# Isolation and Characterization of a Soluble Form of the LDL Receptor, an Interferon-Induced Antiviral Protein (43749)

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*Abstract.* **Interferons (IFNs) act by inducing several intracellular antiviral proteins. We report here that IFNs also induce an extracellular soluble protein that inhibits vesicular stomatitis virus (VSV) infection. This protein accounts for 25%–50% of the total antiviral activity elicited by IFN. The antiviral protein was purified to homogeneity from culture supernatants of IFN-treated cells by several chromatographic steps, to give a single 28-kDa active polypeptide. Upon sequencing, this novel protein corresponded to the N-terminal ligand-binding domain of the human 160-kDa low-density lipoprotein receptor (LDLR). In addition, we find that IFN induces the cell surface LDLR and this phenomenon may explain previous reports on reduction of serum cholesterol in IFN-treated patients. Viruses produce soluble cytokine receptors that inhibit their respective cytokines, thereby assisting virus infection. It appears now that host cells employ similar molecules for the opposite role of controlling virus infections.**

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Interferons (IFNs) are virus- and mitogen-induced cytokines that act on cells and induce resistance against viral infection (1). IFNs inhibit many types of viruses by inducing several intracellular antiviral proteins (2). Among these are the Mx protein (3), 2′,5′-oligoadenylate synthetase, and protein kinase (4, 5). Recently a new antiviral protein, RNA-binding protein 9–27 (RBP9–27), was identified (6). Many other IFN-induced proteins were detected, but their role in establishing the antiviral state in cells is either indirect or unknown (7).

The low-density lipoprotein receptor (LDLR) consists of 822 amino acid residues and exhibits a molecular weight of 164,000. It is made of several domains, some of which are shared with other proteins. The N-terminal ligand-binding domain consists of 292 amino acid residues, arranged in seven cysteine-rich imperfect repeats. This domain is followed by two additional extracellular domains, a single transmembrane domain and a short cytoplasmic domain (8, 9). The only known function of this receptor is to internalize LDL and VLDL.

This study describes a new IFN-induced protein which is secreted to the culture medium and inhibits the cytopathic effect of vesicular stomatitis virus (VSV). After purification and sequence analysis, we find that this protein corresponds to the cysteine-rich ligand-binding domain of LDLR.

## Materials and Methods

**Cells and Reagents.** Human WISH amnion cells (CCL 25) from the American Type Culture Collection (ATCC) (Rockville, MD) were grown at 37°C in 5% $CO_2$ in humidified air in MEM supplemented with 10% fetal bovine serum (FBS). Other cell lines used in this study included HeLa (ATCC, CCL 2.1) and SV80 skin fibroblasts (10). Vesicular stomatitis virus (VSV, Indiana strain, ATCC VR-158) was grown and plaque assayed on mouse L cells. All cells were free of mycoplasma. Recombinant human interferons were produced and purified to homogeneity as previously described (11, 12). Interferon titers were calibrated against NIH standards. Monoclonal antibodies to IFN-α and IFN-γ (13, 14) and monoclonal antibody C7 (hybridoma CRL 1691, ATCC) were produced in as-

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citic fluids and purified by ammonium sulfate fractionation. Rabbit polyclonal antibody was raised against pure IFN-β. [$^{125}$I]protein A was obtained from Amersham International (Amersham, UK). Heat-inactivated serum was used throughout this study.

**Production of Crude sLDLR.** Human WISH cells were grown to confluency on Fibracell discs (Sterilin, Aldershot, UK) in MEM supplemented with 10% fetal bovine serum (FBS) and Hepes (20 m*M*) in 5-liter spinner flasks. After an initial growth phase, the cells were induced with IFN-γ (30 U/ml) at 37°C in MEM supplemented with a protein-free serum substitute (ADC-1, 1:50; Biological Industries, Beit Haemek, Israel), Hepes (20 m*M*) and insulin (0.2 μg/ml). The culture medium was collected after 17–24 hr, clarified and concentrated by ultrafiltration (mol wt 10,000 cut off, Minitan; Millipore, Bedford, MA). The cell culture was repeatedly induced by IFN-γ and harvested at 24-hr intervals.

**Chromatographic Procedures.** The concentrated cell culture supernatant was adjusted to sodium borate buffer (pH 8, 20 m*M*, Buffer A) and fractionated on a TSK-DEAE column (E. Merck, Darmstat, Germany) by a stepwise increase of NaCl concentration in Buffer A. The protein peak which contained antiviral activity was pooled, desalted by ultrafiltration (YM-10 membrane, mol wt cut off 10,000; Amicon, Beverly, MA) and loaded on a hydroxyapatite Biogel HTP column (Bio-Rad, Richmond, CA). The column was washed with water and eluted by a stepwise increase of sodium phosphate concentration at pH 6.8. The concentrated active peak was fractionated by an anion exchange HPLC column (Superformance TMAE—650S, E. Merck), in a manner similar to the TSK-DEAE step. The active peak was brought to 1.5 *M* NaCl, loaded on a phenyl Sepharose column (Pharmacia, Uppsala, Sweden), preequilibrated with 1.5 *M* NaCl in Buffer A and the unbound active protein peak was collected. The column was then washed with Buffer A and finally with 50% acetonitrile in Buffer A. The unbound fraction was loaded on an Aquapore RP-300 HPLC column (Applied Biosystems, Foster City, CA) in Hepes buffer (pH 7.5, 20 m*M*) and resolved by an acetonitrile gradient. Active fractions were pooled, diluted and rechromatographed on the Aquapore RP-300 column. Antiviral activity was measured in each fraction. RP-HPLC columns were monitored by a system based on fluorescamine (15), while other columns were monitored at 280 n*M*. Protein concentration was measured with fluorescamine and bovine serum albumin as a standard (16).

**Microsequence Analysis of Proteins.** Microsequencing was performed on a pulsed liquid phase system with on-line HPLC and data analysis according to the manufacturer's instructions (Model 475 protein sequencer, Applied Biosystems).

**Bioassay of AVH.** This assay is similar to the cytopathic effect (CPE) inhibition assay that is used for measuring IFN activity (17), except that the virus is added immediately after the addition of the AVH rather than after preincubation with IFN for 2–18 hr. Briefly, serially 2-fold diluted samples of the antiviral factor are added to confluent monolayers of WISH cells in 96-flat bottom well plates, together with neutralizing monoclonal anti-IFN-γ antibody 166-5 (final concentration: 250 neutralizing units/ml; Ref. 14). The cells are immediately challenged with VSV (220 PFU/cell). After incubation at 37°C for about 20 hr, the cytopathic effect in control wells is observed microscopically and the cells are then stained with crystal violet. The antiviral activity is calibrated against NIH reference standard of human IFN-β or IFN-α.

## Results

**Evidence for an IFN-Induced Antiviral Protein and its Bioassay.** The titration of the antiviral activity of IFN is performed by preincubation (2–24 hr) of a cell culture (in a 96-well plate) with a series of 2-fold diluted IFN, followed by a virus challenge. Our IFN assay system is based on human WISH amnion cells and VSV. We observed that after 18 hr of incubation, removal of the IFN-containing medium prior to challenge with virus significantly reduces the antiviral titer. Replacement of the culture medium with a fresh, IFN-

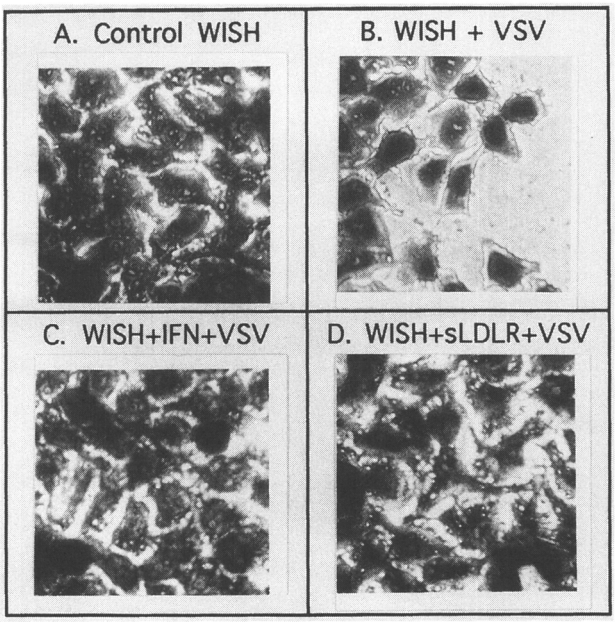


**Figure 1.** Morphology of protected and VSV-infected WISH cells. Monolayers of WISH cells in 96-well plates were treated with medium alone (A and B), IFN-β, 12 U/ml (C) or partially purified AVH (sLDLR) from the TSK-DEAE step, 10 U/ml, mixed with anti-IFN-γ monoclonal antibody (D). VSV (220 PFU/cell) was added to Well B, C, and D; the wells were incubated for 20 hr and stained. The photographs were taken by light microscopy at ×200 magnification.

containing medium did not fully restore the level of antiviral protection. This phenomenon appears to be general, as it was seen with several other cell types, including fibroblasts (SV80) and HeLa cells. On the basis of these experiments, we concluded that IFN-treated cells produced an extracellular antiviral factor distinct from IFN. It was tentatively named Antiviral Helper Factor (AVH). Calculation of the observed titer after replacement of IFN indicated that in the case of IFN-α and IFN-γ at least half of the total antiviral activity elicited by IFN is mediated by AVH.

Concentrated, serum-free cell culture supernatant of IFN-γ-treated WISH cells, mixed with neutralizing monoclonal anti-IFN-γ antibody was used for initial characterization of AVH. Microscopic observation of AVH-protected WISH monolayers after VSV challenge revealed a morphology identical to that of control and IFN-protected cells (Fig. 1). Neutralizing monoclonal antibodies directed against IFN-α and a polyclonal antibody to IFN-β had no effect on the antiviral activity. No protection from VSV was seen when cells were preincubated with AVH for 6–18 hr and washed prior to VSV challenge, hence AVH was not an interferon. It was therefore concluded that AVH was a new IFN-induced antiviral protein. Based on the above observations, we devised a procedure for its quantitative bioassay, resembling in principle the assay of IFN, but performed in the presence of anti-IFN antibodies.

**Production and Purification of the Antiviral Helper Protein.** Conditions for optimal production of crude AVH by WISH cells in a serum-free medium

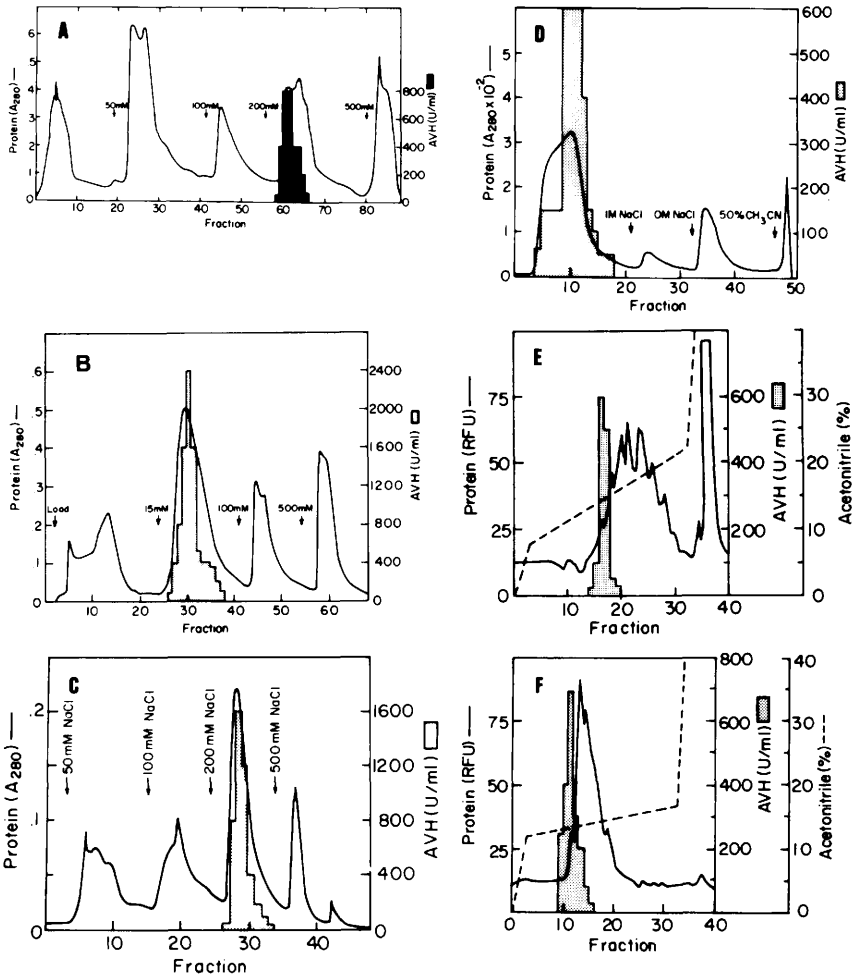


**Figure 2.** Isolation of AVH by tandem chromatography. (A) Concentrated (40-fold) culture supernatant of IFN-γ-treated WISH cells (500 ml, 2.5 g protein) was applied to a TSK-DEAE column (2.5 × 33 cm). The column was eluted at 8 ml/min and fractions of 12 ml were collected. (B) Pooled active fractions (200 m*M* NaCl peak) of step A were desalted, concentrated and applied to a hydroxyapatite (Biogel HTP, 2.5 × 4 cm) column. The column was eluted at 2 ml/min and 2.5 ml fractions were collected. (C) Pooled active fractions (15 m*M* Na phosphate peak) of Step B were loaded on a Superformance TMAE (1 × 15 cm) column. The column was eluted at 1 ml/min and fractions of 2.5 ml were collected. (D) Pooled active fractions (200 m*M* NaCl peak) of Step C, adjusted to 1.5 *M* NaCl were loaded on a Phenyl Sepharose (1.6 × 6.5 cm) column. The column was washed at 1 ml/min with 1.5 *M* NaCl in Buffer A and the unbound material was collected. (E) Pooled unbound fractions from Step D were loaded on a reversed-phase HPLC (Aquapore RP-300, 4.6 × 30 mm) column at pH 7.5. The column was washed and eluted at 0.5 ml/min and fractions of 1 ml were collected. (F) Pooled active fractions from Step E were diluted 2-fold with Hepes buffer and rechromatographed on the Aquapore column. About 2 μg of pure AVH was obtained.

were worked out. Maximal induction of AVH was obtained with 30 U/ml of IFN-γ after 18–24 hr, while culture media of noninduced WISH cells contained low levels of AVH. Several other types of human cells (HeLa, foreskin fibroblasts, etc.) were also tested and they all gave comparable levels of AVH. It was found that the cells could be repeatedly induced by IFN to produce AVH and that the levels of AVH were the same even after 50 consecutive daily replacements of the culture medium.

Preliminary experiments showed that AVH was nondialyzable and sensitive to heat, low pH, and trypsin. Size exclusion HPLC gave a single peak of activity, with a molecular weight of about 40 kD. It was therefore concluded that AVH was an IFN-induced extracellular protein. We developed a multistep procedure for the purification of AVH. Crude culture supernatant of WISH cells (typically 20 l, 2.5 g protein) was clarified and then concentrated by ultrafiltration. It was subsequently fractionated by a series of chromatographic steps, including TSK-DEAE, hydroxyapatite, superformance TMAE and phenyl Sepharose, ending with two steps of RP-HPLC at neutral pH with an acetonitrile gradient. Each step was monitored by the bioassay, which was performed in the presence of neutralizing anti-IFN-γ monoclonal antibody 166–5 (Fig. 2). The analysis of aliquots from the final purification step performed by SDS-PAGE under reducing conditions and staining with silver gave a band of molecular weight 28 kD in biologically active fractions (Fig. 3).

**Identification of AVH.** Protein microsequence analysis of pure 28-kDa AVH (0.6 μg) gave a single sequence of 14 amino acid residues at levels of 12 to 2 pmol (initial yield 51%). Only cycles 3 and 10, which were found later to correspond to Cys residues, were ambiguous. A comparison of the sequence to the NBRF protein data bank gave 100% identity with the N-terminal sequence of the human LDL receptor (LDLR). However the N-terminal sequence of this soluble 28-kDa LDL receptor (sLDLR) was shorter by three amino acid residues than was that of the (predicted) human LDLR (Fig. 4).

An independent purification procedure was used in order to confirm the relation between the antiviral activity and sLDLR. Monoclonal antibody C7 is directed against an epitope within the cysteine-rich, ligand-binding domain of bovine and human LDLR, which comprises the N-terminal 292 amino acid residues of the receptor (18). Partially purified sLDLR from the first step of chromatography (TSK-DEAE) was loaded on a C7 monoclonal antibody column; the column was washed and eluted at a high pH. The eluted fractions were immediately neutralized and tested for antiviral activity. It was found that all the antiviral activity was bound to the column and then

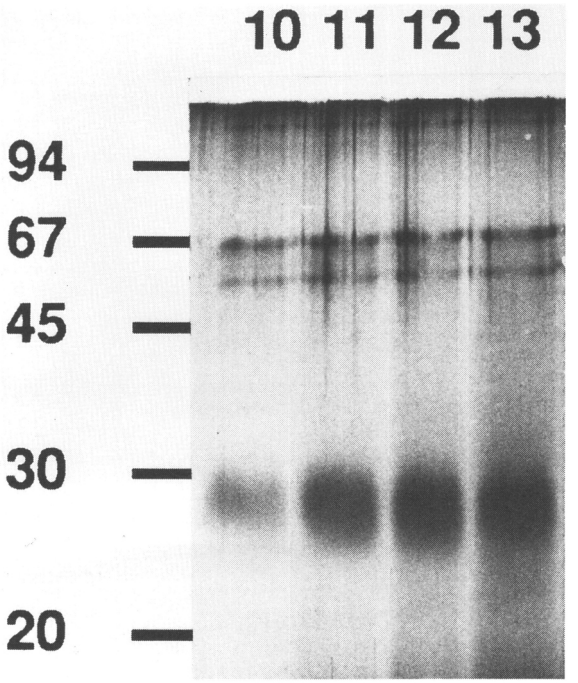


**Figure 3.** Analysis by SDS-PAGE of purified AVH. Aliquots (0.5 ml, 1 to 8 μg protein) of biologically active fraction of the final HPLC step (Fig. 3F) were evaporated and analyzed on a 13% polyacrylamide gel under reducing conditions. The gel was stained with silver. Lane numbers correspond to the HPLC fractions of Fig. 3F. Molecular weight markers are indicated on the left margin.

eluted at pH 11, with a recovery of 32% and an 80-fold purification. These results, obtained by a second and independent purification procedure, confirmed the existence of a 28-kDa sLDLR that displays antiviral activity.

## Discussion

The present study describes a novel interferon-induced antiviral protein, sLDLR, that exhibits independent antiviral activity by a novel, as yet unknown mechanism. The activity of this agent in vitro is highly significant, since its removal reduces the antiviral titers of IFN-γ and IFN-α against VSV by at least 50%. This antiviral protein is different from the previously described ones, as it is secreted to the extracellular medium. The identity of sLDLR as an IFN-induced antiviral protein that is secreted to the culture medium was determined by two independent experiments: isolation by chromatography followed by protein sequencing and immunoaffinity chromatography with a specific anti-LDLR monoclonal antibody column.

The mechanism of action of sLDLR is different from that of IFN itself, since unlike IFN, sLDLR must be continuously present in the cell culture in order to exert its action. Our recent studies indicate that sLDLR interferes at later stages of the infectious cycle, by inhibiting either assembly or budding of the virions.

Soluble proteins corresponding to ligand binding

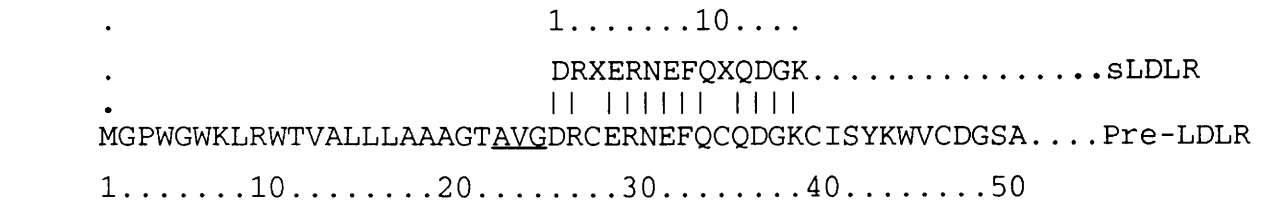


**Figure 4.** The amino acid sequence of AVH and its identity with that of human LDLR. Protein microsequencing was performed on a pool (0.6 μg) of aliquots from fractions 10–12 of Fig. 3F. Position 3 and 10 in the resulting sequence (labeled X in the upper row) were not identified, as expected for a Cys residue. The proposed signal peptide of human LDLR consists of the first 21 amino acid residues of pre-LDLR. The signal peptide based on the sequence of sLDLR is longer by these three residues (underlined).

domains of many polypeptide hormone receptors and cytokine receptors were described previously, including soluble receptors to insulin, EGF, IL-2, growth hormone, IFN-γ, IL-6 (19–23), and more. It appears that the existence of soluble variants of cell surface receptors in body fluids is a general phenomenon. Previous studies have shown that some viruses produce soluble receptor-like molecules that block their respective cytokines (e.g., IFN-γ, IL-1, and TNF), thereby assisting virus infection (24). It now appears that host organisms employ a similar type of molecule for the opposite function of controlling virus infections.

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1. Billiau A. Redefining interferon: The interferon-like antiviral effects of certain cytokines (interleukin-1, interferon β-2, interferon-γ) may be indirect or side effects. Antiviral Res **8**:55–70, 1987.
2. Samuel CE. Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology **183**:1–11, 1991.
3. Horisberger MA, Gunst MC. Interferon-induced proteins: Identification of Mx proteins in various mammalian species. Virology **180**:185–190, 1991.
4. Kerr IM, Brown RE. pppA2′p5′A2′p5′A: An inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. Proc Natl Acad Sci USA **75**:256–260, 1978.
5. Ohtsuki K, Nakamura M, Koike T, Ishida N, Baron S. A ribosomal protein mediates eIF-2 phosphorylation by interferon-induced kinase. Nature **287**:65–67, 1980.
6. Constantoulakis P, Campbell M, Felber BK, Nasioulas G, Afonina E, Pavlakis GN. Inhibition of Rev-mediated HIV-1 expression by an RNA binding protein encoded by the interferon-inducible 9-27 gene. Science **259**:1314–1318, 1993.
7. Weil J, Epstein CJ, Epstein LB, Sedmak JJ, Sabran JL, Grossberg SE. A unique set of polypeptides is induced by γ interferon in addition to those induced in common with α and β interferons. Nature **301**:437–439, 1983.
8. Sudhof TC, Goldstein JL, Brown MS, Russell DW. The LDL receptor gene: A mosaic of exons shared with different proteins. Science **228**:815–822, 1985.
9. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science **232**:34–47, 1986.
10. Todaro GJ, Green H, Swift MR. Susceptibility of human diploid fibroblast strains to transformation by SV40 virus. Science **153**:1252–1254, 1966.
11. Chernajovsky Y, Mory Y, Chen L, Marks Z, Novick D, Rubinstein M, Revel M. Efficient constitutive production of human fibroblast interferon by hamster cells transformed with the IFN-beta 1 gene fused to an SV40 early promoter. DNA **3**:297–308, 1984.
12. Mory Y, Ben BJ, Segev D, Cohen B, Novick D, Fischer DG, Rubinstein M, Revel M. Efficient constitutive production of human IFN-gamma in Chinese hamster ovary cells. DNA **5**:181–193, 1986.
13. Novick D, Eshhar Z, Rubinstein M. Monoclonal antibodies to human alpha-interferon and their use for affinity chromatography. J Immunol **129**:2244–2247, 1982.
14. Novick D, Eshhar Z, Fischer DG, Friedlander J, Rubinstein M. Monoclonal antibodies to human interferon-γ: Production, affinity purification and radiommunoassay. EMBO J **2**:1527–1530, 1983.
15. Böhlen P, Stein S, Stone J, Udenfriend S. Automatic monitoring of primary amines in preparative column effluents with fluorescamine. Anal Biochem **67**:438–445, 1975.
16. Stein S, Moschera J. High performance liquid chromatography and picomole-level detection of peptides and proteins. Methods Enzymol **79**:7–16, 1981.
17. Rubinstein S, Familletti PC, Pestka S. Convenient assay for interferons. J Virol **37**:755–758, 1981.
18. Yamamoto T, Davis CG, Brown MS, Schneider WJ, Casey ML, Goldstein JL, Russell DW. The human LDL receptor: A cysteine-rich protein with multiple Alu sequences in its mRNA. Cell **39**:27–38, 1984.
19. Gavin III Jr, Buell DN, Roth J. Water-soluble insulin receptors from human lymphocytes. Science **178**:168–169, 1972.
20. Weber W, Gill GN, Spiess J. Production of an epidermal growth factor receptor-related protein. Science **224**:294–297, 1984.
21. Rubin LA, Kurman CC, Fritz ME, Biddison WE, Boutin B, Yarchoan R, Nelson DL. Soluble interleukin 2 receptors are released from activated human lymphoid cells *in vitro*. J Immunol **135**:3172–3177, 1985.
22. Leung DW, Spencer SA, Cachianes G, Hammonds RG, Collins C, Henzel WJ, Barnard R, Waters MJ, Wood WI. Growth hormone receptor and serum binding protein: Purification, cloning and expression. Nature **330**:537–543, 1987.
23. Novick D, Engelmann H, Wallach D, Rubinstein M. Soluble cytokine receptors are present in normal human urine. J Exp Med **170**:1409–1414, 1989.
24. Gooding LR. Virus proteins that counteract host immune defenses. Cell **71**:5–7, 1992.