Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 321-329

LOCALIZATION OF THE INSULIN-BINDING SITE TO THE CYSTEINE-RICH REGION OF THE INSULIN RECEPTOR α-SUBUNIT

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Received September 16, 1988

SUMMARY Affinity-purified insulin receptor was photoaffinity labeled with a cleavable radioactive insulin photoprobe. Exhaustive digestion of the labeled α-subunit with endoproteinase Glu-C produced a major radioactive fragment of 23 kDa as a part of the putative insulin-binding domain. This fragment could contain either residues 205-316 or 518-633 of the α-subunit. Rat hepatoma cells and Chinese hamster ovary cells were transfected with cDNA encoding a human insulin receptor mutant with a deletion of the cysteine-rich region spanning amino acid residues 124-319. Insulin binding by these cells was not increased in spite of high numbers of the mutant insulin receptors being expressed. A panel of monoclonal antibodies which was specific for the receptor α-subunit and inhibited insulin binding immunoprecipitated the photolabeled 23-kDa receptor fragment but not the receptor mutant. A synthetic peptide containing residues 243-251 was specifically bound by agarose-insulin beads. We therefore suggest that the 23-kDa fragment contains residues 205-316, and that insulin binding occurs, in part, in the cysteine-rich region of the α-subunit.

Insulin receptors are oligomers composed of two 130-kDa α-subunits and two 90-kDa β-subunits (see Reviews 1 and 2). Insulin binding to the α-subunit leads to the autophosphorylation and activation of the β-subunit tyrosine kinase (3-5). Whereas the sites of ATP binding and phosphorylation of the β-subunit have been identified (6,7), little is known about the insulin-binding site(s) or domains of the α-subunit. We have previously established the feasibility of using a novel cleavable radioactive insulin photoprobe, [^{125}I]-AZAP-insulin, to identify putative insulin-binding sites on the α-subunit (8). We now report the application of this technique,

Abbreviations: HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylene diamine tetra-acetate; TPCK, tosylphenylchloromethyl ketone; [^{125}I]-AZAP-insulin, N-[4-(4'-azido-3'-[^{125}I]iodo-phenylazo)-benzoyl]-3-aminopropyl insulin.

0006-291X/88 $1.50

in combination with deletion mutagenesis of the receptor α-subunit, to study the insulin-binding domain of the insulin receptor.

Materials and Methods

Materials: The following materials were obtained from the sources indicated: bovine insulin (Zn crystals) from Connaught-Novo Laboratories Ltd. (Toronto), near carrier-free (2200 Ci/mmole) N-[4-(4'-azido-3'-[^{125}I]iodo-phenylazo)-benzoyl]-3-aminopropyl-N-oxy-succinimide ester (Denny-Jaffe reagent) from New England Nuclear, Staphylococcus aureus V8 endoproteinase Glu-C (EC 3.4.21.19) from Boehringer Mannhei Canada, TPCK-trypsin (EC 3.4.21.4) from Worthington Diagnostic Systems Inc., AffiGel 10, β-mercaptoethanol and reagents for SDS-PAGE from Bio-Rad Laboratories (Canada) Ltd., iodoacetamide and PMSF from Sigma Chemical, sodium dithionite from Fisher Scientific Company. Synthetic peptides were prepared by iAF BioChem International, Inc. (Laval, Quebec), and purified by HPLC. Their identity was confirmed by amino acid analysis. Cell culture supplies were obtained from the Tissue Culture Laboratory, University of California, San Francisco. Tissue culture cells were grow as previously described (9,10).

Photoaffinity Labeling and Isolation of the Labeled Receptor α-subunit: Human placental insulin receptor was solubilized and affinity purified on insulin-AffiGel 10 as described (11). [^{125}I]-AZAP-insulin was prepared and purified by HPLC as previously described (8) except that no carrier protein BSA was used. Approximately 10 ug of receptor protein were incubated with about 10^7 cpm of the photoprobe for 16 hr at 4 C, and then photolyzed (12). Azo cleavage was carried out as described (8). The photolabeled receptor was reduced and alkylated in 8 M urea according to the method of Crestfield (13). After desalting through Sephadex G25 in 1 M acetic acid containing 0.1% SDS and 1 mM PMSF, the reduced-alkylated radioactive receptor preparation was subjected to SDS-PAGE in a 7.2% slab gel. The radioactive 130-kDa α-subunit band on the gel located by autoradiography was excised and extracted twice into 2 mL of 10% methanol. Recovery of radioactivity from the gel band was about 85%. The extract was lyophilized.

Enzyme Digestion and SDS-PAGE of Digest: The lyophilized reduced-alkylated α-subunit was redissolved in 50 mM ammonium bicarbonate and digested with either trypsin or the endoproteinase Glu-C at 37 C. SDS-PAGE analysis of the enzyme digest was carried out in a gradient slab gel of 9-26% (14). Autoradiograms of the dry gel were obtained using Kodak X-Omat AR films and Lightning-Plus enhancing screens.

Preparation of Receptor Deletion Mutants and Transfection: cDNA (15) and genomic DNA (16) for the human insulin receptor were cloned into an eukaryotic expression vector (pRSV) (17) under the control of a Rous sarcoma virus LTR promoter. This plasmid, pRSV-IR, was modified by deleting the sequence between the SstI sites at position 499 and 1115 in the sequence of Ullrich et al (18). The deletion was made directly on the expression vector by SstI digestion and snapback ligation. This procedure removed amino acids 124 to 329 from the encoded receptor protein. Rat hepatoma (HTC) and Chinese hamster ovary (CHO) cells were transfected with this vector by the calcium phosphate precipitation/glycerol shock method together with plasmid pSV2-NEO (19) which contains a selectable marker for neomycin resistance. The cells were cultured in medium containing the antibiotic G418 at 600 ug/mL. Resistant colonies were isolated, and subcloned by limiting dilution. Cell lines expressing high numbers of receptors measured by radioimmunoassay (20) were selected and these were assayed for insulin binding as previously described (9).

Immunoprecipitation of Labeled Receptor Fragment and [^{35}S]methionine Labeled Insulin Receptors: The labeled receptor fragment produced by endoproteinase Glu-C digestion was extracted from the SDS-PAGE gel with 10% methanol. The methanol extract was made 0.01% BSA and 0.04 M phosphate buffer, pH 7.4. The extract was concentrated 10-fold and its medium replaced with phosphate-buffered saline using a Millipore Ultrafree unit (10,000 NMWL-membrane). After removal of excess SDS from the concentrate by repeat cooling in ice and by dialysis against, aliquots of 100 uL (approximately 200 cpm in radioactivity) of were used for immunoprecipitation as described (20), except that Sepharose-Protein A was used in place of the formalin-fixed Staphylococcus aureus. The immunoprecipitate was analyzed by SDS-PAGE in a gradient slab gel of 5-15%.

HTC cells transfected with either the intact insulin receptor or the deletion utant receptor (approximately 20 x 10^6 cells) were cultured in 100-mm tissue culture ishes in 12 mL of methionine-free growth medium plus 2.5 mCi of [^{35}S]methionine for 8 h. The cells were then washed in buffer, and the insulin receptors solubilized nd purified on wheat germ agglutinin affinity columns (21). The solubilized eceptors were immunoprecipitated and analyzed by SDS-PAGE in slab gels of 7.5 % as escribed (21).

Results and Discussion

We have previously demonstrated (8) that photolabeling of the insulin receptor with the radioactive [^{125}I]-AZAP insulin followed by azo cleavage to release the insulin transferred the radioactivity to the receptor α-subunit. Radioactive fragments produced by enzymic digestion of the α-subunit should represent parts of its insulin-binding domain. In the present study, exhaustive digestion of the reduced and alkylated radioactive 130-kDa subunit with the endoproteinase Glu-C vielded a major radioactive band of approximately 23 kDa on SDS-PAGE with or without reduction with dithiothreitol (Lane 2, Figure 1). This band was hydrolyzed by trypsin to a band which migrated in SDS-PAGE ahead of insulin at the dye front (Lane 3, Figure 1).

Glutamoyl bonds are hydrolyzed by endoproteinase Glu-C in ammonium bicarbonate buffer (22). There are 49 glutamic acid residues in the amino acid sequence of the α-subunit (18). Glutamoyl bonds involving hydrophobic amino acids with bulky side chains are resistant to hydrolysis by endoproteinase Glu-C (22). Since the reduction and alkylation of the α-subunit introduced a bulky side chain to cysteinyl residues, glutamoyl bonds involving alkylated cysteinyl residues would also be resistant to hydrolysis. Thus the largest fragment expected from the digestion of the labeled α-subunit would be 77 residues, produced by the hydrolysis of two susceptible glutamoyl bonds at residues 239 and 316. The unexpected finding of a 23-kDa labeled band as the major product of digestion by endoproteinase Glu-C suggests that it may contain at least one or more resistant glutamoyl bonds. Resistant glutamoyl bonds could be produced by the photocrosslinking of the iodophenyl group in the photoprobe to a glutamic residue or to its adjacent residues. Based on this reasoning, an analysis of the sequence for the presence of two adjoining glutamoyl fragments that would yield amino acid sequences large enough to account for the production of the 23-kDa band reveals one sequence of 112 residues

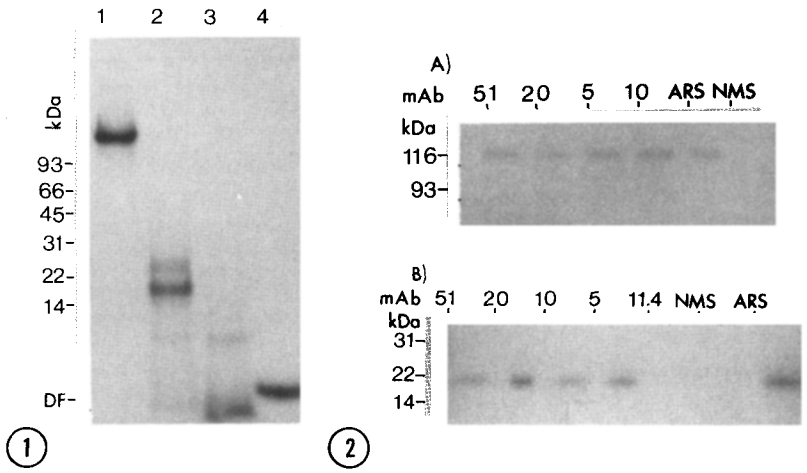

Figure 1 Enzymic digestion of the photolabeled 130-kDa α-subunit after reduction and alkylation. Lane 1, control incubation without enzyme; Lane 2, incubation with 50 ug of endoproteinase Glu-C at 37 C for 4 hr, followed by a further addition of 50 ug of enzyme and incubated for another 4 hr; Lane 3, digestion was carried out as in Lane 2 except that the digest was further incubated with 100 ug of TPCK-trypsin for 3 hr at 37 C; Lane 4, ^{125}I-insulin (unreduced) as molecular weight marker. Positions of stained molecular weight markers are indicated. DF indicates the dye front.

Figure 2 Immunoprecipitation of (A) the 130-kDa α-subunit obtained from the reduction and alkylation of the photolabeled insulin receptor, and (B) the labeled 23-kDa fragment derived from the endoproteinase digestion of the reduced and alkylated 130-kDa α-subunit. The panel of four monoclonal antibodies used was the same as that used in Figure 2. Normal mouse serum (NMS) and an irrelevant monoclonal antibody (11.4) were used as controls. ARS was IgG prepared from a human autoimmune serum against the insulin receptor.

(from residue 205 to 316), and one of 116 residues (from residue 518 to 633). Both sequences contain trypsin-sensitive sites which is consistent with the observation that the 23-kDa band was hydrolyzed by trypsin to a band which was smaller than insulin and migrated to the dye front.

A panel of monoclonal antibodies that have been shown to bind to the receptor and inhibit insulin binding (23) was found to immunoprecipitate the labeled 130-kDa α-subunit (Figure 2A) and its 23-kDa fragment generated by endoproteinase digestion (Figure 2B). However, they did not immunoprecipitate the labeled tryptic fragment.

In both the CHO and HTC cell lines expressing wild type receptors insulin binding and receptor content were increased 10-20 times when compared to non-transfected cells (Table I). In contrast, insulin binding was not increased in cells expressing the mutant receptor deleted of the cysteine-rich region in spite of an increase of more than 10-fold in insulin receptor content. Immunofluorescent labeling of intact cells showed that the mutant receptor was expressed on the cell

Table I. Insulin Binding and Insulin Receptor Content in Transfected Cells

Cell Line	Binding (%Bound/mg protein)	Receptor Content (ng receptor/mg protein)	(sites/cell)
HTC-NEO	3.5	14	3,500
HTC-IR	24.0	160	37,000
HTC-IRd	1.6	180	45,000
CHO-NEO	1.0	7	3,400
CHO-IR	21.0	130	43,000
CHO-IRd	1.1	67	25,000

HTC-NEO and CHO-NEO are control cell lines containing no human receptor genes. HTC-IR and CHO-IR are cell lines transfected with the wild type human receptor, while HTC-IRd and CHO-IRd were transfected with the deletion mutant. Specific ^{125}I-insulin binding was carried out as previously described (9). Insulin receptor content was determined by radioimmunoassay using a rabbit polyclonal antiserum that recognizes multiple antigenic determinants on both the α and β subunits (20).

surface (data not shown). The panel of monoclonal antibodies immunoprecipitated ^{35}S]methionine-labeled receptors from HTC cells expressing wild type receptors, but not labeled receptors from HTC cells expressing the deletion mutant receptor (Figure 3). A polyclonal antibody which reacted with multiple sites on the α and β subunits of the insulin receptor immunoprecipitated receptors from both of these transfected cell lines (data not shown). The inability of the monoclonal antibodies to precipitate the mutant receptor could be due to conformational changes of the receptor or losses of epitopes resulted from the deletion. Since these antibodies

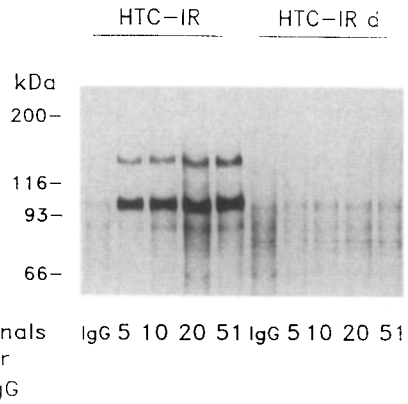

Figure 3 Autoradiograph of insulin receptors immunoprecipitated from cells transfected with either wild type insulin receptor (HTC-IR) or the deletion mutant (HTC-IRd). Immunoprecipitation was carried out using either non-immune mouse IgG (IgG) as a control or a panel of four monoclonal antibodies against the α-subunit of the insulin receptor: 5, antibody MA-5; 10, antibody MA-10; 20, antibody MA-20; and 51, antibody MA-51.

were able to precipitate the labeled 23-kDa receptor fragment which had been denatured by reduction and alkylation, it seems likely that they were reacting with discrete epitopes of amino acid sequences which were not present in the mutant receptor. Therefore it is reasonable to suggest that the 23-kDa fragment contained residues 205-316 in the cysteine-rich region.

Previous studies have raised the possibility that the cysteine-rich region the insulin receptor α-subunit may be involved in insulin binding. Thus it was reported that a large labeled receptor fragment of 60 kDa, produced by chymotryptic digestion of insulin receptors affinity-labeled with ^{125}I-insulin, underwent a mobility shift in SDS-PAGE after reduction suggesting the presence of multiple disulfide bonds near or at the insulin-binding site (24). Ullrich et al (25) suggested that the cysteine-rich region of the α-subunit may contribute to the ligar binding specificity. The LDL receptor also has cysteine-rich regions that are believed to play a role in LDL binding (26).

When compared with the amino acid sequence of IGF-I receptor, the sequence 205-316 in the insulin receptor contains the longest non-homologous stretch of amin acid residues, a total of 18. It is known that the hydrophobic sequence FFY (residues B24-B26) at the carboxyl terminal of the B chain of insulin is part of the receptor-binding domain (27). Therefore the sequence PPYYHFQDW (residues 243-251) this region of the receptor could provide the required hydrophobic interaction with insulin at its receptor-binding domain. Binding of [^{125}I]-AZAP-insulin to this sequence would result in the photocrosslinking of either the glutamic acid at residu 239 or its neighboring residues making the glutamoyl bond resistant to hydrolysis by endoproteinase Glu-C. The presence of a histidine within this short sequence is of particular interest. Results from the study of the effect of pH on insulin binding to rat liver plasma membranes suggest that the B-22 arginyl residue of insulin, whi is located in the receptor-binding domain of the hormone, interacts with a histidyl residue on the insulin receptor (28). In addition, the pH-dependent inhibition of insulin receptor binding activity by diethyl pyrocarbonate also points to an involvement of receptor histidyl residues in the binding of insulin (29).

In order to test the possibility that the sequence 205-316 may be part of the insulin-binding site, we studied the binding two synthetic peptides, Peptide I

Table II. Binding of Synthetic Peptide II to Insulin-AffiGel 10

	Addition	Amount Bound (cpm) ± S.E.
(A)	Glycine-AffiGel 10	3537 ± 1059
	Insulin-AffiGel 10	20765 ± 1562
	" " + Peptide II	12361 ± 122
	" " + Peptide I	19658 ± 1480
(B)	Insulin-AffiGel 10	6824 ± 196
	" " + Peptide II	3829 ± 50
	" " + Insulin Receptor	4451 ± 276

Synthetic Peptide II (CPPYYHFQDWR-NH_2), 5 ug, was iodinated with 500 uCi of carrier-free sodium iodide using Chloramine-T. The iodination mixture was purified through Sep-Pak C-18, the iodinated peptide eluted with 100% acetonitrile. (A): Twenty microliters of packed insulin-AffiGel 10 beads used in the affinity purification of the insulin receptor (11) were incubated overnight in the coldroom with approximately 200,000 cpm of iodinated Peptide II in 200 uL of 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.1 % bovine serum albumin, and 0.1% Triton X-100. (B): Insulin-AffiGel 10 beads, 15 uL, were incubated with the labeled Peptide II as in (A) except for only 6 hr. Where indicated 2 ug (6 uM) of non-radioactive Peptide II or Peptide I (YQDLHHKCKNSR-NH_2), or 1 ug (0.01 uM) of purified insulin receptor were present in the incubation mixture. After incubation, the gel beads were washed three times with 1 mL of 50 mM Tris-HCl, pH 7.4, containing 1 M NaCl and 0.1% Triton X-100. Radioactivity remained bound to the washed beads was determined by counting in a gamma counter. The values shown were average of duplicates.

containing residues 260-270, and Peptide II containing residues 243-251, to agarose-insulin beads. The binding of radioiodinated Peptide II to the beads was inhibited about 48% by non-radioactive Peptide II at a concentration of about 6 uM, and that peptide I was without effect (Table II). The binding of Peptide II was inhibited also by purified insulin receptor at about 0.01 uM. These results show that Peptide II interacted specifically with insulin, but its apparent affinity for insulin was much lower when compared with insulin receptor. This relatively weak interaction is expected since the small peptide very likely lacks the overall structural features present in the receptor that are required for optimal interaction. None of the monoclonal antibodies used in this study was found to bind Peptide II under the conditions where binding of the 23-kDa fragment by these antibodies was observed. This is consistent with the finding that they did not immunoprecipitate the small labeled tryptic fragment.

Considered together, data obtained in the present study support the concept that part of the insulin-binding site of the insulin receptor is located in the cysteine-rich region of the α-subunit. Most likely the binding site is within the amino acid sequence 205-316. The specific binding of Peptide II containing the sequence 243-251 to insulin lends further support to this conclusion. The use of a

radioactive insulin photoprobe of near carrier-free specific activity precluded our attempt in this study to obtain sufficient quantities of the subunit fragments for amino acid analysis or sequencing to identify definitively the insulin-binding sequence. Experiments are in progress in our laboratories to obtain the putative insulin-binding fragment in sufficient amounts through labeling with mass quantities of insulin photoprobe for sequence analysis, and to mutagenize the insulin receptor at its putative binding site.

Acknowledgements

This work was supported in part by a grant from the Medical Research Council Canada to CCY, the American Diabetes Association to DMH, the National Institutes of Health grant DK 26667 to IDG, the Elise Stern Haas Fund and the Department of Medicine at Mount Zion Hospital and Medical Center. We thank Dr. Barry Posner, McGill University, Montreal, for the gift of the human autoimmune serum ARS.

References

1. Czech, M.P. (1985) Ann. Rev. Physiol. 47, 357-381.
2. Goldfine, I.D. (1987) Endocrine Rev. 8, 235-255.
3. Kasuga, M., Karlsson F.A., and Kahn. C.R. (1982) Science 215, 185-187.
4. Petruzelli, L.M., Ganguly, S., Smith, C.J., Cobb M.H.; Rubin, C.S., and Rosen, O.M. (1982) Proc. Natl. Acad. Sci. USA 79, 6792-6796.
5. Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H., and Ponzio, G. (1983) Proc. Natl. Acad. Sci. USA 80, 945-949.
6. Tornqvist, H.F., Pierce, M.W., Frackelton, A.R., Nemenoff, R.A., and Avruch, J. (1987) J. Biol. Chem. 262, 10212-10219.
7. Chou, C.K., Dull, T.J., Russell, D.S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O.M. (1987) J. Biol. Chem. 262, 1842-1847.
8. Ng, D.S., and Yip, C.C. (1985) Biochem. Biophys. Res. Commun. 133, 154-160.
9. Iwamoto, Y., Wong, K.Y., and Goldfine, I.D. (1981) Endocrinology 108, 44-51.
10. Ebina, Y., Edery, M., Ellis, L., Standring, D.N., Beaudoin, J., Roth, R.A., and Rutter, W.J. (1985) Proc. Natl. Acad. Sci. USA 82, 8014-8018.
11. Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y., and Itakura, K. (1983) J. Biol. Chem. 258, 5045-5049.
12. Yip, C.C. (1984) In: Larner J, Pohl SL (ed) Methods in Diabetes Research, Vol. I Laboratory Methods. John Wiley and Sons, New York, pp 3-14.
13. Crestfield, A.M., Moore, S., and Stein, W.H. (1963) J. Biol. Chem. 238, 622-627.
14. DeWald, D.B., Adams, L.D., and Pearson, J.D. (1986) Anal. Biochem. 154, 502-508.
15. Shaw, D.J., and Bell, G.I. (1985) Nucl. Acid Res. 13, 8661.
16. Mamula, P.W., Wong, K.Y., Maddux, B.A., McDonald, A.R., and Goldfine, I.D. (1988 Diabetes, in press.
17. Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I., and Howard, B.H. (1982) Proc. Natl. Acad. Sci. USA 79, 6777-6781.
18. Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M., and Ramachandran, J. (1985) Nature 313, 756-761.
19. Southern, P.J., and Berg, P. (1982) J. Mol. Appl. Gen. 1, 327-341.
20. Goldfine, I.D., Maddux, B.A., Wong, K.Y., Pezzino, V., Papa, V., Brunnetti, A., and Vigneri, R. (1987) Diabetes 36(Suppl. 1), 156A.
21. Forsayeth, J., Maddux, B., and Goldfine, I.D. (1986) Diabetes 35, 837-846.
22. Houmard, J., and Drapeau, G.R. (1972) Proc. Natl. Acad. Sci. USA 69, 3506-3509.
23. Forsayeth, J.R., Montemurro, A., Maddux, B.A., DePirro, R., and Goldfine, I.D. (1987) J. Biol. Chem. 262, 4134-4139.
24. Boni-Schnetzler, M., Scott, W., Waugh, S.M., DiBella, E., Pilch, P.F. (1987) J. Biol. Chem. 262, 8395-8401.

25. Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel. W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., and Fujita-Yamaguchi, Y. (1986) EMBO J. 5, 2503-2515.
26. Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W., and Schneider, W.J. (1985) Ann. Rev. Cell Biol. 1, 1-40.
27. Wood, S.P., Blundell, T.L., Wollmer, A., Lazarus, N.R., and Neville, R.W.J. (1975) Eur. J. Biochem. 55, 531-542.
28. Halperin, M.L., Cheema-Dhadli, S., Desai, K.S., Yip, C.C., and Jungas, R.L. (1987) Clin. Inv. Med. 10, 395-400.
29. Pilch, P.F. (1982) Biochemistry 21, 5638-5644.