of RED. RED purified by immunoaffinity and ion exchange chromatography (20 μg in 50 μl) was injected onto a Superose 6 column (1.0 x 30 cm) equilibrated by immunoaffinity and ion exchange chromatography (20 fig in 50 ml; 0.4 ml/min. Fractions (0.8 ml) were collected and analyzed by stained gel is shown. 42 nM is an upper limit for the true Kd. Similar results were obtained for NGF-R purified from A875 cells (19) and an RED isolated from human amniotic fluid (26). NGF-R on intact A875 cells binds NGF with a Kd = 2 nM (18). Hence, the baculovirus-derived RED binds NGF with an affinity approximately the same as that measured for NGF-R expressed in mammalian cells.

Analytical Gel Filtration and Sucrose Density Gradient Ultracentrifugation of RED—The native molecular weight of RED was assessed by gel filtration (Fig. 7). The RED eluted from an analytical Superose 6 column with an apparent molecular weight of 118,000, which is much greater than either the molecular weight (22,355) predicted from the cDNA sequence or the apparent molecular weight on SDS-PAGE (46,000). The RED Stokes radius (a = 4.3 nm) was calculated from these data by the method of Laurent and Killander (27).

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RED was preincubated with NGF (7.7 μM) and then subjected to ultracentrifugation through a sucrose gradient containing NGF (0.20 or 0.46 μM). Under these conditions, the sedimentation coefficient was 3.8 ± 0.2 (three determinations).

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consistent with a previous study (31) in which NGF-R synthesized in the presence of tunicamycin, an inhibitor of N-linked glycosylation, bound NGF.

Our hydrodynamic analysis of the RED provides new insight into an earlier analysis of the NGF-R from rabbit sympathetic ganglia (32). Much of the asymmetry of the NGF-R (f/f₀ = 1.8) is due to the extracellular domain (f/f₀ = 1.7). The apparent molecular weights of the NGF-R and the RED are 135,000 (32) and 44,000. The molecular weights predicted from the cDNA sequence are 49,689 and 22,355 (8). Since the NGF-R includes about 10,000 Da of oligosaccharides (5), it appears that both molecules are dimers and that the extracellular domain is partially or completely responsible for dimer formation. The extracellular domains of the insulin receptor, the mannose 6-phosphate receptor, and influenza hemagglutinin form the proper dimer or trimer structure (33-35). The insulin receptor extracellular domain is a highly asymmetric protein (33), but similar analyses of other receptor extracellular domains are not yet available.

These results and the primary sequence of the NGF-R suggest a simple model for the three-dimensional structure of the RED. We propose that the 4 cysteine-rich, homologous segments of the RED fold into four independent protein domains. If these four domains were arranged in a linear pattern like a string of beads, then the RED would be an elongated, extended molecule consistent with the hydrodynamic analysis. A RED dimer might consist of two parallel strings of beads like IgG heavy chains.

The RED migrates on SDS-PAGE with an apparent molecular weight of 46,000, much greater than the 22,355 predicted from the cDNA sequence. We do not think that this anomalous molecular weight is due to any insect cell-associated modification, since RED expressed in COS cells has an apparent molecular weight of 48,000. In addition, as this manuscript was being prepared, Zupan et al. (26) reported the isolation of an extracellular NGF-R shed into human amniotic fluid. Even after glycosidase treatment, this protein had an apparent molecular weight of 43,000. Glycophorin A persists as a dimer following denaturation (36), but it is unlikely that RED also would since it lacks a hydrophobic transmembrane domain. The most likely explanation is that elements of the asymmetric structure persist even after denaturation and cause the anomalous migration of RED on SDS-PAGE.

The RED secreted into the culture medium, but not that extracted from cells, migrated on SDS-PAGE as a doublet. This pattern was particularly evident on nonreducing gels, with no NGF-induced aggregation of RED molecules. Such large quantities of protein will facilitate the identification and isolation of these putative factors. Also, the large quantities of RED will allow the detailed structural analyses needed to understand the mechanism of signal transduction.

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