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PURIFICATION AND FUNCTIONAL STUDIES OF INSULIN RECEPTOR SUBSTRATE pp180

BY

QING FEI

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Purification and Functional Studies of Insulin Receptor Substrate pp180" submitted by Qing Fei in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Wheat germ agglutinin-purified rat liver glycoproteins in the presence of insulin, Mn^{2+}, and ATP yield two tyrosine phosphorylated proteins: 180,000 molecular weight and 95,000 molecular weight. The tyrosine phosphorylated 95,000 molecular weight protein is the β-subunit of insulin receptor and the 180,000 molecular weight protein includes an insulin receptor substrate pp180. With two dimensional gel electrophoresis, an approximate isoelectric point of 7.2 was determined for pp180.

As the EGF receptor is more acidic ($pI < 6.8$) than pp180, cation exchange chromatography permitted pp180 to be isolated devoid of the receptor. Further, antiphosphotyrosine affinity chromatography purified to homogeneity cation-exchange-purified pp180. Based on the two chromatographic methods, cation exchange chromatography and antiphosphotyrosine affinity chromatography, a preparative purification procedure to generate pp180 for partial peptide sequence was developed.

The possible function of pp180 has been studied. It was found that a protein tyrosine phosphatase activity was associated with partially purified (cation exchange chromatography) pp180. Phosphoamino acid analysis confirmed that the phosphatase activity is specific for phosphotyrosine. As affinity chromatography-purified tyrosine phosphorylated pp180 does not have phosphotyrosine phosphatase activity, the enzyme in cation-exchange-purified pp180 is likely one of the low molecular weight polypeptides found in this preparation.

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ABBREVIATIONS

G protein	guanine nucleotide-binding protein
EGF	epidermal growth factor
PDGF	platelet-derived growth factor
DER	Drosophila EGF receptor homologue
IGF-1	insulin-like growth factor-1
CSF-1	colony-stimulating factor-1
FGF	fibroblast growth factor
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
rER	rough endoplasmic reticulum
cAMP	3', 5'-cyclic adenosine monophosphate
Cys	cysteine
Tyr	tyrosine
Thr	threonine
Ser	serine
YXXM	tyrosine-X-X-methionine
ATP	adenosine 5'-triphosphate
CTP	cytidine 5'-triphosphate
GTP	guanosine triphosphate

GDP	guanosine diphosphate
GTPase	guanosine 5'-triphosphatase
PKC	protein kinase C
IR	insulin receptor
PI	phosphatidylinositol
PI3-kinase	phosphatidylinositol 3-kinase
PI-PLC	phosphatidylinositol-phospholipase C
SH2 domain	Src-homology-2 domain
GAP	GTPase activating protein
PTPase	protein tyrosine phosphatase
DTT	dithiothreitol
HEPES	4-(2-Hydroxyethyl)-1-piperazinethan-sulfonsaure
SDS	sodium dodecyl sulfate
Tris	tris (hydroxymethyl) aminoethane
EDTA	ethylene diamine tetracetate
TEMED	N',N',N',N'-Tetra-methylethylene diamine
NP-40	nonidet P-40
WGA	wheat germ agglutinin
PMSF	phenyl methyl sulfonyl fluoride
IEF	isoelectric focusing
NEPHGE	nonequilibrium pH gradient gel electrophoresis
kDa	kilodalton

^{32}P	^{32}P-phosphate
CPM	counts per minute
rpm	revolutions per minute
PAGE	polyacrylamide gel electrophoresis
°C	degree centigrade
g	gram
m	milli(10^{-3})
u	micro(10^{-6})
n	nano(10^{-9})
s	second
O.D.	optical density
v/v	volume to volume
w/v	weight to volume
M	molar
pI	isoelectric point
MW	molecular weight

CHAPTER 1 INTRODUCTION

1.1 General Signal Transduction System

Cells communicate. An efficient communication system that conveys information from one cell to another is the fundamental function of any multicellular organism. The flow of information upon cell stimulation by a ligand, such as a hormone or growth factor, is accomplished by a cascade of regulatory reactions that involve a number of specific enzymes, regulatory proteins and other regulatory factors. Based on the data accumulated to date, there are four basic processes involved in transmembrane receptor signal transduction systems. The first is ligand-modulated ion channel activity. The second is ligand-regulated enzymatic activity at the intracellular domain of the receptor. The third is the ligand-regulated receptor-G protein system. The fourth is a group of receptors, which are normally inactive on their own, but act indirectly through molecules that associate with their intracellular domains (Taga et al., 1993). Among the four basic transmembrane receptor signal transduction systems, the second includes protein tyrosine kinases. The transmembrane receptor tyrosine kinase family can be divided into three major groups: EGF receptor-like, insulin receptor-like, and PDGF receptor-like (Cadena et al., 1992). One group of receptors that does not fit into any of these categories is trk and related proteins. Recently trk was identified as the NGF receptor (Meakin et al., 1992).

Members of the insulin-receptor family are the most intensively studied. This is

in part because of the therapeutic effects of insulin in diabetes mellitus. Insulin exerts its action by interacting with its receptor. After insulin binding, the receptor's intracellular domain is autophosphorylated and the intrinsic tyrosine kinase activity is increased. This is the signal to initiate the sequence of intracellular events (Zick., 1989). The cellular effects of insulin are multiple. They include modulation of: 1) transport of molecules across the plasma membrane; 2) intracellular levels of cyclic nucleotides; 3) activities of key enzymes in intermediary metabolism; 4) rates of protein synthesis; 5) DNA and RNA synthesis, including specific gene expression; and 6) cellular growth and differentiation (Draznin et al., 1989). From initiation of the signal to the cell response, much progress has been made in the understanding of molecular mechanisms of insulin signalling. Although the spectrum of responses to insulin are known, the detail of each step of the signalling path is still largely unknown. In this chapter, the structure of insulin and the insulin receptor, the relationship between their structure and their function and the research on insulin signalling will be reviewed.

1.2 Insulin Signal Transduction System

1.2.1 Insulin

Insulin is a polypeptide hormone which is synthesized in pancreatic β-cells. The initial precursor of insulin is preproinsulin which consists of proinsulin and a signal peptide (Chan et al., 1976). After synthesis on the rough endoplasmic reticulum (rER), the preproinsulin crosses the membrane into its luminal compartment (Kreil et al., 1981). Following translocation and signal peptide cleavage, the proinsulin molecules fold and

form disulphide bonds to acquire their native structure, which contains A and B chain as well as a connecting peptide (C-peptide). Further, the proinsulin molecules are passed through the cis Golgi apparatus into condensing vacuoles where they are cleaved to yield insulin and C-peptide (Steiner et al., 1984). Insulin and C-peptide are stored together in the secretion granules and released from the pancreatic β-cell by exocytosis (Steiner et al., 1986, Rhode et al., 1987).

1.2.2 Insulin Receptor Structure and Function

Insulin initiates its cellular action by binding to its cell surface receptor (Shoelson et al., 1989). The mature receptor is composed of two identical α-subunits (Mr=135,000) and two β-subunits (Mr=95,000). $\alpha\beta$-disulphide bonds and $\alpha\alpha$-disulphide bond hold the $\alpha_2\beta_2$ together to form the heterotetrameric receptor. The two α-subunits are entirely located at the extracellular face of the plasma membrane and contain a characteristic cysteine-rich region (residues 281-291) and Phe-Phe doublet (residues 88-89). Both of them are critical to insulin binding (Olefsky., 1990). Recent mutation studies showed that the four Cys residues at the C-terminal region of the α-subunit are required for normal heterotetrameric structure and that Cys 647 plays a major role in the normal covalent association of the α- and β-subunits (Cheatham et al., 1992).

In contrast to the α-subunits, the two β-subunits are integral membrane glycoproteins. They can be divided into extracellular, transmembrane and intracellular domains. The extracellular portion of the β-subunit is connected to the α-subunit via disulphide bonds. The transmembrane domain crosses the cell membrane once with a

stretch of 23 hydrophobic amino acids. The intracellular portion of the β-subunit can be further subdivided into a juxtamembrane domain, a tyrosine kinase domain, and the carboxyl terminal tail (Haring et al.,1991; Taylor et al., 1992).

The juxtamembrane domain of the β-subunit has 12 amino acids (966-977) with high homology to an analogous region of the LDL-receptor. It is required for the internalization of the insulin receptor and possibly for association with specific substrate proteins (Chen et al., 1990). Tyrosine-972 is weakly phosphorylated and appears to be important for substrate binding (Haring et al., 1991).

The kinase domain of the insulin receptor contains an ATP binding site and several autophosphorylation sites. Upon insulin binding, the receptor autophosphorylates initially on either tyrosines 1158 or 1162, while autophosphorylation of tyrosine 1163 occurs late in the autophosphorylation process (Dickens et al., 1992). Tyrosine 1162 is in an equivalent position to a major autophosphorylation site of the protein product of the viral Src oncogene and has been found to be autophosphorylated in the receptor. Tyrosine 1162 and 1163 are the major determinants of kinase activity (Shoelson et al., 1989). Insulin receptors only become fully active after all three tyrosines, 1158,1162 and 1163, have been phosphorylated. This is supported by point mutation studies which showed that full activation of the insulin receptor tyrosine kinase is dependent on insulin-stimulated trisphosphorylation of the kinase domain (Wilden et al., 1992). Apart from the autophosphorylation sites, the kinase domain also contains a region which is associated with ATP-binding. Within the ATP-binding region there is a lysine residue which is critical for the kinase activity. Replacement of this lysine with another amino

acid results in the formation of a receptor which binds insulin normally, but is totally devoid of kinase activity (Chou et al., 1987).

The carboxyl terminal tail of the β-subunit shows less homology to other kinases and is hydrophilic. This region also contains two autophosphorylation sites, Tyr-1328 and Tyr-1334. Mutation of the two carboxyl-terminal tyrosines results in an insulin receptor with normal metabolic signalling but enhanced mitogenic signalling properties (Takata et al., 1991; 1992).

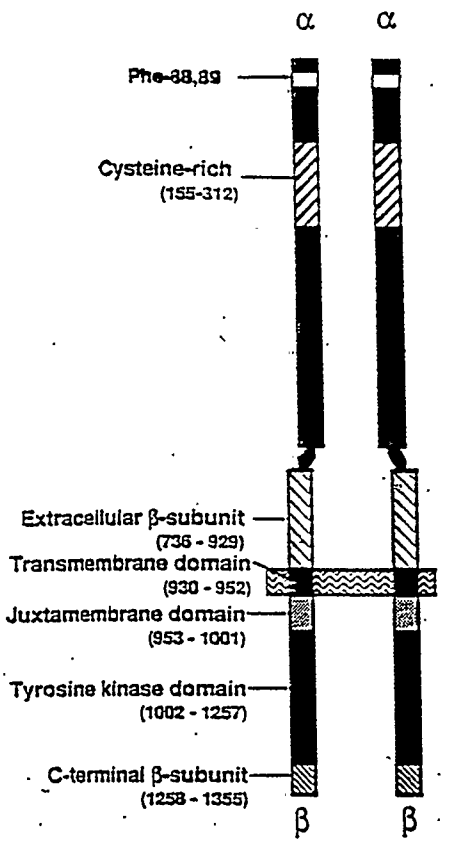


Fig.1 Map of structural domains in the human insulin receptor. The various structural domains of the proreceptor are depicted in this figure (Taylor et al., 1992).

The immediate consequence of insulin binding is a change in conformation of the receptor α-subunit as suggested from the alterations in the affinity of anti-IR antibody for the α-subunit and in tryptic lability of insulin receptor α-subunit in response to hormone binding (Donner et al., 1983). The conformational change in the intracellular domain happens in more than one stage. The first stage is a conformational change in the carboxyl terminus in direct response to insulin binding to the α-subunit. This was suggested by an experiment in which antibodies directed to the receptor C-terminal sequences identified an insulin-induced conformational change; this is, the antibody demonstrated altered affinity for the ligand bound form and unbound form of the receptor (Baron et al., 1990). The changes in the carboxyl terminal, induced by insulin binding, can be separated from those induced in the kinase domain after ATP binding and autophosphorylation. A mutant receptor in which the autophosphorylation sites were mutated still retained the C-terminal conformational change in response to insulin binding. Further, accessibility of receptor autophosphorylation sites to antibodies that interact specifically with the kinase domain increased after ligand binding (Perlman et al., 1989; Baron et al., 1992). In summary, insulin binding to the receptor α-subunit leads to a short-lived, preactivated receptor that becomes competent to bind ATP. The ensuing receptor autophosphorylation induces another conformational change which affects the major part of the cytoplasmic domain and which could lead to unmasking of the receptor catalytic domain or of binding sites for cellular proteins, and allows enzyme-substrate interaction.

Receptor aggregation is also essential for activation of the insulin receptor kinase.

Human polyclonal antireceptor antibodies and mouse monoclonal antibodies can bind specifically to the insulin receptor and mimic biological effects of insulin (Heffetz et al., 1986). These antibodies can be presented to the receptor either as bivalent or monovalent ligands. It was demonstrated that bivalent antireceptor antibodies act as partial agonists of the receptor kinase, while the monovalent forms inhibit insulin binding are devoid of activity. When two monovalent antibodies are cross-linked, however, they regain the capacity to stimulate the insulin receptor kinase activity (O'Brien et al., 1987). These data suggested that receptor occupancy by monovalent antibody is necessary but insufficient for activation of the receptor kinase, whereas microaggregation or clustering of the receptor's extracellular domains is the necessary trigger for kinase activation. Microaggregation of the receptor which is induced upon binding of antireceptor antibodies could take place between subunits within the same receptor tetramer or between subunits of two adjacent receptors.

In the absence of insulin, the insulin receptor kinase exhibits low rates of autophosphorylation and kinase activity. The extracellular, transmembrane and intracellular portions, each contributes an important role to maintain this inactive state. A mutant receptor truncated to remove the α-subunit and extracellular extension of the β-subunit is constitutively active in autophosphorylation, which suggests that the α-subunit and extracellular extension of the β-subunit is the structure that maintains the inhibited state of the intracellular extension, and this inhibition can be moderated by the binding of insulin to the α-subunit (Ellis et al., 1987). This conclusion is further supported by the observation that selective tryptic cleavage of the α-subunit to remove

the insulin binding domain activates β-subunit autophosphorylation. The removal of the extracellular/transmembrane portion of the β-subunit is apparently even more liberating to β-subunit autophosphorylation than is the binding of insulin to the α-subunit (Shoelson et al., 1988).

The insulin signal transduction pathway starts at insulin binding to its cell surface receptor. After insulin binding, the activated receptor will pass on the signal by interaction with a "signal" enzyme or by phosphorylation of substrate protein. Pathway divergence may happen here.

1.2.3 Receptor Signal Transduction Pathway

In general, there are two major signalling pathways for the activated receptor to function. One is the insulin receptor tyrosine kinase that phosphorylates cellular proteins. The other is the non-covalent interaction of the phosphorylated receptor with other cellular proteins (Kasuga., 1990).

1.2.3.1 Receptor Substrate Phosphorylation

Subsequent to insulin binding, the tyrosine kinase activity of the β-subunit is increased. The activated tyrosine kinase phosphorylates endogenous cellular substrates. Proteins that interact with the insulin receptor and have tyrosines in a proper primary and secondary structure are likely to be phosphorylated. Many proteins are phosphorylated in the presence of insulin receptors and their phosphorylation is stimulated by insulin (Zick., 1989, Kasuga., 1990). Proteins of Mr185,000 (pp185),

120,000 (pp120), 15,000(pp15) and 180,000 (pp180) as well as several others were believed to be endogenous cellular substrates which are phosphorylated in an insulin dependent fashion (Roth et al., 1992, Goren et al., 1990, Miralpeix et al., 1992).

A) pp185

Using anti-phosphotyrosine antibodies, White et al (1985) reported a phosphotyrosine containing protein of relative Mr185,000 (pp185), which appears during the initial responses of hepatoma cells to insulin binding. This protein does not bind to the lectin, wheat germ agglutinin, or to anti-insulin receptor antibodies. It is a water soluble protein and has a different tryptic phosphopeptide map from the insulin receptor (White et al., 1987, Condorelli., et al 1989). Tyrosine phosphorylation of pp185 exhibits a dose-response curve similar to that of receptor autophosphorylation. Similarly, insulin responsive phosphotyrosine-containing pp185 was also detected in 3T3-L1 adipocytes, human epidermal carcinoma cell KB, and mouse neuroblasts (Kadowaki et al., 1987, Shemer et al., 1987). Not only the tyrosine kinase activity associated with the insulin receptor but also the insulin-like growth factor-1 receptor can stimulate the tyrosine phosphorylation of pp185 (Kadowaki et al., 1987, Shemer et al., 1987). Insulin mimickers also activated the tyrosine kinase activity of the insulin receptor and induced the tyrosine phosphorylation of pp185 (Momomura et al., 1989). When the mutant insulin receptor, which lacks tyrosine kinase activity, was transfected and expressed in CHO cells, the tyrosine phosphorylation of pp185 in response to insulin was not detected (Maegawa et al., 1988). These data suggested that the tyrosine kinase activity of the insulin receptor is responsible for the tyrosine phosphorylation of pp185. Tyrosine

phosphorylation of pp185 reaches maximum within 30s, then it starts to be dephosphorylated, despite continued insulin receptor autophosphorylation (Shemer et al., 1987). The unstable phosphorylation of pp185 in intact liver raises the possibility that the tyrosine phosphorylated form of pp185 serves as a labile, transient intermediary element that initiates the persistent metabolic effects or growth-promoting effects of insulin.

pp185 has been purified and partial sequence has been obtained (Rothenberg et al.,1991). Antipeptide antibodies were raised, which specifically recognize a single, 185 kDa insulin-stimulated phosphotyrosyl protein in liver, skeletal muscle, adipose tissue, and several cultured cell lines (Rothenberg et al., 1991). Based on the partial peptide sequence, pp185 has been cloned (Sun et al., 1991). The cloned protein, called IRS-1, shows no significant amino acid sequence identity to known proteins. IRS-1 contains 34 tyrosine residues, 14 of which are adjacent to acidic residues and might be potential phosphorylation sites (Shoelson et al., 1992). During insulin stimulation, the IRS-1 undergoes tyrosine phosphorylation and binds phosphatidylinositol 3-kinase (Cantley et al., 1990). The activity of phosphatidylinositol 3-kinase was found to be increased during insulin stimulation (Ruderman et al., 1990, Folli et al., 1992). Bacterial fusion proteins containing the src homology-2 domains of the 85-kDa subunit of the PI3-kinase bound quantitatively to tyrosine phosphorylated, but not unphosphorylated, IRS-1, and this association was blocked by phosphotyrosine-containing synthetic peptides. Moreover, the phosphorylated peptides and the SH2 domains each inhibited binding of PI3-kinase to IRS-1 (Myers et al., 1992). Accordingly, it is suggested that the

interaction between PI3-kinase and IRS-1 is mediated by tyrosine phosphorylated motifs on IRS-1 and the SH2 domains of pp85 subunit. Furthermore, formation of the IRS-1-PI3-kinase complex was found to be specifically inhibited by synthetic peptides containing phosphorylated YXXM motifs (Backer et al., 1992, Yonezawa et al., 1992). Therefore, it is likely that the IRS-1 which is tyrosyl phosphorylated on YXXM motifs binds and activates the PI3-kinase. Thus, pp185 acts as a multisite 'docking' protein to which signal transducing molecules containing src-homology-2 domains could bind.

B) pp120

When rats were treated with dexamethasone, insulin stimulated the phosphorylation of its own receptor as well as of a phosphoprotein of molecular weight 120kDa (pp120) (Rees-Jones et al., 1985). pp120 is found in a plasma membrane-enriched fraction, is detergent-soluble, and binds to wheat germ agglutinin (Perrotti et al., 1987). Phosphorylation of pp120 in response to insulin stimulation occurs on a similar time course as that of the receptor itself. IGF-1 is only 1% as potent as insulin in stimulating the phosphorylation of pp120 (Kadowaki et al., 1987). This tyrosine-phosphorylated glycoprotein has been identified as HA4 which localizes primarily to the bile canalicular domain of the hepatocyte (Margolis et al., 1988). Anti-pp120 and anti-HA4 were found to cross-react, and phosphopeptide maps for each of the corresponding antigens were identical. Recently, pp120/HA4 has been purified to homogeneity (Margolis et al., 1990). The partial peptide sequences of pp120/HA4 have been obtained, and they were found to match amino acid sequences of a hepatocyte membrane protein called ecto-ATPase (Margolis et al., 1990). It was concluded that pp120/HA4

and ecto-ATPase are the same protein (Margolis et al., 1990). The identification of pp120/HA4 as ecto-ATPase serves to link insulin action through the receptor tyrosine kinase activity to bile metabolism.

C) pp15

Bernier et al (1987) found that insulin stimulated the tyrosine phosphorylation of a 15,000 molecular weight phosphoprotein (pp15) when 3T3-L1 adipocytes were treated with phenylarsine oxide, a phosphatase inhibitor. The sequence analysis of tryptic fragments of pp15 revealed that pp15 is a member of the family of fatty acid-binding proteins (Hresko et al., 1988). It is possible that tyrosine phosphorylation of a lipid binding protein may be important in insulin regulation of lipid metabolism. Further studies are needed to determine the role of pp15 in insulin signalling and to understand how phosphorylation of pp15 modulates lipid metabolism.

D) pp180

By using anti-phosphotyrosine antibody, it was found that in the presence of insulin, phosphorylation of glycoproteins from human placental membranes yielded the 95kDa β-subunit of insulin receptor and a 180 kDa protein (Machicao et al., 1987). Phosphoamino acid analysis indicated that the phosphorylation of both proteins occurred only on tyrosine residues (Machicao et al., 1987). The same protein was observed by another group in muscle and liver (Komori et al., 1989). Later, Goren et al (1990) analyzed immunoprecipitated proteins from in vitro phosphorylation reactions and also found that in addition to phosphorylated insulin receptor β-subunit, a phosphorylated 180,000 molecular weight protein was detected in human placenta, rat liver, rat skeletal

muscle, rat heart and rat brain plasma membrane. Experimental evidence suggests that pp180 phosphorylated in each of these tested tissues is the same monomeric glycoprotein (Goren et al., 1990). pp180 is extracted with detergent, binds to wheat germ agglutinin-agarose, is sensitive to glycosidase digestion and behaves like a 150,000 molecular weight protein in denaturing gel in the absence of dithiothreitol (Goren et al., 1990). The phosphorylation of pp180 stimulated by insulin receptor kinase has a very quick time course and a similar insulin dose-dependency as insulin receptor autophosphorylation. Removal of insulin receptors with monoclonal antibodies prevented subsequent pp180 phosphorylation. Insulin-activated receptors increased the extent, but not the rate, of pp180 phosphorylation; the increased phosphate was incorporated into tyrosine and appeared in three or four of pp180's 12 tryptic phosphopeptides (Goren et al., 1990). Although both pp180 and EGF receptor have 180,000 molecular weight and are tyrosine phosphorylated, chromatograms of tryptic phosphopeptides from pp180 in reverse-phase high pressure liquid chromatography (Goren et al., 1993) and functional studies showed that pp180 is entirely different from the EGF receptor (Goren et al., 1993). Also, the lectin absorption property as well as the chromatographic behavior of tryptic phosphopeptides make pp180 distinguishable from pp185 (Goren et al., 1991).

E) Guanosine-triphosphate (GTP)-binding protein

A role for GTP-binding proteins in post-kinase signal transduction has long been discussed. The involvement of a G-protein in insulin action has been suggested in studies demonstrating that pertussis toxin (Goren et al., 1985) or antibody to GTP-binding ras-p21 protein can block certain actions of insulin (Luttrell et al., 1988). The evidence that

G-proteins can serve in vitro as substrates for the insulin receptor kinase was observed by several laboratories (O'Brien et al., 1987, Rothenberg et al., 1988, Krupinski et al., 1988). Recently a 40kDa G-protein has been identified which copurified with the insulin receptor and has an increased GTP binding effect corresponding to insulin stimulation (Kellerer et al., 1991). It is still unclear which effector systems might be activated by these G-proteins.

Ras-p21 is involved in insulin signalling. Stimulation of the insulin receptor resulted in an increase in the GTP-bound state of ras-p21 (Burgering et al., 1991, Maassen et al., 1992). Insulin-induced ras-p21 activation is sensitive to phenylarsine oxide, a putative inhibitor of tyrosine phosphatase (Medem et al., 1991). The mechanism by which insulin stimulates the formation of ras-GTP is still unclear. It may involve either an activation of the GDP/GTP exchange rate or an inhibition of the GTPase reaction by inhibiting GAP or NF1. Ras-GTP transmits its signal probably via the Ser/Thr kinase Raf (Maassen et al., 1992).

1.2.3.2 Receptor Interaction with Signal Protein

In addition to phosphorylation of substrates, the activated insulin receptor could also interact with enzymes such as phospholipases which would hydrolyze membrane lipids to generate products acting as signal transduction messages. The search for an insulin-dependent "second messenger" began in the early 1970's. Larner(1971) first reported the existence of an insulin-sensitive substance in skeletal muscle that could modulate glycogen synthetase in vitro. Similar substances which have the same property

were subsequently identified in a variety of cell types. Two structurally similar substances that were released from hepatic plasma membranes in response to insulin were isolated in 1986 (Saltiel et al 1986b). It was found that inositol was a component in the enzyme-modulating substance and it was suggested that the enzyme modulators might be unusual derivatives containing inositol phosphate. Furthermore, phosphatidylinositol-phospholipase (PI-PLC) was found to reproduce the effect of insulin in generating the enzyme modulators. The PI-PLC digestion generated substances which were chromatographically, electrophoretically, and chemically identical to those produced by insulin treatment. This evidence suggested that the modulators may contain a basic inositol phosphate glycan structure and the enzyme modulators were produced as a result of a hormone-stimulated hydrolysis of a new membrane-associated glycosyl-PI (Saltiel et al 1986a). The chromatographic, chemical, and electrophoretic behavior of the metabolically labelled compounds was identical to that of the biologically active enzyme modulators. Thus, the structure of membrane-associated glycosyl-PI was suggested to consist of inositol phosphate glycosidically linked to the C-1 position of glucosamine, which in turn is glycosidically linked to additional monosaccharides. The mechanism whereby the insulin receptor is coupled to the stimulation of PLC activity remains a critical unknown.

1.2.3.3 Insulin Receptor and IGF-1 Receptor

Since IGF-1 is structurally related to insulin, and IGF-1 receptors have a very similar structure and subunit composition to insulin receptors, insulin can regulate

metabolic effects on growth not only through the insulin receptor but also via the IGF-1 receptor (Straus., 1989). Insulin stimulates growth and proliferation of a variety of types of mammalian cells. The growth-stimulatory effects of insulin are mediated by high affinity binding to insulin receptors and by low affinity binding to IGF-1 receptors. Human fibroblasts are a cell type that expresses insulin receptors and IGF-1 receptors. Dose-response curves for the growth effects of insulin in fibroblasts are very broad. This very broad dose-response profile suggests that insulin might stimulate the growth of these cells at low concentrations by binding to the high-affinity insulin receptor and at higher concentrations by binding to the IGF-1 receptor (Rechler et al., 1985). Insulin stimulates glycogen synthesis in the liver cells in a biphasic manner: one phase that represents 20-30% of the maximal response occurs over a lower insulin concentration range whereas the second phase occurs in a high concentration range (Verspohl et al., 1988). These results together indicate that at low concentrations insulin stimulates glycogen synthesis via the insulin receptor while at high concentrations insulin stimulates glycogen synthesis via an IGF-1 receptor. In addition, recent research showed that activated IGF-1 receptor regulated tyrosyl phosphorylation of nuclear proteins (Oemar et al., 1991). This evidence suggest that tyrosyl phosphorylation of nuclear proteins may be a step in the transduction of mitogenic signals.

1.2.3.4 Receptor Internalization

The binding of insulin to cell-surface receptors initiates a complex and rapid cascade of events that leads to the expression of insulin's biological action as well as the

endocytotic uptake and intracellular processing of insulin-receptor complexes (Kahn et al., 1985). After this internalization, insulin is mainly degraded, while most of the receptors are recycled back to the membrane (Marshall et al 1983). The protein tyrosine kinase activity of the insulin receptor is necessary for insulin mediated receptor down-regulation (Russell et al., 1987, Carpentier et al., 1992). Mutation of tyrosine residues 1162 and 1163 of the insulin receptor also affects insulin and receptor internalization (Reynet et al., 1990). Point mutations of tyrosine residues in the juxtamembrane region of some plasma membrane receptors have shown that this region is required for insulin receptor internalization (Backer et al., 1990). Endocytotic uptake and intracellular routing may regulate events involved in terminating the biological actions of insulin.

1.2.3.5 PTPase and Insulin Signalling

Protein tyrosine phosphorylation of the insulin receptor and its substrates is an essential regulatory mechanism for the regulation of many cellular functions. However, protein tyrosine phosphatases catalyze the reverse of tyrosine phosphorylation. The dephosphorylation of the insulin receptor and its substrates will also play a central role in the regulation of insulin action. In studies with cultured cells (Haring et al., 1984) or a permeabilized adipocyte model (Mooney et al., 1989), dissociation of insulin from the receptor is followed by a rapid dephosphorylation of the β-subunit and deactivation of the receptor kinase. Several PTPases have been found to be active against the autophosphorylated insulin receptor in vitro, including purified CD45 and PTPase 1B (Tonks et al., 1988, 1990). The catalytic domains of several PTPases expressed in a

recombinant bacterial system, including CD45, LAR, and RPTP-2, RPTP-δ were also found to dephosphorylate the autophosphorylated insulin receptor (Goldstein et al., 1992). In addition to the insulin receptor, phosphorylated insulin receptor substrates are also highly regulated by dephosphorylation. IRS-1 is one of the typical examples. pp185 is phosphorylated in seconds following stimulation of the receptor by insulin (Rothenberg et al 1991). This phosphorylation is transient and dephosphorylation occurs rapidly despite the continued presence of insulin and receptor activation (White et al., 1987, Rothenberg et al., 1991).

Potential substrate specificity for PTPase in vivo has been suggested by a few reports in the literature. The best studied example is that of CD45 which has been reported in the activation of the $p56^{lck}$ kinase by the specific dephosphorylation of Tyr-505 in vivo (Ostergard et al., 1989, Mustelin et al., 1990). Direct effects of PTPase activity on insulin signalling in intact cells has also been demonstrated by microinjection of purified PTPase 1B into Xenopus oocytes, which blocked insulin-stimulated S6 peptide phosphorylation and retarded insulin-induced oocyte maturation (Cicirelli et al.,1990, Tonks et al., 1990). Conversely, PTPase inhibitor studies suggested that the enhancement of the phosphorylation state of the insulin receptor can serve to augment insulin signalling (Fantus et al., 1989, Shechter et al., 1990).

Liver tissue is a rich source of PTPase activity and multiple enzymes have been described in particulate and cytosolic liver fractions that will dephosphorylate the insulin receptor (Goldstein etal., 1991). Three peaks of membrane PTPase activity were separated by poly (L-lysine) chromatography of the particulate fractions (Gruppuso etal.,

1991). PTPase I and II were most active with P-Tyr-reduced, carboxyamidomethylated and maleylated lysozyme, whereas PTPase III showed greater activity with P-Tyr-myelin basic protein. The difference between PTPase I and PTPase II is that PTPase I reacted with anti-peptide antibodies directed towards human placental PTPase (Gruppuso et al., 1991). PTPase-1B was recently identified among these enzymes in the membrane fraction of rat liver by immunoblotting with a specific anti-peptide antibody (Gruppuso et al., 1991). Moreover, autophosphorylation sites in the insulin receptor tyrosine kinase domain can auto-dephosphorylate in the presence of ADP (Gruppuso et al., 1992). The auto-dephosphorylation was insensitive to known PTPase inhibitors. This finding raises the possibility of a novel mechanism for termination of the insulin receptor signal. Although much progress has been made in studying the major mechanism of signal integration by protein phosphorylation and dephosphorylation, the detailed molecular events still need to be intensively investigated

1.3 Research Objectives

1) To determine the physical-chemical characteristics of pp180

Since previous reports described pp180 as an insulin receptor substrate isolated by wheat germ-agglutinin chromatography followed by antiphosphotyrosine immunoprecipitation, it is possible that the tyrosine phosphorylated 180 kDa protein contains proteins other than pp180, such as the EGF receptor. In order to separate pp180 from other 180 kDa proteins, its physical-chemical properties such as isoelectric point were determined which enabled a further purification of pp180.

2) To isolate insulin receptor substrate pp180

Based on the isoelectric point of pp180, which was determined to be slightly basic, Carboxymethyl Sepharose cation exchange chromatography was employed to separate pp180 from other 180 kDa proteins which have acidic isoelectric points. After separation by cation exchange chromatography, pp180 was further isolated from polypeptides which have the same isoelectric point as pp180 by antiphosphotyrosine affinity chromatography. By using cation exchange chromatography and antiphosphotyrosine chromatography, pp180 was purified to near homogeneity.

3) To scale up purification protocols and purify pp180 preparatively

The cation exchange chromatographic procedure that was designed based on isoelectric point was modified to enable preparative scale purification of pp180. Based on the preparative scale purification, pp180 was accumulated for partial peptide sequence determination which would enable the identification of pp180.

4) To determine the possible function of pp180

pp180 is an insulin receptor substrate. Preliminary evidence indicates that pp180 has protein tyrosine phosphatase activity. Since wheat germ agglutinin-purified glycoproteins which contain pp180 showed PTPase activity, then experiments were performed to determine whether pp180 functions as a protein tyrosine phosphatase.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Wheat germ agglutinin-agarose was purchased from Vector Laboratories. Carboxymethyl-CL-6B cation exchange agarose was from Pharmacia. Monoclonal anti-phosphotyrosine-agarose was from Sigma. Isoelectric chromatofocusing agarose (PBE94) was from Pharmacia. Protein A agarose was from Sigma. Sephadex G-25 was from Pharmacia. Immobilon-P membrane was purchased from Millipore. Immobilon-CD (Millipore) membrane was a gift from Dr. R. Aebersold (University of British Columbia). Nitrocellulose paper was purchased from Millipore. Crystalline pork insulin was from Dr. D.A.Brodie (Novo Laboratories, Toronto, Canada). Antiphosphotyrosine antibody was raised in rabbits immunized with O-Phosphotyramine-Carboxymethyl-keyhole limpet haemocyanin (Neufeld., et al 1989). Mouse monoclonal antiphosphotyrosine antibody (clone PT-66) was purchased from Sigma Chemical. Rabbit anti-mouse IgG/IgM antibody was from Jackson Immuno Research. Protein A, prepared from Staphylococcus aureus (provided by Dr. P. Lee, University of Calgary, Canada) was stored as 10% (w/v) suspension (Goren., et al 1989). γ -^{32}P-ATP (4500 Ci/mmol) was purchased from ICN. Sodium (^{125}I) iodide was from Amersham. Aprotinin was from Boehringer Mannheim. Bacitracin was from Behring Diagnostics (Lot 101417). Tosylphenylchloromethyl ketone-treated trypsin (TPCK-trypsin, type III) was from Sigma. Tyrosine phosphorylated synthetic peptide was a gift from Dr.D.Cool (University of Washington, USA). X-OMAT (XR) diagnostic film was from Kodak.

Bacto-agarose was from DIFCO Laboratories (USA). SDS-PAGE molecular weight standards and isoelectric focusing protein standards were from Sigma. Bio-Lyte 4/6, 6/8, 8/10, and 3/10 were purchased from Sigma. Polybuffer 96 and 74 was purchased from Pharmacia. Quick Stain was a gift from Dr.R.Aebersold (University of British Columbia).

2.2 Preparation of cell extract

Livers from male rats (150-250g) were removed, rinsed in 0.9% NaCl, and immediately frozen in liquid nitrogen. The frozen liver was ground in a cooled (4°C) porcelain mortar and pestle. The ground liver was homogenized in a Brinckman Polytron homogenizer (90s, setting 4). Homogenization buffer was 50 mM HEPES, 0.1% (v/v) Triton X-100, pH 7.4 (buffer A) which contained 5mM EDTA, 0.1mg/ml aprotinin, 1mM phenyl methyl sulfonyl fluoride, and 0.1mg/ml bacitracin. Homogenates were centrifuged at 12,000 rpm (Beckman Ultracentrifuge, Rotor Type Ti70.1), 10 minutes, 4°C. Triton X-100 was added to the supernatant at a final concentration of 1% (v/v) which was sufficient to solubilize all plasma membrane proteins (Hjelmeland., 1990). The detergent-protein mixture was stirred 45 minutes, 4°C, and then ultracentrifuged 45,000 rpm, 90 minutes, 4°C. The supernatant was loaded to a wheat germ-agglutinin chromatography column.

2.3. Wheat Germ Agglutinin Chromatography

2.3.1 Analytical Wheat Germ-agglutinin Chromatography

The supernatant from ultracentrifuged cell extract (from 4-5g rat liver) containing solubilized glycoproteins was passed through wheat germ agglutinin-agarose (0.3ml) in a 10 ml polypropylene column three times. The column was washed with 50 column volumes of buffer A. Glycoproteins were eluted with 0.5M N-acetyl glucosamine in buffer A. Six 200-300 ul fractions were collected. Protein concentrations in fractions (20ul aliquots) were determined with the Bradford protein assay (Bradford., 1976. Bio Rad). Bovine serum albumin was used to prepare the calibration curve. Protease inhibitor phenyl methyl sulfonyl fluoride was added to the glycoprotein preparation to 1mM final concentration. Protein fractions were stored at -70°C as 50 ul aliquots.

2.3.2 Preparative Wheat Germ-agglutinin Chromatography

The solubilized glycoproteins from 15g rat liver were passed through wheat germ agglutinin-agarose (1.0ml) in a 10ml polypropylene column three times. The column was washed with 25 column volumes of buffer A and 25 column volumes of buffer B (50mM HEPES, 0.1% Triton X-100, pH 6.8). Glycoproteins were eluted with 0.5M N-acetyl glucosamine in buffer B. 1ml fractions were collected. Protein concentrations in fractions (20ul aliquots) were determined with the Bradford (Bio Rad) protein assay (Bradford., 1976). Bovine serum albumin was used to prepare the calibration curve. Protease inhibitor phenyl methyl sulfonyl fluoride was added to the glycoprotein preparation to 1mM final concentration. Protein fractions were stored at -70°C.

2.4 Phosphorylation Reaction

Phosphorylation reaction was done according to Goren et al (1991). 5-10 ug of total wheat germ-agglutinin purified rat liver glycoproteins were preincubated with 5mM $MnCl_2$ in the presence (10^{-8}M) or absence of insulin for 10 minutes at 20°C. After a 10 minute preincubation, γ-^{32}P-ATP (500uM, 0.02Ci/M) was added. Incubation was continued for another 10 minutes. The volume of reaction mixture was 40 ul buffered by 50 mM HEPES, pH 7.4. Following incubation, 5ul 2mM sodium vanadate, 5ul 50mM EDTA was added to terminate the phosphorylation reaction.

2.5 Immunoprecipitation

The phosphorylation reaction mixture (50ul) was incubated for 60 minutes, at 4°C, with antiphosphotyrosine antiserum (1/100 dilution) or with monoclonal anti-phosphotyrosine ascites fluid (PT66,1/2000 dilution). 20ul 50% (v/v) protein A-agarose in buffer A was added and incubation was continued for a further 30 minutes, 4°C. Monoclonal antiphosphotyrosine-immunoprecipitations were incubated with anti-mouse IgG/IgM (2ug/ml), 60 minutes, 4°C prior to protein A addition. Immunoprecipitates were washed twice with 0.1ml wash buffer (0.5M NaCl, 0.1% sodium dodecyl sulphate, 1% Triton X-100 in buffer A) and once with 0.1ml buffer A. Anti-phosphotyrosine-immunoprecipitated proteins were resuspended in 40ul 10mM p-nitrophenyl phosphate or 10mM phenyl phosphate in buffer A.

2.6 SDS-PAGE

7.5% acrylamide gels contained 3ml glycerol, 7.5ml 30% (w/v) monoacrylamide/0.8% (w/v) bisacrylamide in distilled water, 15ml resolving gel buffer (0.75M Tris, 0.2%SDS, 4mM EDTA, pH 8.9), 4.3ml distilled water, 20ul TEMED and 300ul 10%(w/v) ammonium persulfate. Stacking gel was overlaid on the resolving gel. The stacking gel contained 2ml 30%(w/v) monoacrylamide/0.8% (w/v) bisacrylamide, 7.5ml stacking gel buffer (0.1M Tris, 0.2% SDS, 4mM EDTA, pH 6.7), 5.3 ml distilled water, 10ul TEMED and 150ul 10% (w/v) ammonium persulfate. Gel solutions were degassed 15 minutes prior to adding TEMED and ammonium persulfate. The dimensions of resolving gels were 120x160x1.5mm and of the stacking gel were 20x160x1.5mm. After gel polymerization, samples were mixed with 6xSDS solubilization buffer which contained 36%(v/v) glycerol, 10% (w/v) SDS, 9.3% (w/v) DTT, and 0.012% (w/v) Bromophenol Blue in pH 6.8 Tris buffer (0.5M Tris'Cl and 0.4% (w/v) SDS), boiled at 100°C for 2 minutes, and applied to wells in the stacking gel. Electrophoresis was allowed to proceed at 15mA through the stacking gel and 30 mA through the resolving gel. The running buffer used for electrophoresis contained 120mM Tris, 960mM glycine, 17mM SDS, pH 8.3.

2.7 Gel Staining

2.7.1 Coomassie Blue Staining

The gel containing proteins was fixed and stained with fixing solution (40% methanol, 10% acetic acid, and 50% distilled water) containing 0.25% (w/v) Coomassie

Brilliant Blue R-250 5 minutes. The fixed and stained gel was rinsed with 7% (v/v) acetic acid three times, then destained in 7% (v/v) acetic acid overnight or longer until the protein bands appeared blue against a clear background.

2.7.2 Silver Staining

Silver oxide stain according to Schleicher's method (1983) was used to detect the resolved proteins in the second dimension. The gel was soaked in 50% (v/v) methanol, 1hr, 20°C, washed with distilled water 1hr, 20°C, and then re-soaked in 50% methanol 1hr, 20°C. Silver nitrate was prepared in NaOH-NH_4OH solution(20% $AgNO_3$ in distilled water was added to a solution containing 21ml 0.36% (w/v) NaOH and 1.4ml 14.8M NH_4OH with stirring). After staining 15 minutes, gels were washed in distilled water, and then developed in developing solution (0.005% (w/v) citric acid and 0.2% (w/v) formaldehyde in distilled water). When black protein bands were present against a yellow background, developing was terminated by soaking the gel in distilled water.

2.7.3 Sensitive Silver Staining

This method for a sensitive silver stain was first described by Merril et al (1981). After electrophoresis, the gel was fixed and washed with 10% acetic acid, 40% distilled water and 50% methanol. After washing three times, the gel was fixed in 5% (v/v) glutaraldehyde 30 minutes, washed three times with distilled water, and then incubated in fresh $K_2Cr_2O_7$-HNO_3 solution (3.4mM$K_2Cr_2O_7$ plus 3.2mM HNO_3) for three minutes. Prior to addition of silver nitrate(12mM $AgNO_3$), the gel was washed four times with

distilled water. After 30 minute silver nitrate staining, the gel was washed with distilled water, and developed in Na_2CO_3-Formaldehyde (280mM Na_2CO_3 plus 0.05% Formaldehyde). 1% acetic acid was used to stop the developing reaction.

2.8 Autoradiography

After staining and destaining, gels were dried (Bio-Rad Gel Slab Dryer, Model 224) between cellophane sheets. Gels containing ^{32}P-labelled proteins were exposed to X-Omat film (Kodak) in the presence of a Cronex intensifying screen (Dupont). The film was exposed for several days at -70°C and developed. Radioactively-labelled proteins were quantitated either by cutting out proteins from the dried gel and measuring Cerenkov radiation (LKB WALLAC 1215 RACK BETA II Liquid Scintillation Counter), or by scanning the developed X-ray film in a Bio-Rad Densitometer (Model-620).

2.9 Determination of Distribution of Wheat Germ agglutinin-purified Glycoproteins Along pH Gradient

2.9.1 Preparation of ^{125}I-WGA Proteins

Wheat germ agglutinin-purified proteins (30ug) in 60 ul buffer A were iodinated (0.5mCi $Na^{125}I$) in the presence of 6ug chloramine T (Goren et al., 1993). After 90s, the mixture was applied to 0.2 ml wheat germ agglutinin-agarose in a 10 ml polypropylene column. After washing with buffer A (40 ml), ^{125}I-labelled glycoproteins were eluted with 0.5M N-acetyl glucosamine in buffer A. Six 300 ul fractions were collected. Protease inhibitor phenyl methyl sulfonyl fluoride was added to each fraction

to a final concentration 1 mM. Radioactivity of each ^{125}I-labelled glycoprotein fraction was measured (LKB-Wallac 1274 counter).

2.9.2 Isoelectric Chromatofocusing

The type of chromatofocusing matrix was PBE 94 from Pharmacia. Dimensions of the column were 0.8cmx18cm. The column was equilibrated in 0.025M imidazole, pH 7.4 buffer first, then ^{125}I-labelled wheat germ-agglutinin-purified glycoproteins ($2x10^6$CPM) 40 ul were loaded onto the top of the column. Polybuffer 74, pH 4, was used to elute the proteins from the column. Flow rate was 125ul/minute and 2-minute fractions were collected. A total of 145 fractions were collected. The pH (RADIOMETER COPENHAGEN) and radioactivity of each fraction were measured.

To determine the isoelectric point of glycoproteins of pI greater than pH 7, the PBE 94 chromatofocusing matrix was equilibrated in 0.025M ethanolamine-CH_3COOH, pH 9.4. ^{32}P-labelled, wheat germ agglutinin-purified glycoproteins were immunoprecipitated with antiphosphotyrosine antibody. Tyrosine phosphorylated wheat germ agglutinin-purified glycoproteins suspended in 10mM phenyl phosphate in buffer A 100ul (6000 CPM) were applied to the column. Polybuffer 94-CH_3COOH, pH 6.0 was used to elute the proteins from the chromatofocusing column. Flow rate was 125ul/minute and 1-minute fractions were collected. A total of 120 fractions were collected. The pH and radioactivity of each fraction were measured. When pH of the collected fraction dropped to pH 6.0, the eluting buffer was changed to 0.1M CH_3COOH buffer, pH 3. The column was eluted further with this buffer till the pH of the collected

fraction was 3.

2.9.3 Isoelectric Focusing

Isoelectric focusing tube gels were prepared as described previously by O'Farrell (1975). The gel solution contained the following components: urea 4.12g, 3ml distilled water, 1ml 30% (w/v) monoacrylamide/1.8%(w/v) bisacrylamide, 0.4ml Bio-Lyte 3/10, 0.15ml NP-40. After dissolving urea with warming, 5ul TEMED and 30ul 10% (w/v) ammonium persulphate was added to initiate polymerization. First dimensional gels were 1x140mm. Before the sample was loaded, tube gels were subjected to 300V, for 30 minutes between 20mM NaOH (cathode) and 10mM phosphoric acid (anode). Unfractionated lectin purified-glycoproteins were mixed with an equal volume of solubilization buffer containing 9M urea, 4%(v/v) NP40, 5% (v/v) Bio-Lyte 3/10, and 2% (v/v) 2-mercaptoethanol, pH9.5. The glycoprotein-solubilization buffer mixture was loaded onto the tube gel, and the mixture was overlaid with 30ul overlay buffer containing 8M urea, 6% (v/v) Bio-Lyte 6/8, 2% (v/v) Bio-Lyte 3/10, and 2% (v/v) Triton X-100. Electrophoresis proceeded between 20mM NaOH and 10mM phosphoric acid at 800V for 5hr 50 minutes and 1000V for 10 minutes (Tracy., et al 1984). Control gels for first dimension isoelectric focusing included a blank gel to which no protein was applied and a standard gel to which prestained Bio-Rad IEF protein standards were applied. After running, the blank gel was cut into 5mm slices, equilibrated in 1ml distilled water for 16 hr and the pH was measured. The pH gradient formed in the first dimensional gel is based on the measured pH of each slice of blank gel and the migration

of IEF protein standards. The protein standards were phycocyanin (pI 4.65, MW 232,000), β-lactoglobulin B (pI 5.1, MW 18,400), bovine carbonic anhydrase (pI 6.0, MW 31,000), human carbonic anhydrase (pI 6.5, MW 28,000), equine myoglobin (pI 7.0, MW 17,500), human haemoglobin A (pI 7.1, MW 64,500), human haemoglobin C (pI7.5, MW 64,500), lentil lectin (pI 7.8, 8.0, 8.2, MW 49,000), and cytochrome C (pI 9.6,MW 12,200).

2.9.4 Nonequilibrium pH Gradient Gel Electrophoresis

For nonequilibrium pH-gradient gel electrophoresis, the gel solution contained 2.75g urea, 0.7ml 30% (w/v) monoacrylamide/1.8% (w/v) bisacrylamide, 1ml 10% (v/v) NP 40, 0.125ml Bio-Lyte 6/8, 0.125ml Bio-Lyte 3/10, and 1ml distilled water. Following dissolution of urea with warming, 7ul TEMED and 30ul 10% (w/v) ammonium persulfate were added to initiate polymerization. Dimensions of tube gels were 1x140mm. After polymerization, wheat germ agglutinin-purified glycoproteins mixed with an equal volume of lysis buffer (9.5M urea, 2% (v/v) NP-40, 1.6% (v/v) Bio-Lyte 6/8, 0.4%(v/v) 3/1, and 5%(v/v) 2-mercaptoethanol) were loaded on top of the gel. Overlay buffer (8M urea, 0.8%(v/v) Bio-Lyte 6/8, 0.2%(v/v) Bio-Lyte 3/10) was then applied. For nonequilibrium pH gradient gel electrophoresis, the electrodes were the reverse of isoelectric focusing. That is the upper chamber contained 10mM phosphoric acid (anode) and the lower chamber contained 10mM NaOH (cathode). 800 Voltages were applied for 4.5 hr. The pH gradient in the tube gel was determined in the same way as for isoelectric focusing.

2.9.5 Gel Electrophoresis in the Second Dimension

The SDS-slab second dimension gel electrophoresis was prepared as described by Ausubel et al (1990). A 7.5% acrylamide gel contained 15ml 30% (w/v) monoacrylamide/0.8%(w/v) bisacrylamide, 36ml resolving gel buffer (0.75M Tris, 0.2% SDS, 4mM EDTA, pH8.9) 8.6ml distilled water, 40ul TEMED and 600ul 10% (w/v) ammonium persulfate. A stacking gel, which was made from 2ml 30% (w/v) monoacrylamide/0.8% (w/v) bisacrylamide, 7.5ml stacking gel buffer (0.1M Tris, 0.2% SDS, 4mM EDTA, pH6.7), 3ml distilled water, 10ul TEMED and 150ul 10% ammonium persulfate was overlaid on the resolving gel. Dimensions of the resolving gel were 100 x 160 x 1.5mm and dimensions of the stacking gel were 40mmx 160mm x 1.5mm. Isoelectric focusing gels and nonequilibrium pH gradient gels were removed from their tubes by positive air pressure. They were placed on top of the stacking gel of the second dimension gel. 100ul 0.5% (w/v) agarose in distilled water was applied on the top of the tube gel to allow a permanent contact to be made between the tube gel and the SDS-slab stacking gel. Approximately 150ul SDS-solubilization buffer (2% (w/v) Tris, 2%(w/v)SDS, 1% (w/v) dithiothreitol, and 10% (v/v) glycerol) were applied to the top of the agarose-tube gel. Molecular weight standards (Bio Rad) which included myosin (MW 200,000), E.coli β-galactosidase (MW 116,250), rabbit muscle phosphorylase b (MW 97,400), bovine serum albumin (MW 66,200), and hen egg white ovalbumin (MW 45,000) were mixed with the same solubilization buffer, boiled and applied to a well at the end of the stacking gel. Gel electrophoresis in a Bio Rad Protean II apparatus was at 10mA through the stacking gel and 30mA through the resolving gel.

2.10 Determination of Isoelectric Point (pI) of pp180

2.10.1 Isoelectric Focusing of Tyrosine Phosphorylated Glycoproteins between pH 3.5 and 6.8

^{32}P-labelled wheat germ agglutinin-purified glycoproteins were immunoprecipitated with anti-phosphotyrosine antibody. After incubation with protein A, the tyrosine phosphorylated wheat germ agglutinin-purified glycoproteins were resuspended in phenyl phosphate (section 2.5). The resuspended proteins were mixed with IEF solubilization buffer (9M urea, 4%(v/v) NP-40, 5%(v/v) Bio-Lyte 3/10, and 2% (v/v) 2-mercaptoethanol, pH 9.5) in an equal volume and subjected to the isoelectric focusing gel (section 2.9.3). Overlay buffer was applied. Control tubes to determine pH gradient and running condition were the same as in section 2.9.3. After running, tube gels were extruded. Gels which contained tyrosine phosphorylated WGA-purified glycoproteins were sliced into 5mm segments and ^{32}P-labelled proteins were quantitated by Cerenkov radiation (LKB Rack beta II). Alternatively, the first dimension gels were applied to a second dimension SDS-slab gel (section 2.9.5). Following electrophoresis, the SDS-slab-gel was stained with silver nitrate, dried with a Bio-Rad gel slab dryer (Model 224) and then exposed to the X-ray film.

2.10.2 Non-equilibrium pH-gradient electrophoresis of tyrosine phosphorylated glycoproteins between pH 6.5 and 8.8

Tyrosine phosphorylated proteins (40ul, 8-10 ug wheat germ agglutinin-purified glycoproteins) were lyophilized (Speed Vac Concentrator, SAVANT), mixed with 4ul

lysis buffer (Section 2.9.4) and then subjected to nonequilibrium pH gradient gel electrophoresis. The components of the gel solution, solubilization conditions, and control gels were the same as described in section 2.9.4, except that electrophoresis was at 800V for 4.5 hr. After electrophoresis, tube gels were extruded. The isoelectric point of pp180 was determined by slicing the tube gel, counting the radioactivity, and comparing the peaks of radioactivity with the pH at this position. The latter was previously determined by slicing the control gel and measuring the pH as described previously. In addition, the molecular weight of pp180 in the tube gel was determined electrophoretically in a second dimension SDS gel.

2.11 CM-Sepharose-CL-6B Cation Exchange Chromatography

3ml Carboxymethyl-Sepharose-CL-6B (Pharmacia) cation exchange chromatography agarose in a 10ml polypropylene column (Bio-Rad polypropylene, Econo-column) was washed and equilibrated with 50 mM HEPES, 0.1% (v/v) Triton X-100, pH 6.8 (buffer B). Approximately 1 mg wheat germ agglutinin-purified rat liver glycoproteins in buffer B were applied to the column at 4°C. 150 ml buffer B was used to wash the column (0.5ml/minute, pump speed 25, Peristaltic-1, Pharmacia Fine Chemicals). 100 1ml fractions were collected (Gilson fraction collector). Following the wash step, a NaCl step gradient (0.05M-0.5M) was used to elute proteins. There are ten even gradients between 0.05M and 0.5M. Ten 300ul fractions were collected in each gradient. A total of 100 fractions were collected. 20ul 6x Laemmli SDS solubilization buffer was added to 100ul of each presalt fraction No.3, No.4 and every recorded salt

gradient fractions, boiled 2 minutes at 100°C, and the fractions were subjected to SDS-polyacrylamide gel electrophoresis. After gel electrophoresis, proteins were detected by silver oxide stain (section 2.7.2).

2.12 Purification of pp180 on Analytical Scale

Cationic exchange chromatography was used to purify pp180. Two different procedures were compared. In the first procedure, total ^{32}P-labelled glycoproteins were applied to the ion exchange column and eluted proteins were immunoprecipitated. The second procedure involved prior immunoprecipitation of tyrosine phosphorylated glycoproteins before ion exchange chromatography.

2.12.1 Method I

Carboxymethyl-Sepharose-CL-6B cation exchange agarose 3ml in a 10 ml polypropylene column was washed and equilibrated with buffer B. Wheat germ agglutinin-purified rat liver glycoproteins (8-10ug) were ^{32}P-labelled in the presence of insulin as described before (section 2.4). After phosphorylation, the reaction was terminated by adding EDTA and sodium vanadate (section 2.4). The reaction mixture was applied to the agarose beads at 4°C. Buffer B was used to wash off proteins that did not bind to the ion exchange agarose beads. Fractions were collected during the wash. The volume of each fraction was 1ml and 100 fractions were collected. Cerenkov radiation of every fraction was measured and the counts were relatively unchanged from fraction 50 to fraction 100. Cerenkov radiation measured in fraction 100 was less than

100 CPM. Subsequently, a NaCl step gradient (0.05M-0.5M in buffer B) was used to elute the protein bound to the column. From 0.05M-0.5M, 10 incremental gradient steps were chosen. The volume of each NaCl gradient step was 3ml and 10 fractions were collected in each NaCl gradient. After a total of 100 salt fractions were collected, Cerenkov radiation in each fraction was measured.

2.12.2 Method II

Wheat-germ agglutinin purified glycoproteins (20ug) were phosphorylated in the presence of 10^{-8}M insulin, 5mM $MnCl_2$ and 100uM, 4.4Ci/mmol ^{32}P-ATP (section 2.4). After the phosphorylation reaction was terminated by adding 5ul 2mM sodium vanadate and 5ul 50mM EDTA, the ^{32}P-labelled glycoproteins were immunoprecipitated with anti-phosphotyrosine antiserum (section 2.5). Following immunoprecipitation, phenyl phosphate 10mM in buffer A was used to resuspend the tyrosine phosphorylated glycoproteins. 80ul resuspended proteins were applied to CM-Sepharose-CL-6B cation exchange chromatography as described in section 2.10. Proteins which did not bind to the agarose beads were washed out with buffer B. During the wash, eighty 1ml fractions were collected. Cerenkov radiation of each fraction was measured and the counts were relatively unchanged from fraction 20-80. The CPM of fraction 80 was less than 100. Protein bound to ion exchange beads was eluted with the NaCl gradient. Elution conditions and fraction collection were the same as in section 2.10. ^{32}P-radioactivity in all eluted fractions was measured. Fractions in the presalt radioactive peak and salt radioactive peak were checked by SDS-PAGE. 100ul of each of the first ten presalt peak

fractions was mixed with 20ul 6x SDS solubilization buffer. Every 2 fractions in the salt peak (about 300ul each) were combined and lyophilized on a Speed Vac concentrator (SAVANT). After lyophilization, 100ul 1xSDS solubilization buffer was added to each lyophilized sample. All samples were boiled for 2 minutes at 100°C and then applied to SDS-PAGE. Running condition was as in section 2.2.5. After electrophoresis, gel was stained with Coomassie Blue, dried with slab gel dryer (Model 224), and exposed to X-ray film.

2.13 Preparative Purification Protocols

2.13.1 Preparative phosphorylation

Phosphorylation reaction mixture (2ml final volume) contained wheat germ agglutinin-purified glycoproteins (800-1000ug), 5mM $MnCl_2$, 100uM ATP in buffer A. Insulin (10^{-8}M final concentration) was incubated with wheat germ agglutinin-purified glycoproteins for 10 minutes, 20°C, before the addition of ATP. Phosphorylation was allowed to proceed for 10 minutes, 20°C. 400ul 10mM Na_3VO_4 and 1.6ml 50mM HEPES, pH 4.0 was then added to the reaction mixture.

2.13.2 Preparative CM-Sepharose-CL-6B Cation Exchange Chromatography

3ml Carboxymethyl-Sepharose-CL-6B cation exchange beads in a 10 ml polypropylene column were washed and equilibrated with buffer B as described in section 2.4.1. Phosphorylated glycoproteins (800-1000ug) were applied to the column beads. After washing with 50ml buffer B, 10ml 0.2M NaCl in buffer B was used to elute

protein. The 0.2M NaCl eluate was applied to an anti-phosphotyrosine affinity chromatographic column.

2.13.3 Monoclonal Anti-phosphotyrosine Affinity Chromatography

Monoclonal antiphosphotyrosine agarose beads (Sigma, Lot no. 091H 4803) 0.5ml in a 10 ml polypropylene column were equilibrated with buffer A. Phosphorylated proteins from CM-Sepharose-CL-6B cation exchange chromatography 0.2M NaCl eluate were dialysed in buffer A overnight at 4°C. The dialysed proteins were applied to the antiphosphotyrosine affinity column. Protein solution was passed through the column three times. Buffer A 50ml was used to wash out the non-tyrosine phosphorylated proteins. 40mM phenyl phosphate in buffer A was applied to the column. Six 200ul fractions were collected. Phenyl methyl sulfonyl fluoride was added to each fraction to a concentration of 1mM. These 200 ul fractions were then stored at -70°C.

2.13.4 Wheat Germ-agglutinin Chromatography of Tyrosine Phosphorylated Proteins

Wheat germ agglutinin-agarose 0.5ml in a 10ml polypropylene column was washed and equilibrated with buffer A. Tyrosine phosphorylated protein in the phenyl phosphate eluate was applied to the column. The protein eluate was passed through the column three times. The column was then washed with 50 ml buffer A. Tyrosine phosphorylated glycoproteins were eluted with 0.3M N-acetyl glucosamine in buffer A. Six 200 ul fractions were collected.

2.13.5 Sample Preparation for SDS-PAGE

Protein concentrator (Filtron Tech. Corp.) was used to concentrate the tyrosine phosphorylated glycoprotein eluate (section 2.3.3). The concentrator tube (Microsep, 3ml) for molecular weight 100 kDa cut was filled with 2ml protein solution and centrifuged at 700 rpm (Clinic centrifuge, IEC Model CL). When the protein solution was concentrated to about 500ul, distilled water was added to the tube to 1ml and the protein solution was concentrated again. This was repeated two times. Protein solution 200-300ul was mixed with 40-60 ul 6x Laemmli SDS-solubilization buffer and subjected to 7.5% SDS-gel electrophoresis. After electrophoresis, gels were stained with silver nitrate (section 2.7.2).

2.14 Isolation of pp180 for Sequencing

2.14.1 Modified CM-Sepharose-CL-6B Cation Exchange Chromatography

Each of 6ml Pushon scintillation vials (Fisher Scientific) containing 3ml CM-Sepharose-CL-6B cation exchange agarose was washed and equilibrated with buffer B. 400-500 ug Wheat germ agglutinin-purified proteins in 0.5ml buffer B was added. The protein-agarose mixture was rotated at 4°C, 1h. The protein CM-Sepharose mixtures were centrifuged at 700 rpm (Clinic centrifuge, IEC, Model CL). Supernatants were removed and 1ml buffer B was added to each of the cation-exchange resin-containing vials. The suspensions were rotated at 4°C for 15 minutes, centrifuged at 700 rpm (Clinic centrifuge, IEC, Model CL), and the supernatants were removed. The latter wash stage was repeated two times and then 0.5ml 0.2M NaCl in buffer B was added to

each vial. Following 30 minutes rotation, the protein-agarose was centrifuged 5 minutes at 700 rpm (IEC, Model CL) and the supernatant was removed. Phenyl methyl sulfonyl fluoride was added to the supernatant protein solutions to a final concentration of 1mM. Carrier protein (pork insulin) was added to the protein solution to a concentration of 0.1mg/ml. Protein solution was stored at -70°C.

2.14.2 Protein Concentration

Proteins in 0.2M NaCl supernatant approximately 60 ml were pre-concentrated using a microconcentrator. Approximately 2ml protein solution was subjected to each 3ml microconcentrator (100kDa molecular weight cut off, Filtron Tech. Corp.), and centrifuged at 4°C, 700 rpm in a clinical centrifuge (IEC, Model CL) until the protein solution was concentrated to approximately 0.5ml. 1ml distilled water was then added to each microconcentrator. Centrifugation was continued at 4°C, 700 rpm until the protein solution was concentrated to approximately 0.5ml again. 1ml distilled water was added to each microconcentrator again and centrifugation was continued under the same conditions until the protein solution was concentrated to approximately 0.5ml.

A total of 15 ml pre-concentrated protein solution was aliquotted into 3ml. Two of the 3ml protein solutions were concentrated to approximately 500ul. After that, another 3ml pre-concentrated protein solution was added to the concentrated proteins. Concentration was continued until approximately 500ul protein solution was left in the tube. Then the pre-concentrated protein solution (approximately 2 ml) was added to each of the concentrated protein solutions again and the two about 3ml protein solutions were

moved to another two 15ml ultracentrifugation tube. Following transfer, acetone 7ml was added to each 3 ml concentrated proteins (the final concentration of acetone was 70%(v/v)). Carrier protein (pork insulin) was added to the solution to a concentration of 0.1mg/ml. The mixture was left at -20°C, 2hr, centrifuged at 7000rpm (Beckman Ultracentrifuge, Rotor Type Ti70.1) 4°C for 30 minutes. Pellets were combined, and washed gently with 70% acetone in buffer B, centrifuged at 4°C, 7000rpm for 15 minutes. The pellet was dissolved in 500ul 2%-SDS solubilization buffer containing 2% Tris (w/v), 2% SDS (w/v), 1% dithiothreitol (w/v), and 10% glycerol (v/v). The solubilization mixture was incubated at 37°C for at least three hours, then subjected to 5% SDS-PAGE.

2.14.3 SDS-gel Purification for Sequencing

To facilitate transfer of the high molecular weight protein from gel to membrane, 5% SDS resolving gel was used. The gel solution of 5% SDS-resolving gel contained 5ml 30% (w/v) monoacrylamide/0.8%bisacrylamide, 18ml resolving gel buffer (0.75M Tris, 0.2% SDS, 4mM EDTA, pH8.9), 6.8ml distilled water, 20ul TEMED and 300ul 10% (w/v) ammonium persulfate. Dimensions of the resolving gel were 120x160x1.5mm, and dimensions of the stacking gel were 20x160x1.5mm. Solubilized proteins were applied to wells in stacking gel. Electrophoresis at 10mA was applied. After samples entered the stacking gel, electrophoresis was stopped and more sample was loaded into wells. Electrophoresis was re-applied. These steps were repeated until all the solubilized protein had entered the stacking gel. Electrophoresis was kept at 10mA

through the stacking gel and increased to 30mA through the resolving gel.

2.14.4 Transfer of Protein onto Membrane and Identification of Band

Immobilon CD-membrane (12cmx14cm) was prewetted in methanol. Filter paper and membrane was equilibrated in Tris/Glycine transfer buffer (48mM Tris, 39mM Glycine and 0.3mM SDS, pH 8.3). After electrophoresis, the gel was immersed for 5 minutes in the Tris/Glycine buffer. Complete contact CD-membrane to the gel was made and the membrane-gel pair was placed between two filter papers pre-equilibrated in the Tris/Glycine buffer. Transfer(Bio-Rad Tube Cell, Model 175) proceeded at 300mA for 6hr at 4°C. The CD-membrane was washed 5 minutes in distilled water, placed 1 minute in 10ml 25% (v/v)"Quick Stain" solution A and 3 minutes in 25ml 8% (v/v)"Quick Stain" solution B. After a white band appeared against purple background, the membrane was washed with distilled water two times and was sealed in a plastic bag.

2.15 Phosphotyrosine Phosphatase Assay

2.15.1 Phosphorylated Glycoproteins as Substrate

2.15.1.1 Dephosphorylation reaction

Wheat germ agglutinin-purified glycoproteins (8-10ug) were phosphorylated in the presence of 10^{-8}M insulin, 5mM $MnCl_2$, and ^{32}P-ATP (500uM, 0.02uCi/uM). After 10 minutes 20°C incubation, 5ul 50 mM EDTA was added to the 40ul reaction mixture. Subsequent addition of EDTA, 100ul aliquot from each of CM-Sepharose low salt peak fractions was added to individual ^{32}P-labelled protein (45ul). Total of ten fractions were

tested. Dephosphorylation reaction was allowed to proceed for 30 minutes at 4°C. Antiphosphotyrosine immunoprecipitation was then carried out as previously described (2.5). Phenyl phosphate resuspended tyrosine phosphorylated proteins were analyzed by SDS-PAGE as well as autoradiography (2.2.7).

Instead of EDTA which chelates Mn^{2+} and terminates insulin receptor catalysed phosphorylation, Sephadex G-25 beads, which preferentially absorbs low molecular components such as ATP and Mn^{2+}, were used to terminate the kinase reaction (Begum., et al 1992). Phosphorylation reaction mixtures were mixed with 0.7ml Sephadex-G 25 agarose which had been washed and equilibrated with buffer A. After centrifugation in microcentrifuge (SOR-Vall Instrument DuPont 24S) supernatants were pooled together. 100ul test protein solution was added to 40ul aliquots of ^{32}P-labelled proteins. As a control, 100 ul buffer A was applied to ^{32}P-labelled proteins. After 30 minutes incubation at 4°C, the reaction mixture was mixed with 30 ul 6x Leammili sample buffer, boild 2 minutes at 100°C, and subjected to the SDS-polyacrylamide gel electrophoresis.

2.15.1.2 Electroblotting

After electrophoresis, gel was equilibrated in the transfer running solution (0.25mM Tris, 0.7M glycine, pH8.3) for 15 minutes at 20°C. Nitrocellulose paper and filter paper were also prewetted in the same buffer for 15 minutes. After equilibration, gel was completely contacted to the Nitrocellulose paper. The gel-paper pair was placed between two filter papers. Electroblotting proceeded in 300mA 3hr, 20°C. Subsequent to transfer, the Nitrocellulose paper was washed with distilled water, dried and exposed

to X-ray film.

2.15.2 p-nitrophenyl Phosphate as Substrate

10mM p-nitrophenyl phosphate in buffer A was used as a substrate to test protein tyrosine phosphatase activity. The reaction mixture contained 0.1ml 10mM p-nitrophenyl phosphate in buffer A and 0.9ml test sample. 0.9ml buffer A was added to the substrate solution as a control. Reaction mixtures were incubated at 37°C, 30 minutes. The hydrolysis of pNPP was measured in a spectrophotometer (BECKMAN DU-8, Spectrophotometer) at 410 nm (p-nitrophenol extinction coefficient=$1.78x10^4 M^{-1}cm^{-1}$, pH 7.4). Some assays included 0.1mM dithiothreitol in the reaction mixture.

In another set of studies, the reaction mixture (200ul) contained 150ul test sample, 28ul 40mM MES (pH5.0), 2.0 ul 1.6mM dithiothreitol and 20ul 25mM pNPP (Pot., et al 1991). Control test sample was 150ul 40mM MES buffer. Reaction mixtures were incubated at 30°C, 10 minutes. 1ml 0.2N NaOH was added. Absorbance was measured in spectrophotometer at 400 nm wave length (Molar extinction coefficient=$1.78x10^4$ $M^{-1}cm^{-1}$).

2.15.3 Synthetic Phosphopeptide as Substrate

20ul of test fraction was added to 1uM ^{32}P-labelled tyrosine phosphorylated synthetic peptide substrate (gift from Dr.D.Cool, University of Washington, at Seattle) in 40ul buffer C (40mM MES-0.1% Triton X-100, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, pH6.0). Control was the addition of 20ul buffer C to the ^{32}P-labelled

substrate solution. Reaction mixtures were incubated 10 minutes, 30°C. Following incubation, 290ul of charcoal solution (10% Norite A, 1% Celite, 0.9M HCl, 2mM Na_2HPO_4, 0.1M sodium pyrophosphate) was added with mixing. The mixture was centrifuged and radioactivity in 250ul of supernatant was measured.

2.16 Phosphoamino Acid Analysis of Phosphoproteins

^{32}P-labelled proteins separated in SDS-PAGE were detected by autoradiography. The protein bands were cut out and rehydrated in 10% (v/v) methanol. Cellophane was removed. The gel fragments were redried, 1hr, 70°C and the dried gels were added to 2ml 50mM NH_4HCO_3 containing 100ug TPCK-trypsin. The gel solution was incubated 48 hours, 37°C. An equal volume of 12N HCl was added to the supernatant. The mixture was sealed and heated 2 hours at 110°C.

Alternatively, ^{32}P-labelled proteins in Immobilon-P membranes were detected by autoradiography. The protein band was cut out. The membrane which has ^{32}P-labelled protein was placed in 200 ul 6N HCl, sealed, and heated 2hr at 110°C.

The 6N HCl digests were lyophilized, resuspended in distilled water, lyophilized, redissolved again. This was repeated three times to ensure that excess HCl was removed. Following a final lyophilization, the residue was dissolved in 10 ul distilled water and applied to thin layer chromatography sheets (Eastman Chromatogram Cellulose, 16x20cm). The phosphoamino acids were separated with ascending solvent-i-propanol/propionic acid/1M ammonium hydroxide (45/17.5/17.5) as previously described (Neufeld., et al 1989). ^{32}P-labelled phosphoamino acids were detected by

autoradiography. Identity of the phosphoamino acids is based on relative migration of phosphoserine, phosphothreonine and phosphotyrosine amino acid standards which were applied adjacent to protein hydrolyzates. 5mM phosphoamino acid standards (0.6 ul of each) were detected by ninhydrin (1% (w/v) Triketo-hydrindene-hydrate in acetone) stain.

CHAPTER 3 RESULTS

3.1 Identification of pp180 as an IR Substrate

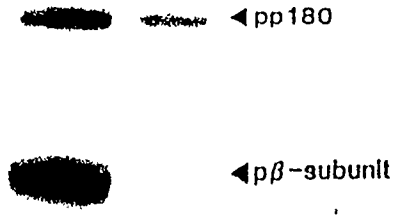
Rat liver glycoproteins were purified over wheat germ agglutinin. The purified glycoproteins were ^{32}P-phosphorylated in vitro in the presence of 5mM Mn^{2+} and 10^{-8}M insulin, immunoprecipitated with antiphosphotyrosine anti-serum, separated in SDS-PAGE, and detected autoradiographically. Two different molecular weight, tyrosine phosphorylated proteins were detected; molecular weight 95,000 and 180,000 (fig.2). The phosphorylated 95 kDa protein was insulin receptor β-subunit. The phosphorylated 180 kDa protein is believed to be mainly phosphorylated EGF receptor and phosphorylated insulin receptor substrate pp180 (Goren et al., 1993). The presence of insulin receptor substrate in tyrosine phosphorylated 180 kDa molecular weight glycoproteins is supported by insulin dose-dependency experiments (Goren et al., 1990), an anti-insulin receptor antibody study (Goren et al., 1990), as well as an in vivo phosphorylation study (Goren et al., 1993). In the absence of insulin, in vitro phosphorylation of rat liver glycoproteins produced less phosphorylated β-subunit and less phosphorylated 180 kDa protein than in the presence of insulin. Insulin increased the phosphorylation of insulin receptor and 180 kDa protein substrate 10 fold and 3 fold, respectively (fig.2).

3.2 Characterization of Insulin Receptor Substrate pp180

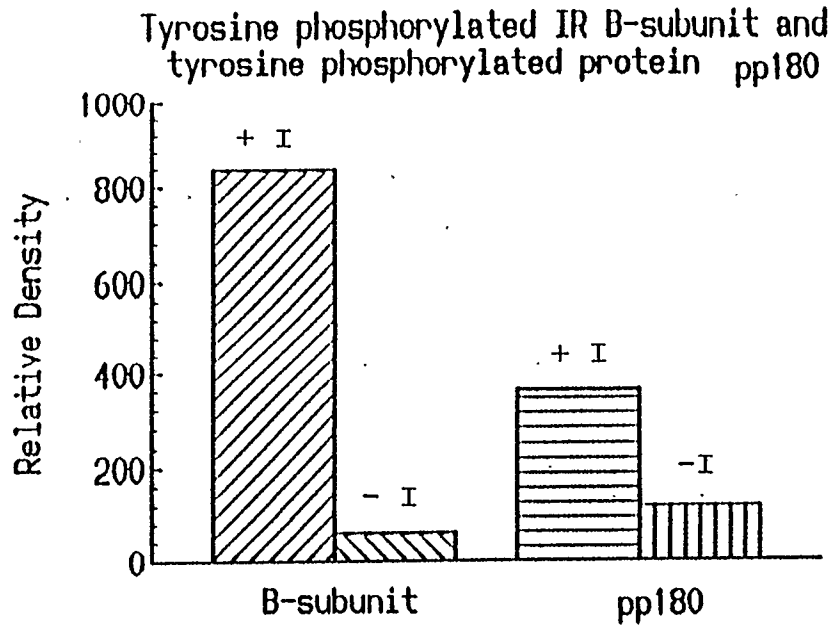
Since pp180 has the same molecular weight as the EGF receptor and they are both

Fig.2 Panel A: Purified glycoproteins were ^{32}P-phosphorylated in vitro in the presence (left lane) and absence (right lane) of insulin. The antiphosphotyrosine immunoprecipitated proteins were resolved in SDS-PAGE and detected by autoradiography. Panel B shows the relative density of the tyrosine phosphorylated protein bands in the autoradiogram.

A


◀pp180
◀pβ-subunit

B


Tyrosine phosphorylated IR B-subunit and
tyrosine phosphorylated protein pp180
Relative Density
1000
800
600
400
200
0
+ I
- I
+ I
-I
B-subunit
pp180

tyrosine phosphorylated, it was necessary to determine the isoelectric point (pI) of pp180 to find out whether it is different from the pI of the EGF receptor. If different, then the method for separation of the two proteins may be based on their ionic properties. Initially, the distribution of total rat liver glycoproteins along a pH gradient was determined.

3.2.1 Separation of Total Rat Liver Glycoproteins According To Their Isoelectric Points

Wheat germ agglutinin-purified rat liver glycoproteins were analyzed by two dimensional gel electrophoresis. The first dimension was either isoelectric focusing or non-equilibrium pH gradient gel electrophoresis. The second dimension was SDS-PAGE. Proteins in second dimension gels were detected by silver oxide staining. Using isoelectric focusing, which separated proteins between pI 3.5 and 7.0 , the second dimension SDS-gel showed that most glycoproteins distributed between pI 3.5 and 5.5 (fig.3A). The molecular weight of these proteins ranged from 180,000 to 30,000.

To determine the isoelectric points of more basic glycoproteins (pI>7), non-equilibrium pH gradient gel electrophoresis was carried out. Two major factors that affect non-equilibrium pH gradient electrophoresis are running time and voltage. pH gradient 5.5-8.6 and 6.2-7.8 were achieved after 4.5 and 9.5 hours at 400 volts and pH gradient 6.8-8.2 was achieved at 800 volts after 4.5 hours (fig.4). The above results showed that at the same voltage, increasing running time leads to a narrowing of the range of the pH gradient that is formed. While with consistent running time, increasing the running voltage reduced the pH gradient in the acidic end, with almost no effect of

the basic end. The non-equilibrium pH gradient gel electrophoresis at 800 volts and 4.5 hours running time allowed more basic membrane glycoproteins to be analyzed. The second dimension SDS gel electrophoresis showed that only a very small amount of protein was present. This protein has a pI approximately 7.2 and it has a molecular weight of 180,000 (fig.3B).

This result was consistent with a chromatofocusing analysis which also showed that most glycoproteins distributed between pI 4.0-7.0 (fig.3C). The molecular weights of these proteins, analyzed by SDS-PAGE, ranged from 180,000 to 30,000. To confirm that there are membrane proteins which have pI beyond 7.0, chromatofocusing between the pH range 6.0-9.4 was performed with insulin stimulated ^{32}P-labelled glycoproteins. The eluate did not show radioactive protein until pH around 7.0. Taken together, the two dimensional gel electrophoresis and chromatofocusing analyses showed that most rat liver glycoproteins purified over wheat germ agglutinin have pI's between 4.0-7.0 and very few have pI above 7.0.

3.2.2 Determination of Isoelectric Point of pp180

Wheat germ agglutinin-purified rat liver glycoproteins (8-10ug) were ^{32}P-labelled in the presence of Mn^{2+} (5mM) and insulin (10^{-8}M). Phosphorylated glycoproteins were immunoprecipitated with antiphosphotyrosine antibody. Phenyl phosphate resuspended tyrosine phosphorylated proteins were applied to isoelectric focusing gel electrophoresis. These gels were either sliced or applied to SDS-second dimension gel electrophoresis. Radioactivity (CPM) and pH of each slice of the isoelectric focusing gel were measured.

Fig.3A Wheat germ agglutinin-purified rat liver glycoproteins were analyzed by two dimensional gel electrophoresis. After isoelectric focusing, the blank tube gel was cut into 0.5cm slice. The pH of each slice was measured (noted at bottom). Isoelectric focusing tube gel, which contained proteins, was analyzed in second dimension by SDS-PAGE. Molecular weight standards (migration noted at left) were applied to second dimension gel. After electrophoresis in the second dimensional, proteins were detected with silver stain.

Fig.3B Wheat germ agglutinin-purified rat liver glycoproteins were separated by nonequilibrim pH gradient gel electrophoresis in the first dimension and SDS-PAGE in the second dimension. Proteins were detected with silver stain. The pH gradient (noted at the bottom) was determined by measuring the pH of 0.5cm slices of a blank first dimension gel. Migration of protein molecular weight standards, applied to SDS-PAGE, was noted at left of gel.

Fig.3C ^{125}I wheat germ agglutinin-purified rat liver glycoproteins were analyzed by isoelectric chromatofocusing in which proteins were separated between pI 4.0-7.4. CPM of each collected fractions were measured (solid line). The pH(x) of some fractions were measured.

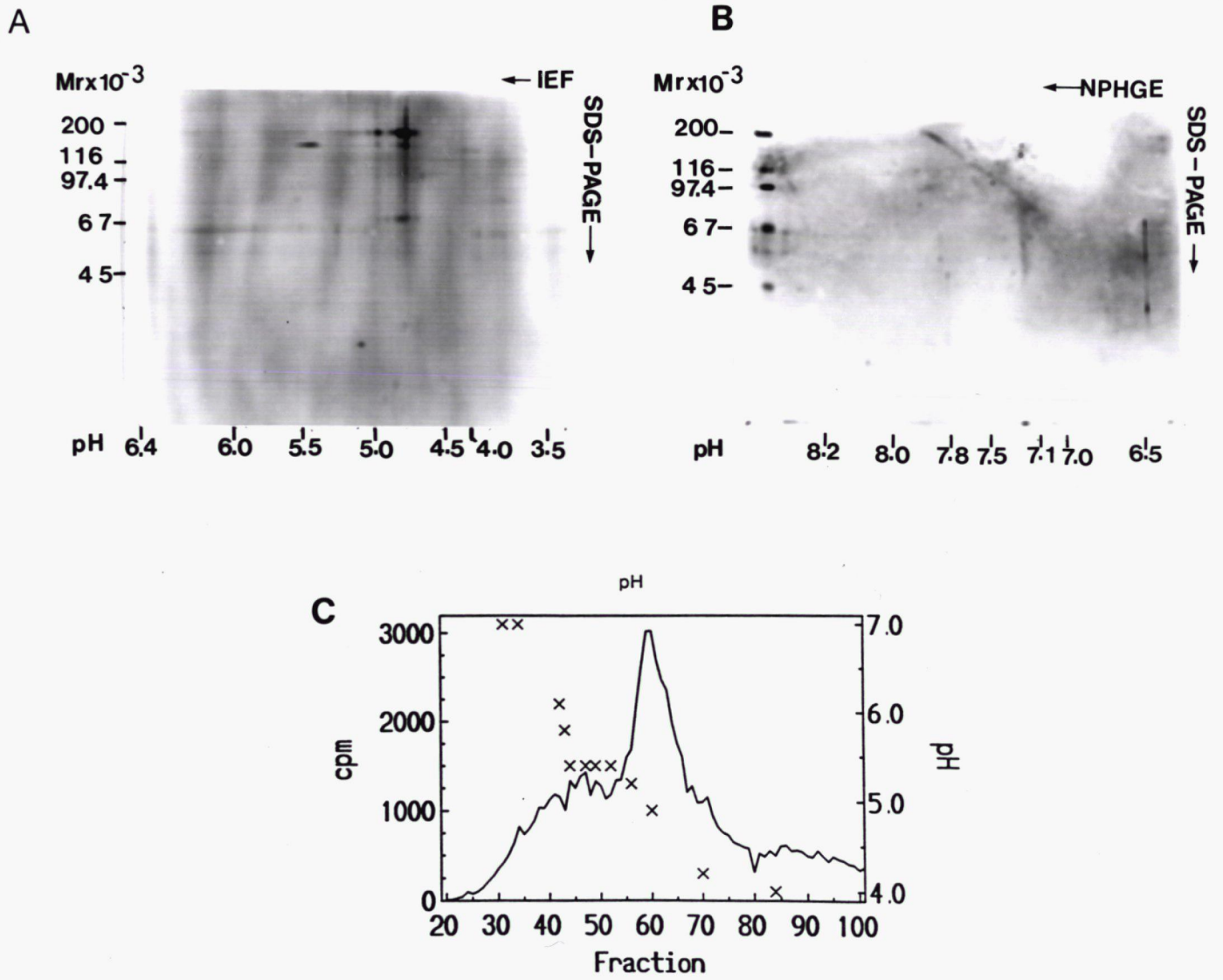
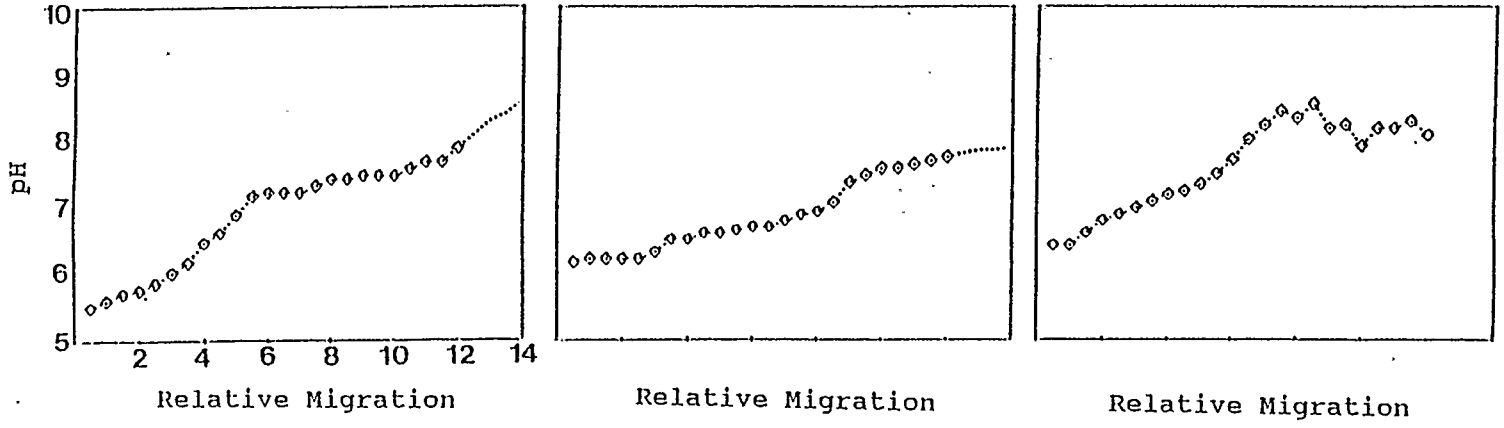

A
Mrx10-3
200
116
97.4
67
45
← IEF
SDS-PAGE →
pH 6.4 6.0 5.5 5.0 4.5 4.0 3.5
B
Mrx10-3
200
116
97.4
67
45
←NPHGE
SDS-PAGE →
pH 8.2 8.0 7.8 7.5 7.1 7.0 6.5
C
pH
cpm
3000
2000
1000
0
7.0
6.0
5.0
4.0
pH
20 30 40 50 60 70 80 90 100
Fraction

Fig.4 pH gradients that were formed under different running conditions in the first dimension for nonequilibrium gel electrophoresis. Curve A was generated at 400 volts, 4.5 hours; Curve B was at 400 volts, 9.5 hours; and Curve C was at 800 volts, 4.5 hours.



Curve A

Curve B

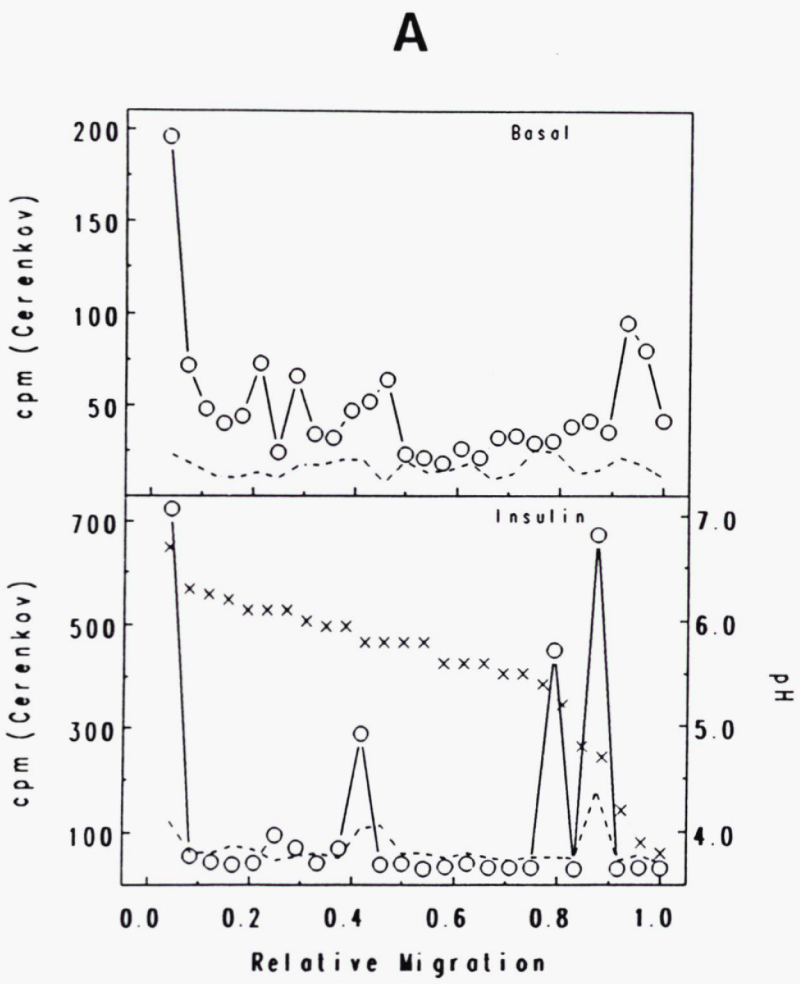
Curve C

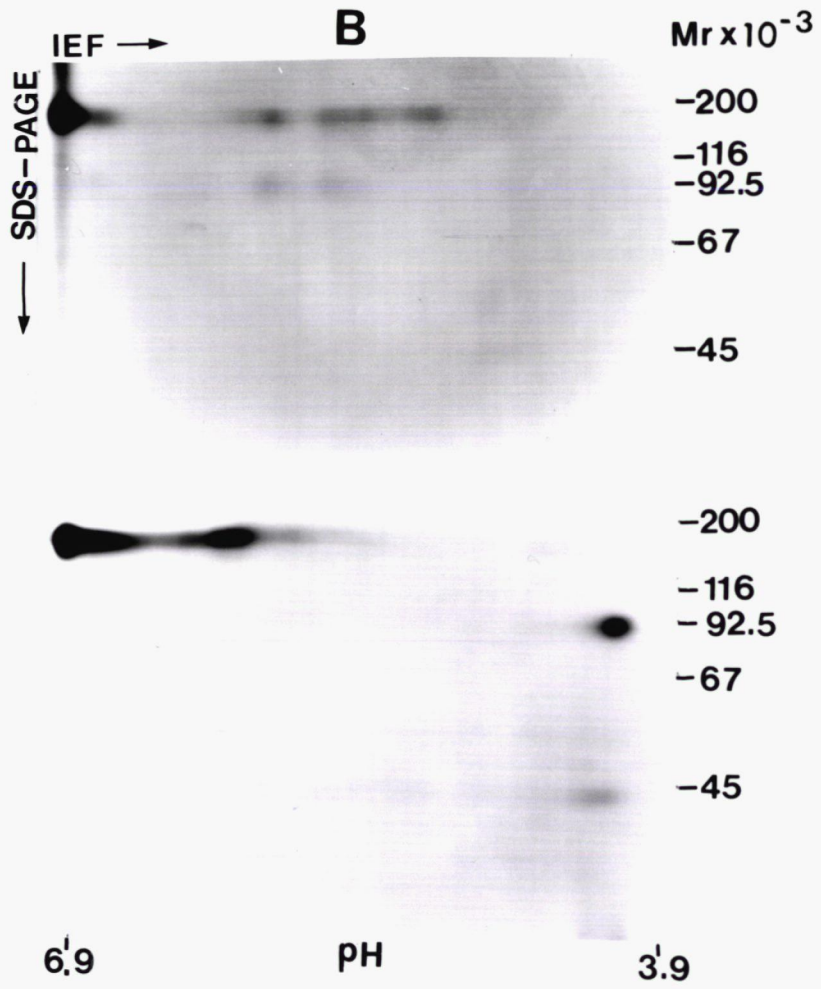
Four phosphotyrosine peaks were present; at pH's 4.5, 5.5, 6.0, and above 7.0 (fig.5A). SDS-PAGE of the isoelectric focusing gel showed that the first peak, pH 4.5, has a molecular weight 95,000; the second peak, pH 5.5, has a molecular weight 40,000; the third peak, pH 6.2, has a molecular weight 180,000; and the fourth peak, pH above 7.0, also has a molecular weight 180,000 (fig.5B). At the 180,000 molecular weight level, the protein which has pI 6.0 is believed to be the EGF receptor. This is the calculated pI based on the amino acid composition of the EGF receptor. The ^{32}P-protein material that did not enter the isoelectric focusing gel is believed to include pp180.

The radioactive content of proteins in the isoelectric focusing gels, compared to insulin-stimulated in vitro phosphorylated protein, was much smaller when in vitro basal phosphorylated proteins were applied (fig.5A). The autoradiogram of second dimension gel of basal-phosphorylated proteins (X-ray film was exposed 14 days) demonstrated less ^{32}P-labelled proteins than in the autoradiogram where insulin-stimulated phosphotyrosine proteins were analyzed (X-ray film was exposed 7 days) (fig.5B). Some radiolabelled protein with 180,000 molecular weight and pI between 5.5 and 4.5 was detected in the autoradiogram of the basal phosphorylated reaction mixture (due to longer exposure of the gel to the X-ray film). The identity of this protein is not known.

To determine the pI of pp180, nonequilibrium pH gradient gel electrophoresis was performed. Rat liver glycoproteins were phosphorylated in the presence of 5mM Mn^{2+} and 10^{-8}M insulin, immunoprecipitated with antiphosphotyrosine antiserum, and tyrosine phosphorylated proteins were suspended with phenyl phosphate. The phenyl phosphate suspended tyrosine phosphorylated proteins were applied to nonequilibrium pH gradient

Fig.5 Two dimension gel electrophoresis of ^{32}P-labelled, antiphosphotyrosine-immunoprecipitated glycoproteins. Purified glycoproteins were phosphorylated in the presence of insulin (A-insulin, B-lower half) or in the absence of insulin (A-basal, B-upper half). They were subjected to isoelectric-focusing (A) and subsequently to SDS-PAGE (B). Fig.4A shows the CPM (o) and pH (x) of each slice of first dimension isoelectric focusing gels. Curve (--) shows the CPM of each gel slice of the first dimension tube gel after resolution of the second dimension gel electrophoresis. Fig.4B shows the autoradiograms of the second dimension gels. Upper gel was exposed 14 days to X-ray film; lower gel was exposed 7 days to X-ray film. Protein molecular weight standards were applied to SDS-PAGE and their migration are noted to the right of the autoradiogram.


A
Basal
Insulin
cpm (Cerenkov)
cpm (Cerenkov)
200
150
100
50
700
500
300
100
7.0
6.0
5.0
4.0
pH
0.0
0.2
0.4
0.6
0.8
1.0
Relative Migration


B
IEF →
SDS-PAGE ↓
Mr x10-3
-200
-116
-92.5
-67
-45
-200
-116
-92.5
-67
-45
6.9
pH
3.9

gel electrophoresis. The radioactivity and pH of each gel slice was measured. The result showed that there were two radioactive peaks at the pI's about 6.0 and 7.2 (fig.6A). In the absence of insulin, the radioactive peaks in the first dimension gel were much smaller than when the protein from an insulin stimulated reaction were applied (fig.6A).

Analysis of the non-equilibrium pH gradient first dimension gel with SDS-PAGE and subsequently with autoradiography indicated that the molecular weight of the protein which had pI 7.2 was 180,000 (fig.6B). Taken together, the two dimensional characterization of rat liver plasma membrane glycoproteins indicated that pp180 is a minor protein and it has a pI of 7.2.

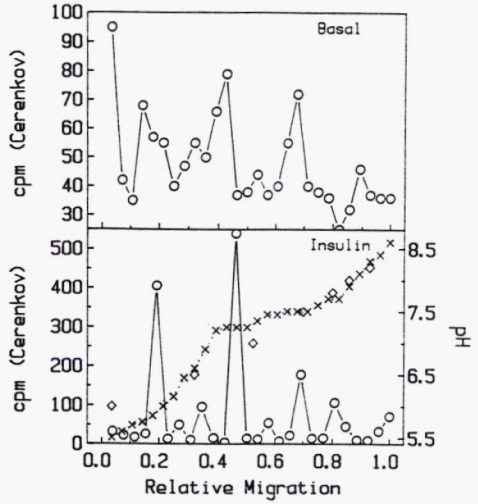
3.3 Purification of pp180

3.3.1 Purification on Analytical Scale

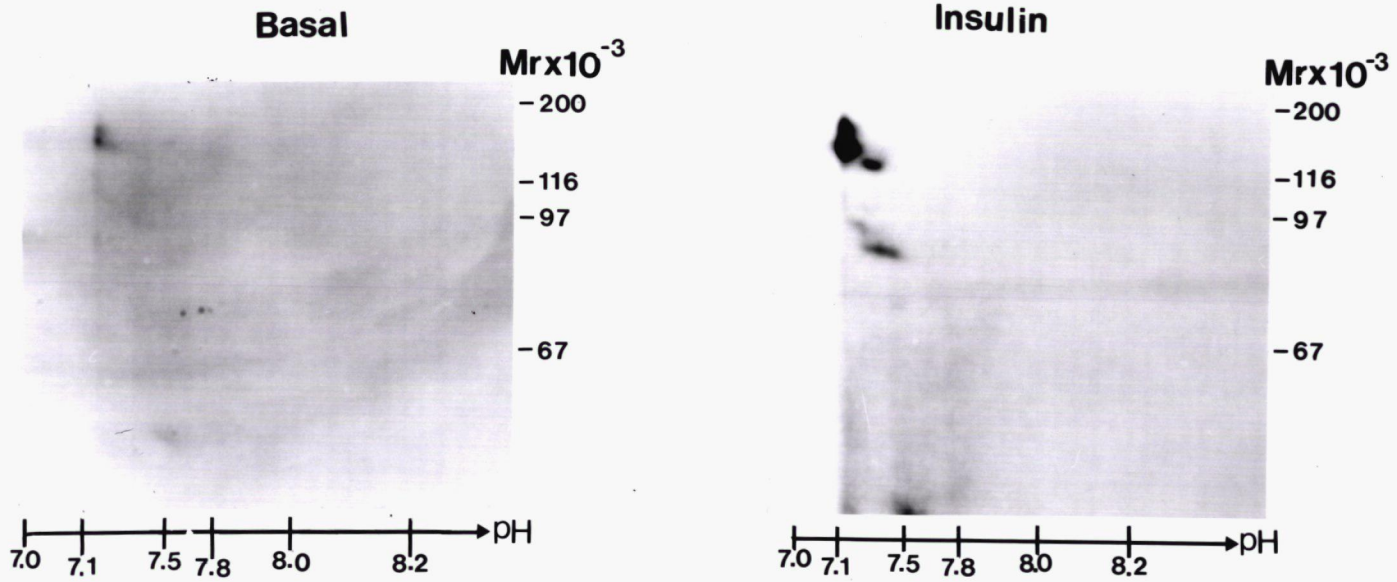
Since two-dimensional gel electrophoresis and chromatofocusing indicated that most rat liver membrane glycoproteins are acidic and pp180 is slightly basic, then Carboxymethyl-Sepharose-CL-6B cation exchange chromatography was employed to isolate pp180. ^{32}P-labelled glycoproteins were applied to the Sepharose beads previously equilibrated to pH 6.8. Proteins which have pI below 6.8 are not anticipated to be retained in the column and would flow through. These proteins are called the presalt proteins. An incremental NaCl gradient (0.05M-0.5M) was used to elute proteins which have pI's above 6.8. Measurement of radioactivity of eluted fractions indicated that there were two radioactive peaks (fig.7). One appeared in the earliest fractions, before

Fig.6 Two dimension gel electrophoresis of ^{32}P-labelled, antiphosphotyrosine immunoprecipitated glycoproteins. Purified glycoproteins were phosphorylated in the presence of insulin (A-lower half, B-right side) or in the absence of insulin (A-upper half, B-left side). They were subjected to isoelectric-focusing (A) and subsequently to SDS-PAGE (B). Fig.5A shows the CPM (o) and pH (x) of each slice of first dimension isoelectric focusing gels. Square point shows the pH's of pH standards in nonequilibrium first dimension gel. Fig.5B shows the autoradiograms of the second dimension gels. Protein molecular weight standards were applied to SDS-PAGE and their migration are noted to the right of the autoradiogram.

A


Basal
Insulin
cpm (Cerenkov)
cpm (Cerenkov)
pH
Relative Migration
0.0
0.2
0.4
0.6
0.8
1.0

B

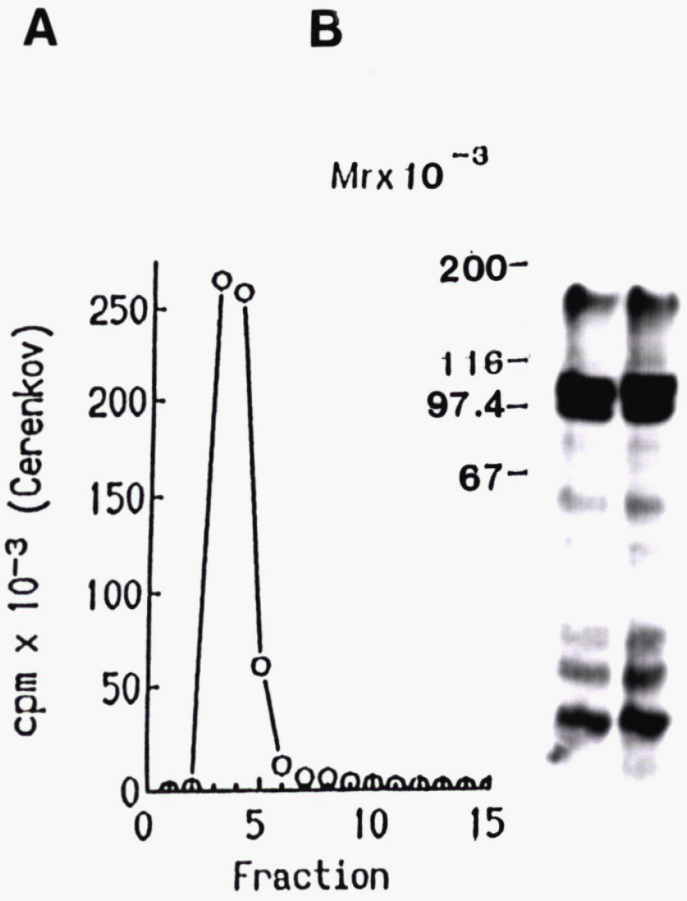

Basal
Insulin
Mrx10-3
Mrx10-3
200
116
97
67
200
116
97
67
pH
pH
7.0
7.1
7.5
7.8
8.0
8.2
7.0
7.1
7.5
7.8
8.0
8.2

application of NaCl, and the other appeared in the eluted fractions between 0.1M and 0.15M NaCl gradient. The radioactive content of the NaCl-eluted peak was much smaller than that of the presalt proteins (fig.7). Antiphosphotyrosine was used to immunoprecipitate fractions from both radioactive peaks. Two tyrosine phosphorylated proteins were present in the presalt proteins and one tyrosine phosphorylated protein was present in the NaCl-eluted peak (fig.7). The molecular weights of the tyrosine phosphorylated proteins in the presalt peak were 95,000 and 180,000. These are believed to be the insulin receptor β-subunit and the EGF receptor. The protein in the NaCl-eluted peak had 180,000 molecular weight. This protein is believed to be pp180, the insulin receptor substrate (fig.7).

Similarly to ^{32}P-phosphorylated proteins, membrane glycoproteins were analyzed by ion exchange chromatography. Both presalt and NaCl-eluted fractions were analyzed by SDS-PAGE and proteins in the gel were detected by silver oxide stain (fig.8). Most glycoproteins, which previously were showed to have pI's below 6.8 (fig.3A), were not retained by the CM-Sepharose column. Small amounts of protein were eluted with NaCl between 0.1M-0.15M. These fractions contained pp180 and several lower molecular weight polypeptides (fig.8).

An estimate of pp180 content in membrane glycoproteins was made. Four to five g rat liver can yield about 1mg glycoproteins from wheat germ-agglutinin chromatography. Based on the intensity of silver-oxide stained pp180 and the limitation for silver-oxide staining, approximately 1ng, (Schleicher et al., 1983). 1mg glycoprotein can yield approximately 200-300ng pp180 following Carboxymethyl-Sepharose

Fig.7 WGA-purified glycoproteins were phosphorylated in the presence of insulin. ^{32}P-labelled glycoproteins were applied to CM-Sepharose-CL-6B cation exchange chromatography at pH 6.8. An incremental NaCl gradient (0.05M-0.5M) was used to elute proteins bound to the column. Radioactivity of each presalt and salt fractions was measured. Panel A shows the radioactive peak of the presalt proteins. Panel B shows the autoradiogram of SDS-PAGE analysis of proteins from presalt fraction 3 and 4. Panel C shows the radioactive peak of the 0.1M NaCl-eluted protein (fractions 10-20). Panel D shows the autoradiogram of SDS-PAGE of 0.1M NaCl-eluted protein immunoprecipitated with antiphosphotyrosine. Migration of molecular weight standards are noted to the left of the autoradiogram.


A
B
Mrx 10 -3
200-
116-
97.4-
67-
250
200
150
100
50
0
cpm x 10-3 (Cerenkov)
0
5
10
15
Fraction

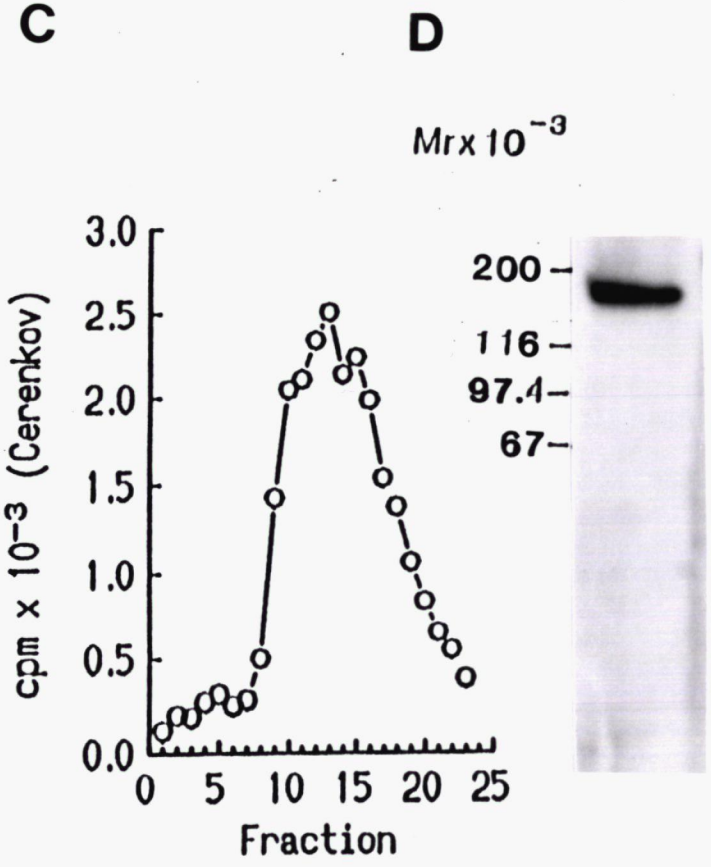
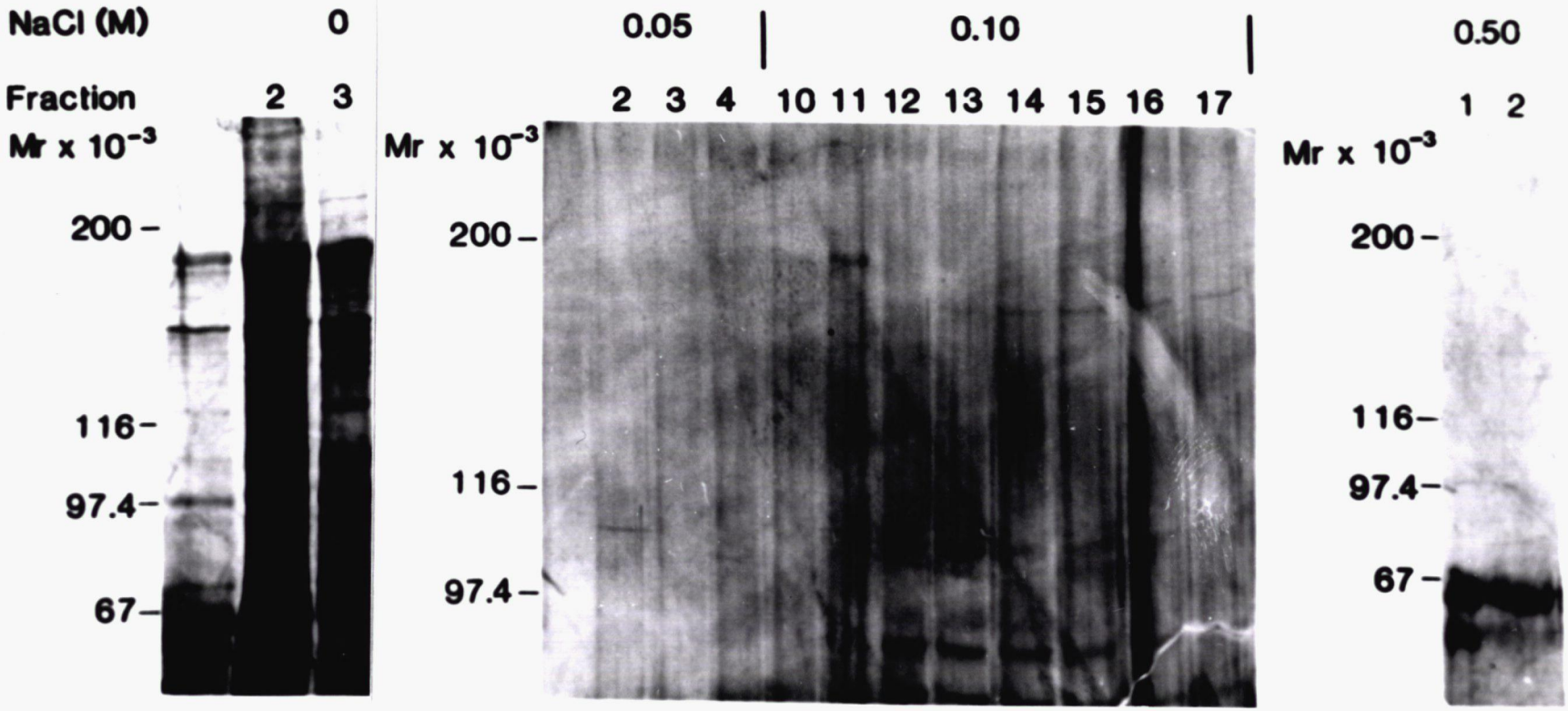

C
D
Mrx 10 -3
200-
116-
97.4-
67-
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cpm x 10-3 (Cerenkov)
0
5
10
15
20
25
Fraction

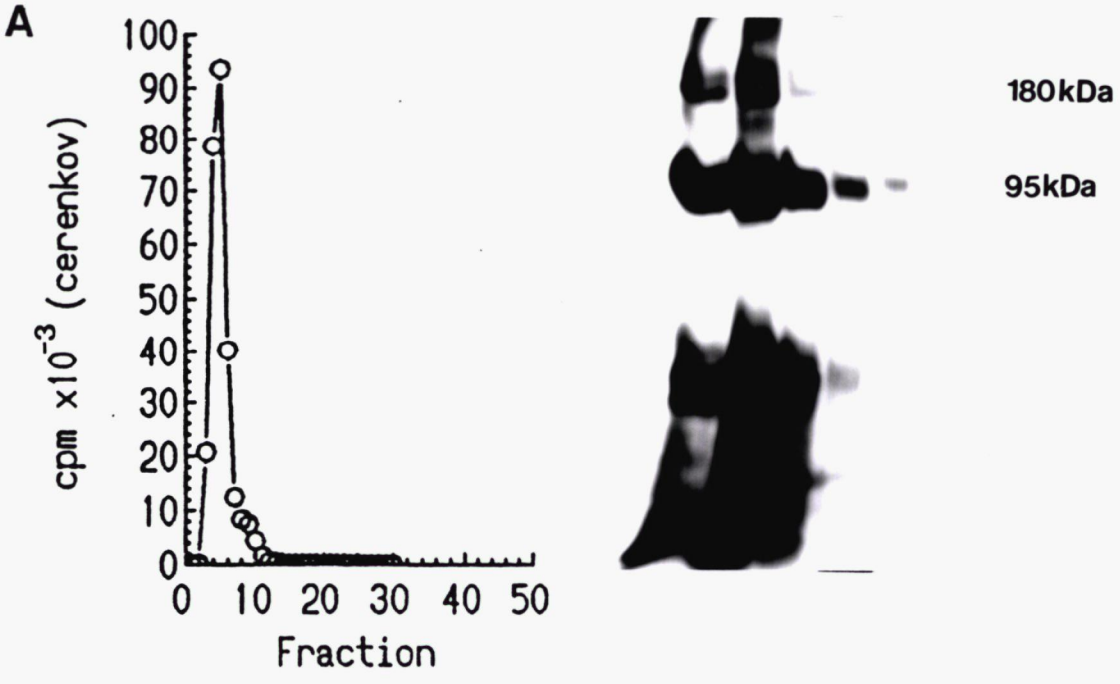
Fig.8 Wheat germ agglutinin-purified glycoproteins were applied to CM-Sepharose-CL-6B cation exchange chromatography previously equilibrated with pH 6.8 buffer. Proteins bound to the column were eluted by an incremental NaCl gradient (0.05M-0.5M). Presalt protein fraction 2 and 3 were analyzed by SDS-PAGE and stained with silver oxide (left side). 0.05M NaCl-eluted fractions (fraction 2-4), 0.1M NaCl-eluted NaCl fractions (fraction 10-17), and 0.5M NaCl-eluted fractions were analyzed by SDS-PAGE with silver stain (right side). Migration protein molecular weight standards are noted to the left of each gel.

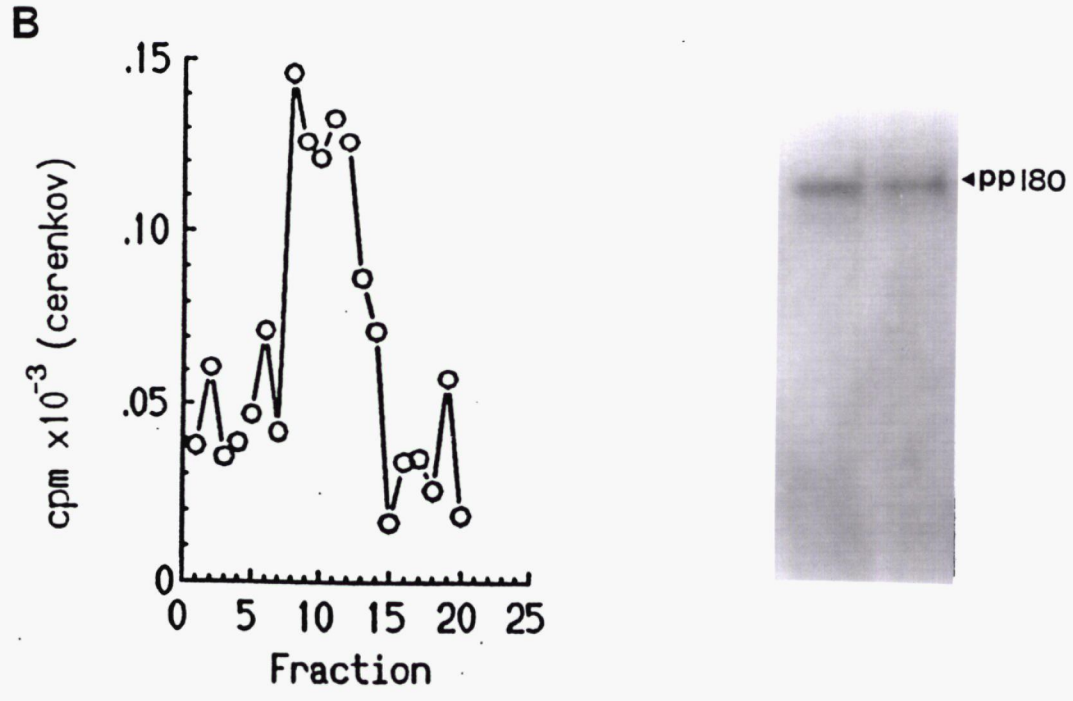

NaCl (M)
0
Fraction
2
3
Mr x 10⁻³
200
116
97.4
67
0.05
0.10
2 3 4 10 11 12 13 14 15 16 17
Mr x 10⁻³
200
116
97.4
0.50
1 2
Mr x 10⁻³
200
116
97.4
67

chromatography. This means pp180 makes up only 0.02-0.03% of total wheat germ agglutinin-purified glycoproteins.

To confirm that pp180 can be purified from wheat germ agglutinin purified-glycoproteins by cation exchange chromatography and antiphosphotyrosine affinity chromatography, an alternative approach to isolate pp180 was studied. ^{32}P-labelled rat liver glycoproteins were first immunoprecipitated with antiphosphotyrosine antibody. The immunoprecipitated tyrosine phosphorylated proteins were suspended with phenyl phosphate. The phenyl phosphate suspended tyrosine phosphorylated proteins were applied to CM-Sepharose-CL-6B cation exchange chromatography. Radioactivity was found in presalt and NaCl-eluted fractions (fig.9). SDS-PAGE of the presalt and NaCl-eluted fractions indicated that there were two proteins in the presalt fraction and one in the 0.1M-0.15M NaCl-eluted peak (fig.9). The molecular weights of the tyrosine phosphorylated proteins in the presalt fraction were 95,000 and 180,000. The molecular weight of the tyrosine phosphorylated protein in NaCl-eluted fraction was 180,000 (fig.9). As previously discussed, it is believed that in the presalt fraction, 95,000 tyrosine phosphorylated protein is the insulin receptor β-subunit and the 180,000 tyrosine phosphorylated protein is the EGF receptor, and the tyrosine phosphorylated 180,000 molecular weight protein in the NaCl-eluted fraction is pp180. These results and the above results suggest that there is only one tyrosine phosphorylated protein in the NaCl-eluted fractions, pp180.

Fig.9 ^{32}P-labelled, antiphosphotyrosine immunoprecipitated, rat liver glycoproteins were applied to CM-Sepharose-CL-6B cation exchange chromatography previously equilibrated at the pH 6.8. Panel A shows the CPM peak of presalt proteins (left side). The presalt protein in presalt peak (fraction 2-8) were analyzed by SDS-PAGE and detected by autoradiography (right side). Panel B shows the CPM peak of the 0.1M NaCl-eluted protein (left side). The 0.1M NaCl-eluted protein was concentrated and analyzed by SDS-PAGE and detected by autoradiography (right side).


A
100
90
80
70
60
50
40
30
20
10
0
cpm x10^{-3} (cerenkov)
0 10 20 30 40 50
Fraction
180kDa
95kDa


B
.15
.10
.05
0
cpm x10^{-3} (cerenkov)
0 5 10 15 20 25
Fraction
◂pp180

3.3.2 Preparative Purification

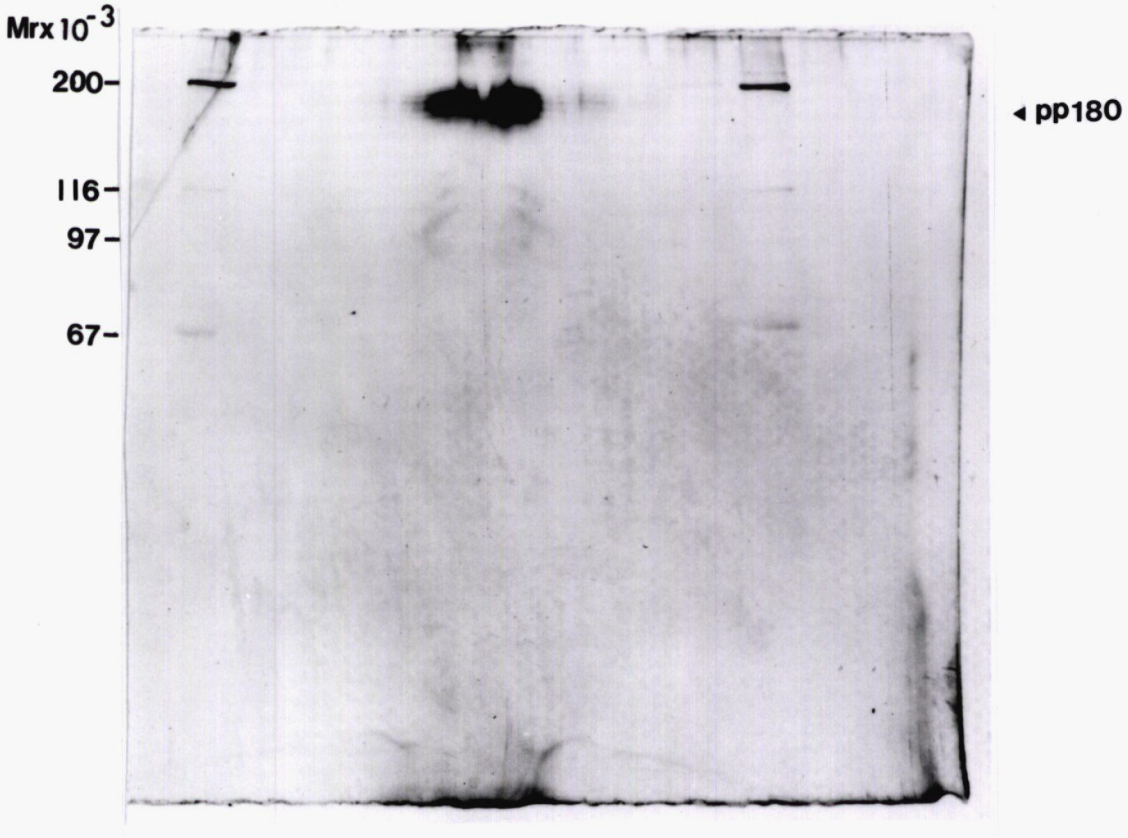
Based on the above procedure for analytical scale preparation, a preparative purification of pp180 was carried out using preparative phosphorylation, antiphosphotyrosine affinity chromatography, ion exchange chromatography and wheat germ agglutinin affinity chromatography. About 1mg rat liver glycoproteins were phosphorylated in the presence of 5mM Mn^{2+} and 10^{-8}M insulin at 20°C, for 10 minutes. Following the phosphorylation reaction, the phosphorylated proteins were kept in a mixture that contained sufficient EDTA and Na_3VO_4 to inhibit both tyrosine kinase and PTPase activity (Tsuda et al., 1991). The phosphorylated glycoproteins were applied to cation exchange chromatography previously equilibrated to pH 6.8 with buffer B. pp180 was eluted with 0.1M to 0.15M NaCl. To separate pp180 from coeluting lower molecular weight polypeptides, which are not tyrosine phosphorylated, the NaCl eluate was dialyzed to remove NaCl and then applied to antiphosphotyrosine affinity chromatography. The column was eluted with 15ml HEPES buffer (pH 7.4), and tyrosine phosphorylated protein was eluted from the column with phenyl phosphate.

To confirm the glycoprotein nature of pp180, pp180 in phenyl phosphate eluate was re-applied to wheat germ-agglutinin agarose chromatography. Protein after the latter purification step was submitted to SDS-PAGE, and the gel was stained with silver oxide. A single protein was detected and its molecular weight was 180,000 (fig.10).

3.3.3 Isolation of pp180 for Sequencing

As shown above, tyrosine phosphorylated pp180 was eluted from the cation

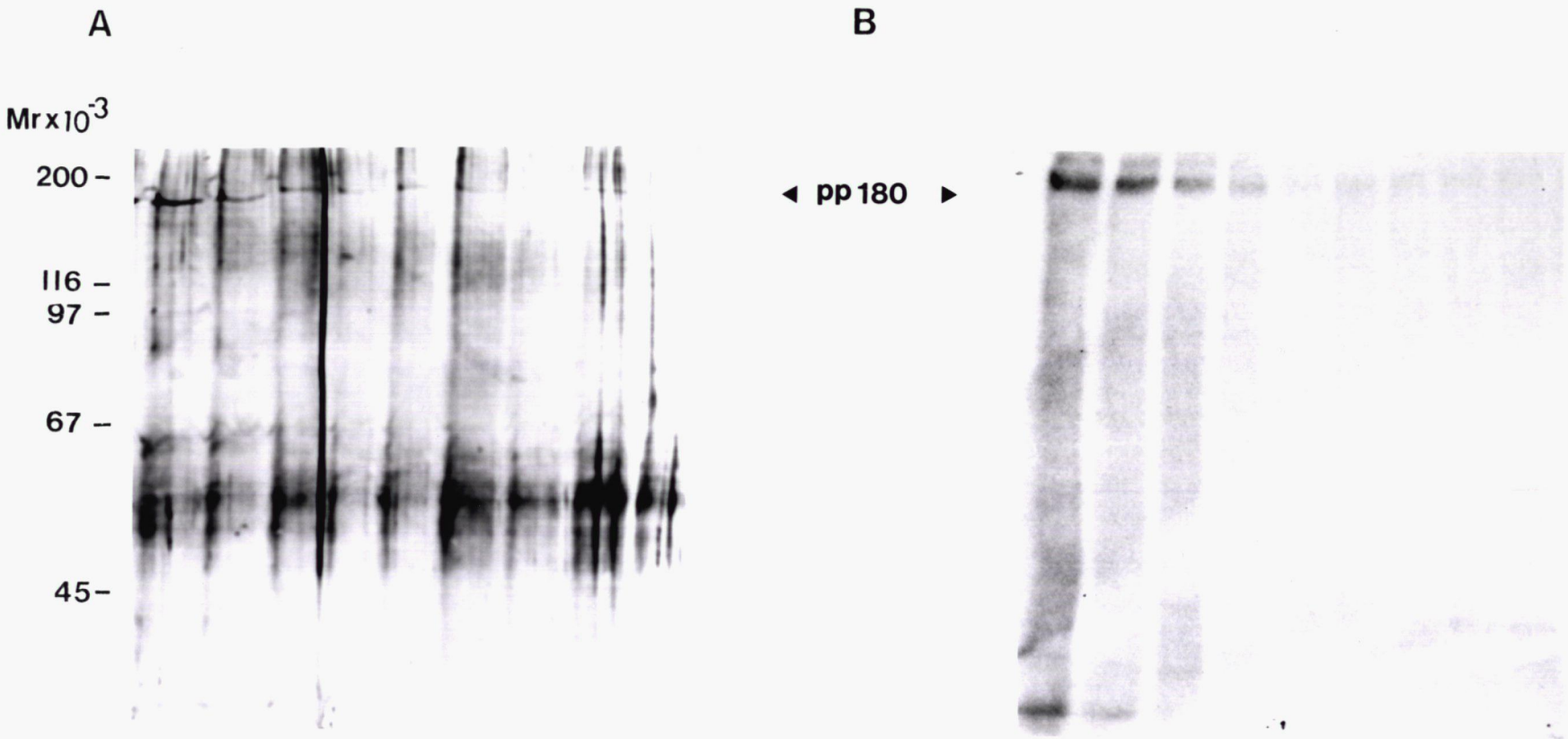
Fig.10 Tyrosine phosphorylated pp180 was purified from rat liver glycoproteins by antiphosphotyrosine affinity chromatography, ion exchange chromatography, and WGA-affinity chromatography. The purified pp180 was subjected to SDS-PAGE and the gel was silver stained.


Mrx10-3
200-
116-
97-
67-
pp180

exchange chromatography in the 0.1M-0.15M NaCl fractions. When ^{32}P-labelled glycoproteins were co-applied with a bulk amount of rat liver glycoproteins to the ion exchange agarose, both the ^{32}P-labelled pp180 and unlabelled pp180 were eluted at 0.1M NaCl eluate. SDS-PAGE of 0.1M NaCl eluate showed that the silver stained pp180 and ^{32}P-labelled pp180 comigrated (fig.11). This result suggested that purification of pp180 for the purpose of sequencing, the procedure of in vitro phosphorylation of wheat germ agglutinin-purified glycoproteins as well as the procedure of antiphosphotyrosine chromatography can be omitted.

Since pp180 is a minor protein in total rat liver glycoproteins, then to isolate sufficient pp180 for sequencing, the Carboxymethyl-Sepharose-CL-6B chromatography procedure was scaled. About 70mg of wheat germ agglutinin-purified rat liver glycoproteins were purified from approximately 50-60 rats. Aliquots of 400-500 ug glycoprotein were mixed and rotated with 3-4ml Carboxymethyl-Sepharose-CL-6B cation exchange agarose previously equilibrated to pH 6.8. Unbound proteins were removed by aspiration of centrifuged supernatant, resuspension in pH 6.8 buffer, and repeating the two steps three times. 0.15 M NaCl in buffer B was used to elute the proteins bound to the ion exchange agarose. Proteins eluted with 0.15M NaCl were concentrated by microconcentrator and lyophilization. Following concentration, the isolated pp180 was purified further by SDS-PAGE. The pp180 isolated in the SDS-gel was transferred onto Immobilon-CD membrane and detected by "quick stain". The pp180 band appeared white against purple background (fig.12). By comparing the intensity of the stained protein with the intensity of the molecular weight protein standards, it is estimated that

Fig.11 ^{32}P-labelled glycoproteins were coapplied with a bulk amount of rat liver glycoproteins to CM-Sepharose-CL-6B cation exchange chromatography at pH 6.8. 0.1M NaCl-eluted proteins (fractions 11-18) were analyzed by SDS-PAGE and the gel was stained with silver oxide (Panel A). Protein molecular weight standards were applied to SDS-PAGE and their migration are noted to the left of the gel. ^{32}P-labelled pp180 in the dried gel was detected autoradiographically (panel B).


A
B
Mr x 10-3
200 –
116 –
97 –
67 –
45 –
◄ pp 180 ►

4-5ug of pp180 was transferred to Immobilon-CD (fig.12).

3.4 Functional Study of pp180

pp180 was purified by ion exchange chromatography. The presence of low molecular weight polypeptides in pp180-containing fractions was indicated (fig.8). Since Initially, the low molecular weight polypeptides were at least believed to be degradation products of pp180, the 0.1M-0.15M NaCl fractions from cation exchange were considered suitable to study pp180 function.

3.4.1 Determination of Protein Tyrosine Phosphatase Activity in Cation Exchange Chromatography Fractions

Using ^{32}P-labelled total glycoproteins as substrate, the PTPase activity of pp180 from ion exchange chromatography was tested. ^{32}P-labelled glycoproteins were incubated 30 minutes with several fractions from ion-exchange chromatography, antiphosphotyrosine immunoprecipitated, and then separated in SDS-PAGE. The 0.05M NaCl-eluted fractions detected by silver staining contained no protein and were used as controls. The autoradiogram indicated that there were less tyrosine-phosphorylated proteins when presalt and 0.1M NaCl fractions were assayed than when the 0.05M NaCl fractions were assayed (fig.13). Of the tested 0.1M NaCl-eluted fractions (14-22), phosphatase activity was present between fractions 14 to 18. Fraction 16 demonstrated the highest phosphatase activity.

Fig.12 pp180 was isolated from rat liver glycoproteins by CM-Sepharose-CL-6B cation exchange chromatography. 0.1M NaCl-eluate which contained pp180 was concentrated and applied to SDS-PAGE. The pp180 band in SDS-gel was transferred to Immobilon-CD membrane and detected by "quick stain". pp180 appeared white against purple background.

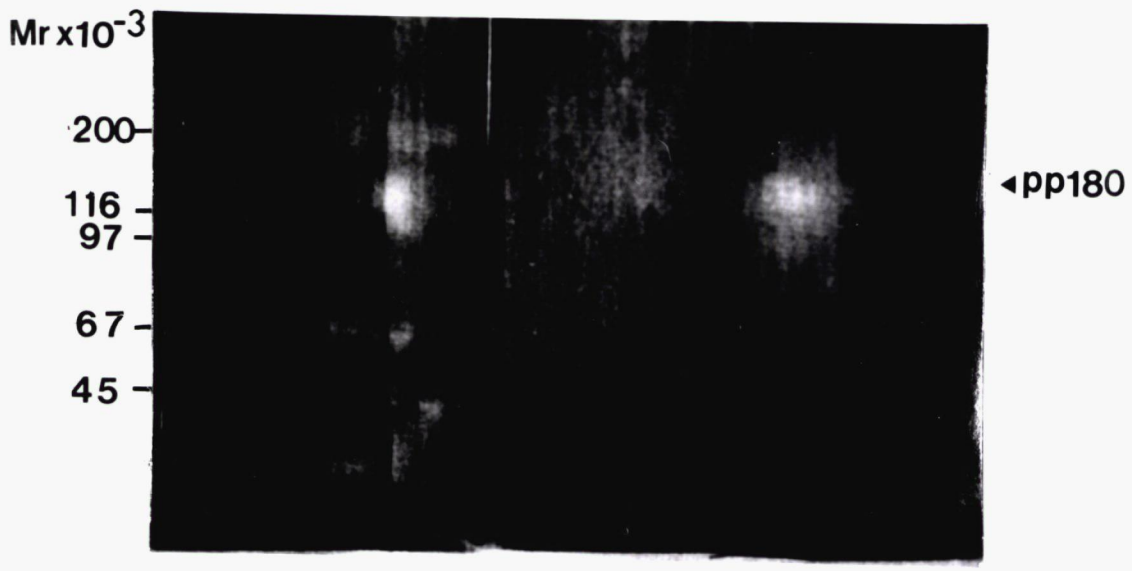
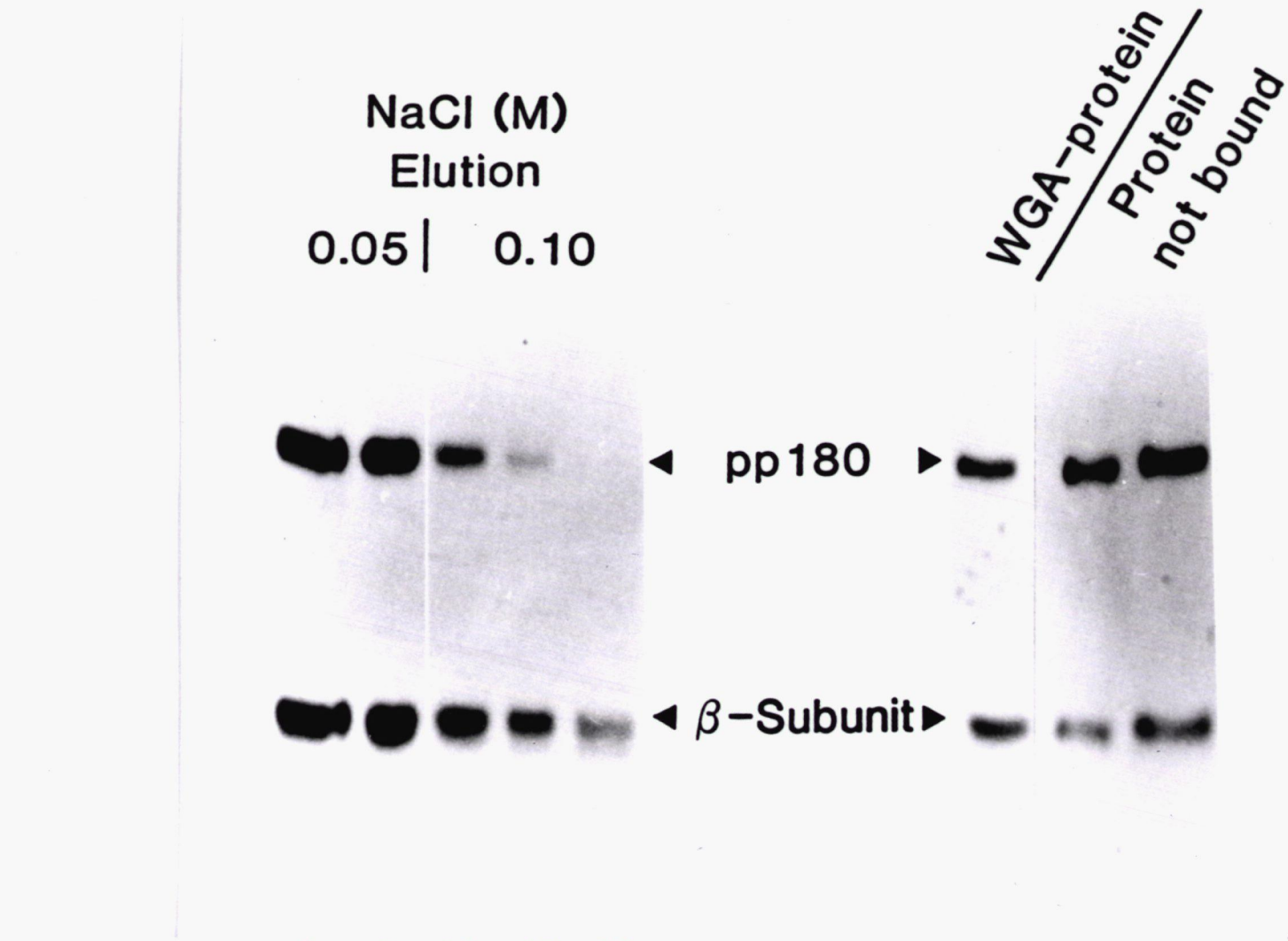

Mr x10-3
200
116
97
67
45
◂pp180

Fig.13 PTPase activities of ion exchange chromatography fractions, in proteins not bound to WGA column, and WGA-purified glycoproteins. Test fractions were added to ^{32}P-labelled rat liver glycoproteins. After incubation at 4°C for 30 minutes, tyrosine phosphorylated proteins were immunoprecipitated with antiphosphotyrosine. The tyrosine phosphorylated proteins were analyzed by SDS-PAGE and detected autoradiographically. Lane 1 and 2 were 0.05M NaCl eluate (fraction 3 and 4). Lane 3-5 were 0.1M NaCl-eluted fractions (fractions 14-16). Lane 6 was WGA-purified glycoproteins. Lane 7 and 8 were the proteins not bound to the WGA column.


NaCl (M)
Elution
0.05
0.10
WGA-protein
Protein
not bound
pp180
β-Subunit
Frac. No
3
4
14
15
16

3.4.2 Detailed Study of Protein Tyrosine Phosphatase Activity in Ion Exchange Chromatography 0.1M NaCl Fractions

3.4.2.1 ^{32}P-labelled Glycoproteins as Substrate

Wheat germ agglutinin-purified glycoproteins were phosphorylated in the presence of 5mM Mn^{2+}, 10^{-8}M insulin and ^{32}P-ATP. The phosphorylation reaction was terminated either by addition of EDTA or by mixing the reaction mixture with Sephadex G-25 (Goren et al., 1993). EDTA chelates Mn^{2+} so that it terminates phosphorylation reactions. Sephadex G-25, which is able to retain low molecular weight chemicals, such as ATP, will also stop insulin receptor kinase activity. Using ^{32}P-labelled proteins from either of the above terminated reactions as substrate, ion exchange chromatography NaCl-peak fractions were tested for PTPase activity. After the reaction mixture was incubated at 4°C for 30 minutes, antiphosphotyrosine antibody was added to the dephosphorylation reaction mixture. SDS-PAGE and autoradiography analysis of the immunoprecipitated tyrosine phosphorylated proteins left in dephosphorylation reaction mixtures demonstrated less proteins in test fraction than in control assays. For controls, buffer A was added to substrate instead of test fraction (fig.14). Phosphoamino acid analysis of dephosphorylated 180 kDa protein band showed a decrease of phosphotyrosine when compared to that of the control reaction mixture (fig.15).

3.4.2.2 p-nitrophenyl Phosphate as Substrate

Every second NaCl-eluted fraction (there were 100 fractions in the 0.05M-0.5M NaCl gradient) was assayed for PTPase activity with the non-specific substrate p-

Fig.14 PTPase activity of ion exchange chromatography 0.1M NaCl-eluted fractions (10-20). The test fraction was added to ^{32}P-labelled wheat germ agglutinin-purified glycoproteins. After 30 minutes incubation at 4°C, the reaction mixture was immunoprecipitated with antiphosphotyrosine. Control (C) was made by incubation the substrate with buffer.

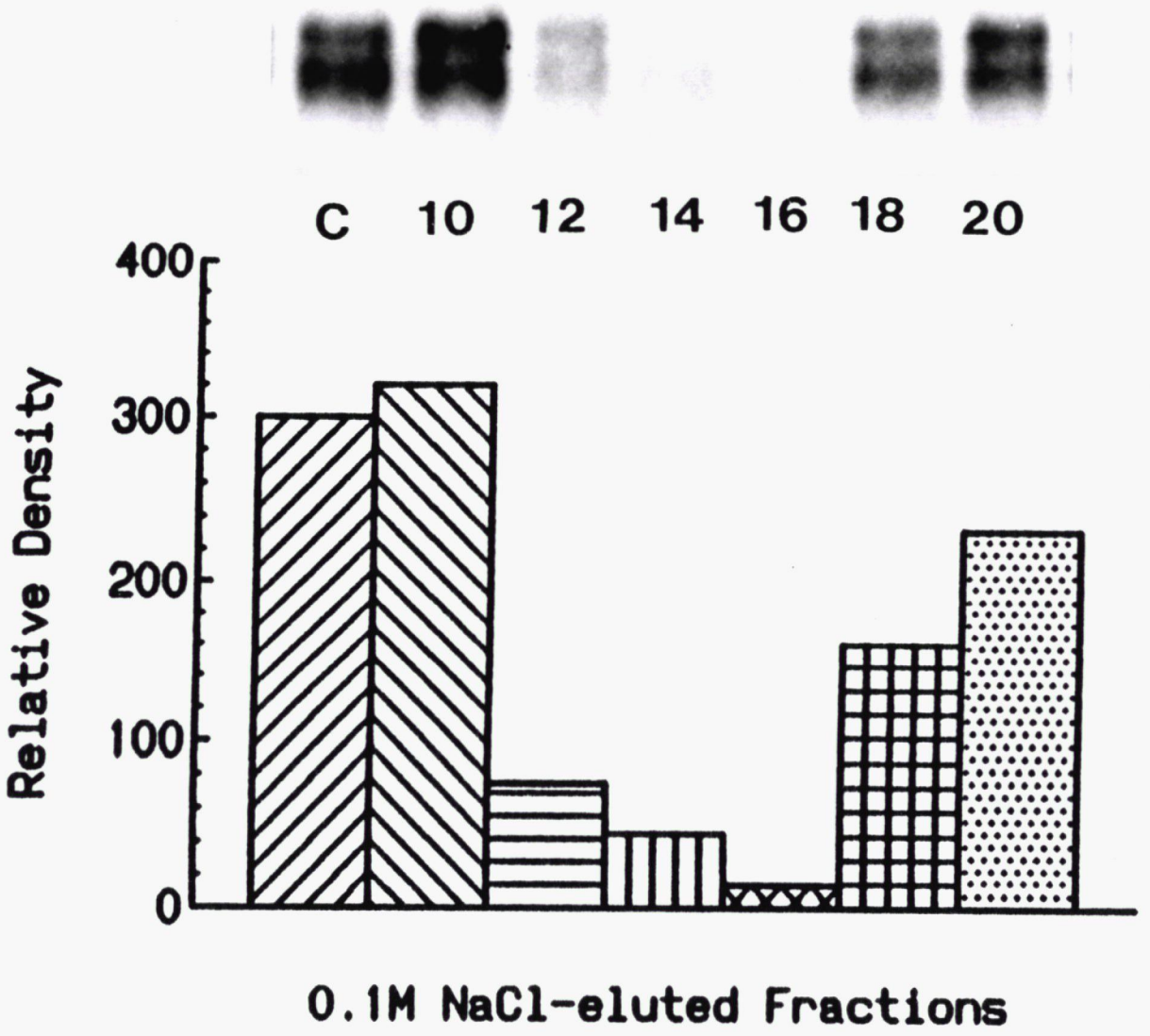

C
10
12
14
16
18
20
400
300
200
100
0
Relative Density
0.1M NaCl-eluted Fractions

Fig.15 Phosphoamino acid analysis. WGA-purified glycoproteins were applied to CM-Sepharose ion exchange chromatography. 0.1M NaCl eluate was analyzed for PTPase activity by using ^{32}P-labelled WGA-purified glycoproteins as substrate. The dephosphorylated proteins were analyzed by SDS-PAGE. The dephosphorylated proteins were transferred to nitrocellulose paper and detected autoradiographically. The dephosphorylated protein band was cut out and hydrolysed by HCl. The ^{32}P-labelled amino acids were analyzed by thin layer chromatography. Lane 1 was from control. Lane 2 was from dephosphorylated protein band. The relative position of phosphotyrosine (pY), phosphothreonine (pT), and phosphoserine (pS) was determined by co-application of phosphoamino acid standards. O presents original where phosphoamino acids were applied.


pY
pT
pS
O
1
2

nitrophenyl phosphate. Not one of these fractions demonstrated hydrolysis above control buffer. Similar results were obtained when 0.1mM dithiothreitol was added. In contrast, both wheat germ agglutinin-purified glycoproteins and proteins not retained by Carboxymethyl-Sepharose hydrolysed p-nitrophenyl phosphate (fig.16). This result suggested 1) that no PTPase activity remained in the cation exchange chromatography agarose 2) or that the PTPase activity was too small to detect by this colorimetric method or 3) that the PTPase will not use p-nitrophenyl phosphate as a substrate.

3.4.2.3 Synthetic phospho-peptide as Substrate

Using a synthetic phosphopeptide YINAS (a gift from Dr.D.Cool, University of Washington, USA) as a substrate, the 0.1 M-NaCl eluate from carboxymethyl-Sepharose-CL-6B cation exchange chromatography was assayed for PTPase activity. Three fractions within the 0.1M NaCl eluate were tested. 20ul test sample was mixed with 1uM synthetic peptide. After 10 minutes, 30°C incubation, the hydrolysed free ^{32}P-phosphate was measured. In comparison to the control assay, which was made by adding 20ul buffer C instead of test fraction, not one of the test samples had any PTPase activity on the phosphopeptide (fig.17). However, when unfractionated wheat germ agglutinin-purified glycoprotein (10ug) was added to the synthetic substrate, dephosphorylation did occur suggesting that unfractionated glycoprotein contains PTPase activity (fig.17).

3.4.3 Investigation of PTPase Activity of Nearly-homogeneous pp180

pp180 in wheat germ agglutinin-purified rat liver glycoproteins was isolated by

Fig.16 PTPase activity of wheat germ agglutinin-purified glycoproteins and proteins in presalt proteins (fractions 2-4) of ion exchange chromatography. Test fractions were incubated with substrate pNPP at 37°C for 30 minutes, and the absorbance of hydrolysed pNPP was measured. Control was made by incubation of buffer with pNPP.

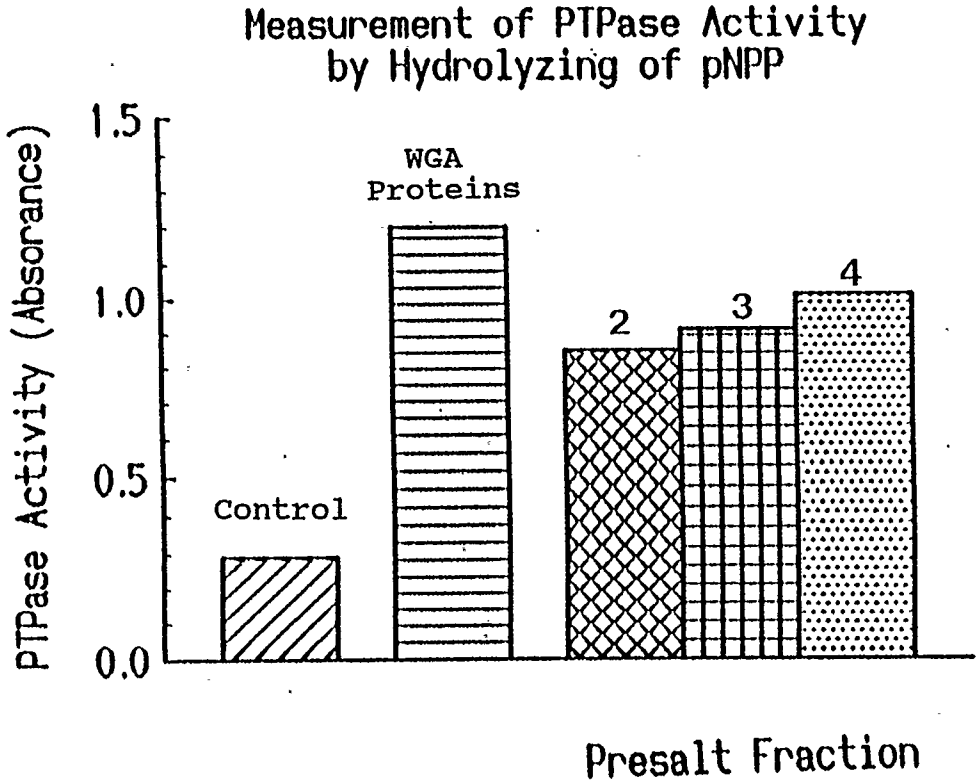
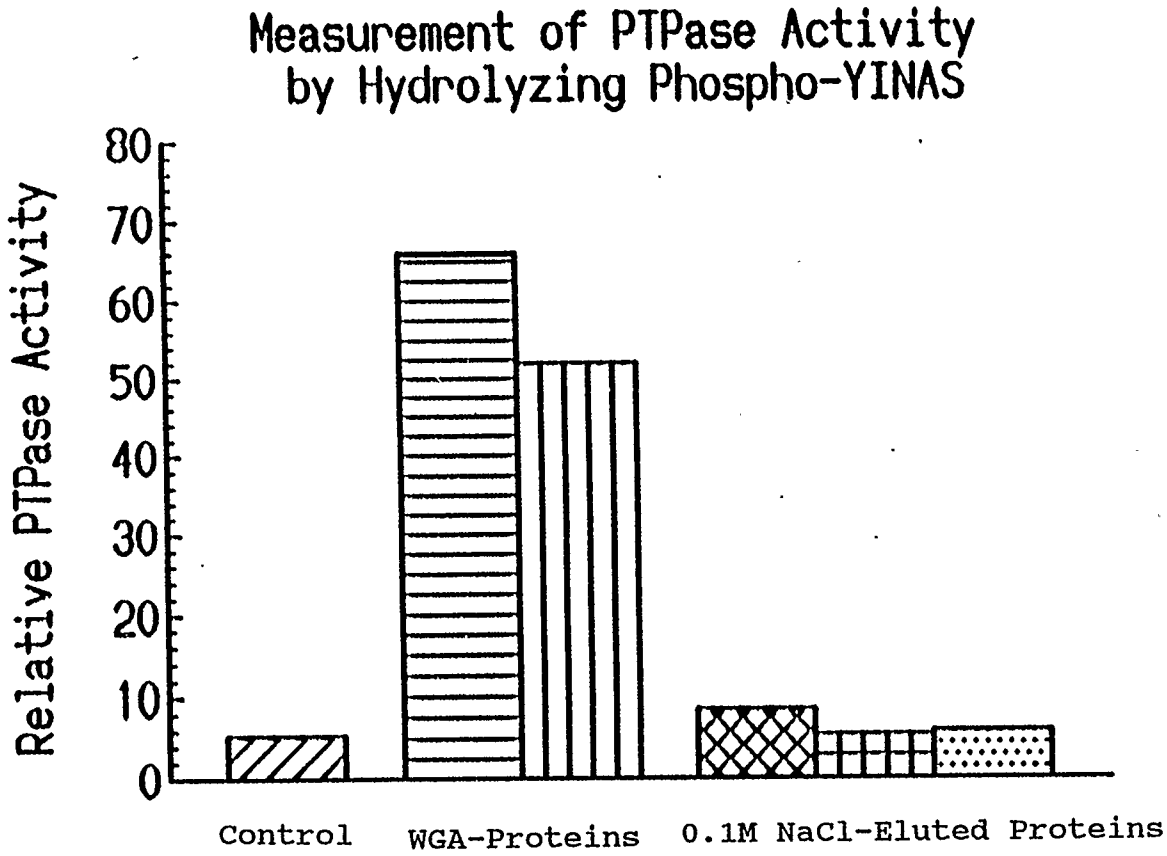

Measurement of PTPase Activity
by Hydrolyzing of pNPP
PTPase Activity (Absorance)
1.5
1.0
0.5
0.0
Control
WGA
Proteins
2
3
4
Presalt Fraction

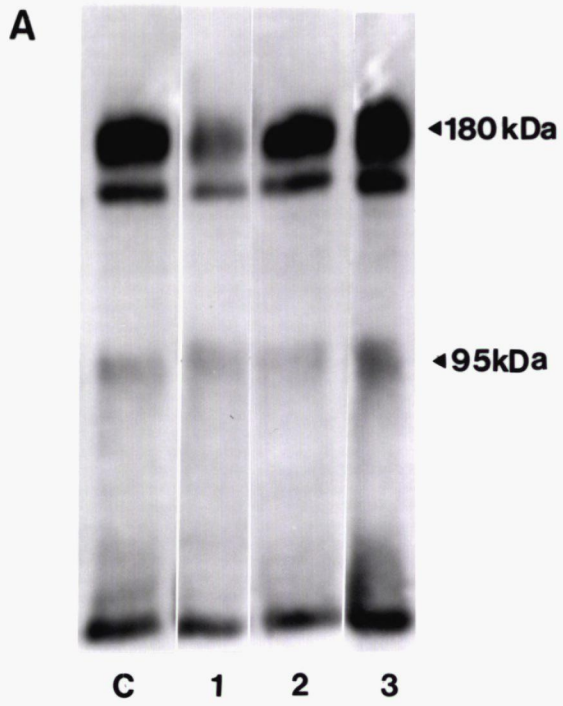
Fig.17 PTPase activity of wheat germ agglutinin-purified glycoproteins and ion exchange chromatography NaCl-eluted fractions by using phospho-YINAS as substrate. After the test fraction was incubated with synthetic phospho-peptide for 10 minutes, the radioactivity of hydrolysed free ^{32}P-phosphate was measured. Control was made by addition of buffer to the substrate solution.

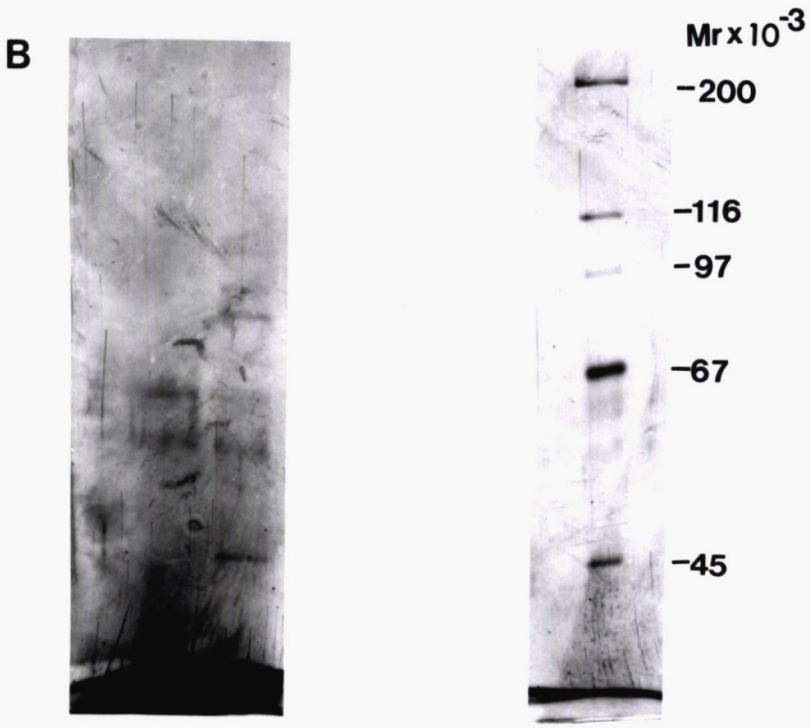

Measurement of PTPase Activity
by Hydrolyzing Phospho-YINAS
Relative PTPase Activity
80
70
60
50
40
30
20
10
0
Control
WGA-Proteins
0.1M NaCl-Eluted Proteins

ion exchange chromatography. Since Carboxymethyl-Sepharose 0.1M-NaCl eluate contained pp180 and lower molecular weight polypeptides, then it is not certain whether pp180 or an other protein had PTPase activity. To clarify whether pp180 has PTPase activity, wheat germ agglutinin-purified rat liver glycoproteins was phosphorylated in the presence of 10^{-8}M insulin and 5mM $MnCl_2$. The phosphorylated glycoproteins were applied to Carboxymethyl-Sepharose ion exchange chromatography. The 0.1M-NaCl eluate from ion exchange chromatography was applied to antiphosphotyrosine chromatography. Phenyl phosphate was used to elute the tyrosine phosphorylated pp180. To eliminate phenyl phosphate from purified tyrosine phosphorylated pp180, the phenyl phosphate eluate was applied to a wheat germ agglutinin column. pp180 was eluted with N-a cetyl glucosamine. Purified pp180 in N-acetyl glucosamine eluate was tested for PTPase activity. Further, the wheat germ agglutinin N-acetyl glucosamine fraction was analyzed by SDS-PAGE. The silver-oxide stained gel showed that only pp180 was present (fig.18C). Thus, by combining ion exchange chromatography with phosphotyrosine- and wheat germ-agglutinin-affinity chromatography, pp180 can be purified to near homogeneity.

Wheat germ agglutinin-purified glycoproteins were phosphorylated in the presence of 5mM Mn^{2+}, 10^{-8}M insulin and ^{32}P-ATP. After phosphorylation was terminated by addition of Sephadex G-25, purified pp180 was added. Following 30 minutes incubation at 4°C, SDS-PAGE was used to analyze dephosphorylated proteins. The autoradiogram of the SDS-PAGE showed that the density of tyrosine phosphorylated proteins where pure pp180 was added was the same as in the control assay where no proteins were

Fig.18 PTPase activity in purified pp180 (tyrosine phosphorylated form), in protein not bound to antiphosphotyrosine column, and in protein not bound to WGA column. Tyrosine phosphorylated rat liver glycoproteins were separated by ion exchange chromatography. The 0.1M NaCl eluate was applied to antiphosphotyrosine chromatography. The unbound protein was collected. The pp180, that bound to the antiphosphotyrosine column, was eluted with phenyl phosphate. The phenyl phosphate eluate was applied to WGA column. The flow through of WGA column was collected. The pp180, that bound to the WGA column, was eluted with N-acetyl glucosamine. Test fractions were added to ^{32}P-labelled rat liver glycoproteins. After incubation at 4°C for 30 minutes, the reaction mixture was analyzed by SDS-PAGE. Panel A shows the PTPase activity of protein not bound to the antiphosphotyrosine column (lane 1), protein not bound to the WGA column (lane 2), and purified pp180 (lane 3). Control was made by addition of buffer to the substrate. Panel B shows the silver stained SDS-PAGE-gel of proteins that did not bind to the antiphosphotyrosine column. Panel C shows the SDS-PAGE of purified pp180 detected with silver stain. Protein molecular weight standards applied to SDS-PAGE was noted in bottom of the middle figure.


A
◂180 kDa
◂95kDa
C
1
2
3

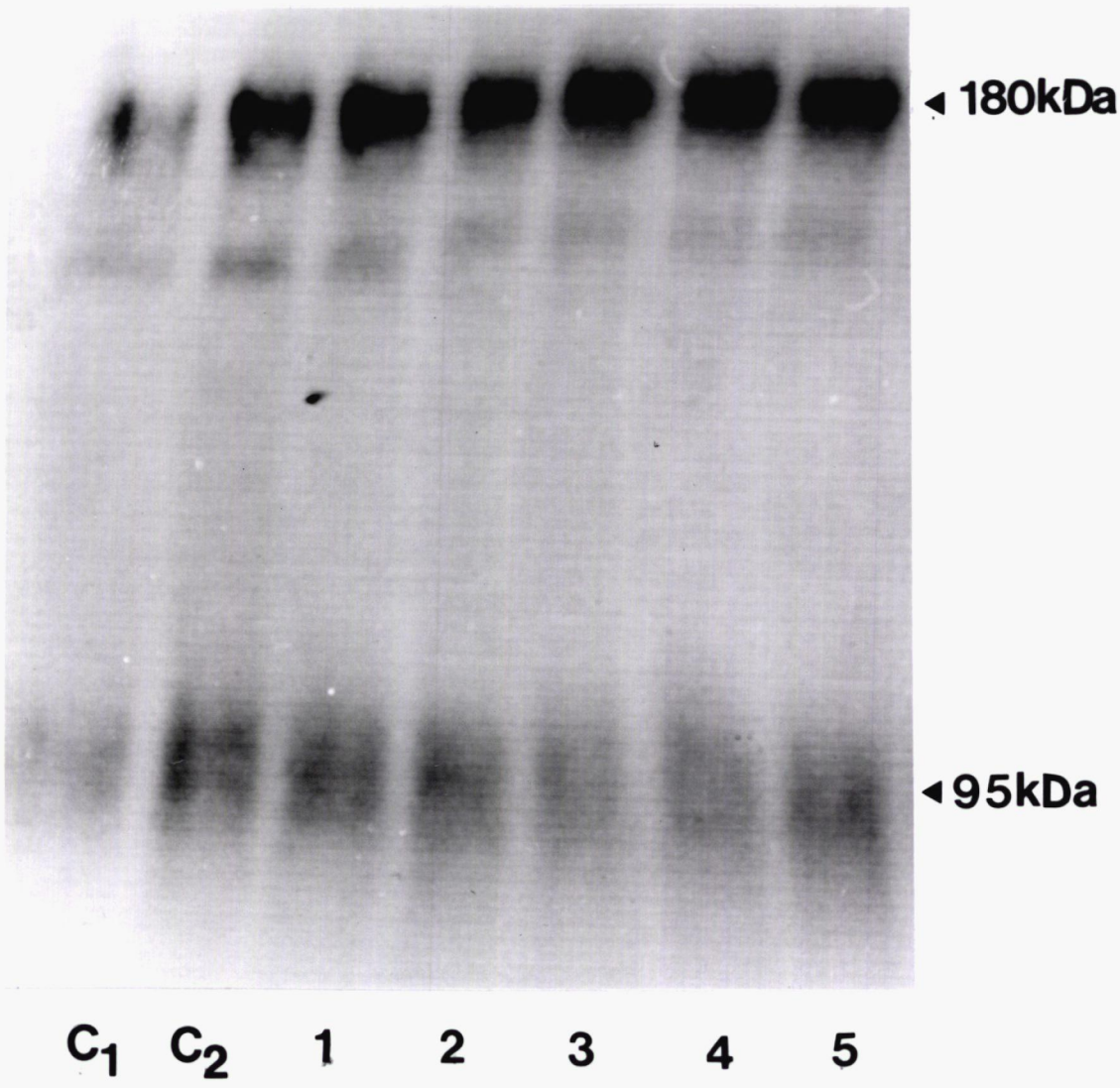

B
Mr x 10-3
-200
-116
-97
-67
-45
C
◂pp 180

added (fig.18A). In addition to the N-acetyl glucosamine fraction, protein not retained by antiphosphotyrosine affinity column was also tested for PTPase activity. In contrast to the protein that bound to antiphosphotyrosine, the unbound eluate had PTPase activity (fig.18A). SDS-PAGE of the unbound eluate indicated the presence of only low molecular weight polypeptides (fig.18C). Taken together, the PTPase activity isolated in the 0.1M NaCl from ion exchange chromatography comes from the lower molecular weight polypeptides.

The study described above was repeated except each N-acetyl glucosamine eluted fraction was tested for PTPase activity. The question addressed was whether the enzyme was missed in the pooled N-acetyl glucosamine eluate. Using ^{32}P-labelled glycoproteins as substrate. pp180 in every wheat germ agglutinin agarose chromatography fraction was added. After the reaction mixture was incubated at 4°C for 10 minutes, the phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. The density of tyrosine phosphorylated protein bands in the test samples was the same as the controls, where buffer A was added (fig.19). In contrast, addition of the unbound proteins from autophosphotyrosine affinity chromatography to ^{32}P-labelled glycoproteins, lowered the radioactive content of the phosphorylated proteins (fig.19).

In summary, when pp180 was initially purified with CM-Sepharose chromatography low salt fraction, there was PTPase activity. However, after further purification, it was found that the PTPase activity was not associated with pp180. It is concluded that the PTPase activity is contained within one of the lower molecular weight polypeptides which copurify with pp180.

Fig.19 PTPase activity of purified pp180. pp180 was purified from tyrosine phosphorylated rat liver glycoproteins by ion exchange chromatography, antiphosphotyrosine chromatography, and wheat germ-agglutinin chromatography. The pp180 in N-acetyl glucosamine-eluted fractions (fractions 1-5) from WGA column was tested for PTPase activity. After the purified pp180 was added to ^{32}P-labelled proteins at 4°C for 30 minutes, the reaction mixture was analyzed by SDS-PAGE and autoradiography. Control (C_1) was the incubation of protein not bound to antiphosphotyrosine with the substrate. Control (C_2) was the incubation of the substrate with buffer.


180kDa
95kDa
C1
C2
1
2
3
4
5

CHAPTER 4 DISCUSSION

4.1 pp180 Is a Insulin Receptor Substrate, Not Epidermal Growth Factor Receptor

In vitro insulin stimulated the phosphorylation of two wheat germ agglutinin-purified rat liver glycoproteins (fig.2). In SDS-PAGE the antiphosphotyrosine immunoprecipitated proteins migrated as 95,000 and 180,000 molecular weight (fig.2). Phosphorylation in the absence of insulin, much less of the two phosphoproteins were obtained (fig.2). This result indicated that most of the phosphorylation was stimulated by insulin through the insulin receptor.

The tyrosine phosphorylated 180,000 molecular weight protein response to insulin stimulation contained insulin receptor substrate (Goren et al., 1990), since preimmunoprecipitation of wheat germ agglutinin-purified glycoproteins with anti-insulin receptor monoclonal antibody MA20 produced a protein mixture where in vitro phosphorylation yielded no tyrosine-phosphorylated proteins (Goren et al., 1990). These findings indicated that not only was pp180 phosphorylation insulin receptor dependent, but that the insulin receptor tyrosine kinase itself catalyzed the reaction. Additional experiments showed that pp180 is phosphorylated in response to insulin in vivo (Goren et al., 1993). Rats were intravenously injected with either insulin or saline solution. When liver plasma membrane glycoproteins were isolated and immunoblotted with antiphosphotyrosine, pp180 was detected in liver membrane from insulin injected rats but not from saline injected rats. This finding indicated that there is 180,000 molecular

weight glycoprotein being tyrosine phosphorylated in rat liver after insulin injection and stimulation. However, whether the tyrosine phosphorylated 180,000 molecular weight glycoprotein is a protein mixture EGF receptor and pp180, the insulin receptor substrate, or they are the same protein is a critical point that needs to be clarified.

Two dimensional gel electrophoresis were performed to show that the 180,000 molecular weight protein contains a novel tyrosine phosphoprotein in addition to the EGF receptor. When the 180,000 molecular weight tyrosine phosphorylated glycoproteins were separated with an isoelectric focusing and SDS-PAGE, the result indicated that there were two proteins which have 180,000 molecular weight and were tyrosine phosphorylated (fig.5). One protein which has pI around 6.5 is proposed to be the EGF receptor and the other which did not enter the isoelectric focusing gel is believed to be pp180. To verify that the protein which did not enter isoelectric focusing gel is pp180, nonequilibrium two dimensional gel electrophoresis was carried out. The second dimension SDS-PAGE gel showed that the protein which did not enter the isoelectric focusing gel is pp180. This protein has pI approximately 7.2 (fig.6).

This result is consistent with HPLC experiments. Pure phosphorylated pp180 and pure phosphorylated EGF receptor from A431 cell membrane were digested with trypsin, and the digests were analyzed by reverse-phase high performance liquid chromatography. The two chromatograms were different. pp180 tryptic phosphopeptides were different in number and in hydrophobicity from those of the EGF receptor (Goren et al., 1993).

Another criteria by which pp180 is different from EGF receptor is functional. EGF receptor is a tyrosine kinase, but when pp180 was tested for kinase activity, it was

not present. To investigate whether pp180 has protein tyrosine kinase activity, the NaCl-eluted protein from ion exchange chromatography was tested for autophosphorylation in the presence of EGF, Mn^{2+} and ^{32}P-ATP. SDS-PAGE and autoradiography to detect the ^{32}P-labelled 180,000 molecular weight protein were carried out. The autoradiogram showed no radiolabelled protein, that is, no protein was autophosphorylated (data not shown). This result was consistent with lack of kinase activity in pp180 isolated by gel permeation chromatography (Goren et al., 1993) as well as experiments where ATP-binding proteins were depleted (Goren et al.,1993). Accordingly, pp180 can not be a protein tyrosine kinase nor the EGF receptor.

Taken together, pp180 is an insulin receptor substrate which differs from the EGF receptor by physical-chemical properties (pI), structure (tryptic phosphopeptides), and function (tyrosine kinase activity).

4.2 Rationale for Purification of pp180

pp180 is a glycoprotein identified as a substrate for insulin receptor tyrosine kinase. Whether the insulin signal transduction pathway passes through pp180, it is necessary to determine its function. As a step prior to studying pp180 function, it is necessary to obtain this substrate in a more pure form. Since pp180 has the same molecular weight as EGF receptor and both of them are glycoproteins and tyrosine phosphorylated, separation of pp180 from EGF receptor is the main task during purification. Based on the isoelectric point of pp180, cation exchange chromatography was chosen as a way of purifying pp180.

Carboxymethyl-Sepharose-CL-6B is agarose to which CM-groups are attached (Porath et al., 1971). Rat liver glycoproteins were applied to the agarose beads which was previously equilibrated to pH 6.8. At this equilibrating condition, most plasma membrane proteins, which are anionic, do not bind to the gel. More basic proteins, however, would bind. To elute these proteins, a NaCl step gradient between 0.05M and 0.5M was used. pp180 was eluted with 0.1M NaCl (fig.7).

In order to ascertain whether the 180,000 molecular weight protein in the 0.1M eluate is insulin receptor substrate, rat liver glycoproteins were ^{32}P-labelled and applied to the ion exchange chromatography. When 0.1M NaCl eluate was immunoprecipitated with antiphosphotyrosine, SDS-PAGE showed that only one protein was obtained. This protein has a molecular weight of 180,000 (fig.7). This result was confirmed by an experiment in which ^{32}P-labelled, antiphosphotyrosine immunoprecipitated rat liver glycoproteins was applied to ion exchange chromatography. The SDS-PAGE of the 0.1M NaCl eluate demonstrated the presence of tyrosine phosphorylated pp180 (Fig.9). On the other hand, silver staining of the 0.1M eluate of phosphorylated but non-immunoprecipitated membrane glycoproteins revealed some lower molecular weight proteins (fig.8). These smaller proteins copurified with pp180.

Since pure pp180 was obtained with the analytical procedure, that is where ^{32}P-labelled rat liver glycoproteins were separated by ion exchange chromatography and the 0.1M NaCl eluate was immunoprecipitated with antiphosphotyrosine, then this purification protocol was upscaled. Following wheat germ agglutinin chromatography, about 1 mg rat liver glycoproteins was isolated. In the presence of 10^{-8}M insulin and

nonradioactive ATP the protein was phosphorylated and applied to cation exchange chromatography. Subsequently, the 0.1M NaCl eluate was applied to antiphosphotyrosine affinity chromatography. This procedure removed pp180 from the non-tyrosine phosphorylated low molecular weight contaminants.

Since insulin receptor substrate IRS-1 has also a molecular weight around 180,000, is not a tyrosine kinase, and is phosphorylated in vivo, then it is necessary to distinguish it from pp180. To do this, pp180 obtained from antiphosphotyrosine chromatography was applied to a wheat germ agglutinin column. As IRS-1 is a cytoplasmic protein which does not bind to wheat germ agglutinin agarose (Rothenberg et al., 1991), while pp180 is a glycoprotein which binds, then whether pure pp180 bind to the lectin column should determine whether they are same protein. After the second lectin-chromatographic procedure, the protein in the N-acetyl glucosamine eluate was analyzed by SDS-PAGE. The silver stained SDS-gel showed only a pp180 band (fig.10). That is, pp180 bound to wheat germ agglutinin and is therefore not IRS-1.

In the preparation of pp180 for sequencing, a bulk purification procedure was used. In this procedure, pp180 was isolated by the cation exchange chromatography of wheat germ agglutinin purified rat liver glycoproteins. Since microsequencing is the purpose of this purification, and since protein separated by electrophoresis can be transferred onto a membrane and cut out, then pp180 was not purified further. In addition, since protein separated by electrophoresis can be transferred onto a membrane, then the transferred protein can be subjected to sequencing protocols. The critical point in this purification procedure is to determine whether when the phosphorylation and

antiphosphotyrosine chromatography steps are omitted, if the pp180 in the ion exchange chromatography low salt eluate (0.1M NaCl) is the same as the ^{32}P-labelled pp180 in response to insulin stimulation. To clarify this, ^{32}P-labelled pp180 (in response to insulin stimulation) and unlabelled pp180 were applied to ion exchange chromatography. SDS-PAGE of the 0.1M NaCl eluate demonstrated that ^{32}P-labelled pp180 comigrated with silver stained pp180 (fig.11).

In the bulk purification procedure, after application of the glycoprotein mixture to the cation agarose beads and washing, pp180 was eluted directly with 0.2M NaCl. Following concentration of the eluate and running on a SDS-gel, the separated proteins were transferred onto an "Immobilon-CD" membrane. It is characterized by a surface that mediates primarily ionic protein/membrane interactions and which allows almost quantitative recovery of adsorbed proteins at relatively mild conditions (Aebersold et al., 1992). By comparing with the amount of applied protein standard, it is estimated that pp180 on Immobilon-CD was approximately 4-5ug (Fig.12).

The 4-5ug pp180 was sent to Dr.Aebersold for microsequencing. Since pp180 is high molecular weight protein, on a molar basis the 4-5ug pp180 was insufficient for sequencing.

4.3 Functional Study of pp180

During the course of these studies, it was observed that SDS-PAGE analyses of ^{32}P-labelled pp180 in 0.1M NaCl eluates suggested the loss of radioactivity from the time the fractions were collected to the time of analysis. That is, it appeared that ^{32}P-labelled

pp180 was dephosphorylated. Accordingly, pp180 was investigated for protein tyrosine phosphatase activity. To test pp180 for this activity, 0.1M NaCl eluate was added to ^{32}P-labelled wheat germ agglutinin-purified proteins in which EDTA was added to terminate kinase activity. After incubation for a period of time,\ tyrosine phosphorylated proteins were immunoprecipitated by antiphosphotyrosine antibody and it showed that the 0.1M salt eluate has the capacity to dephosphorylate both insulin receptor and 180,000 molecular weight protein mixture (fig.13). Phosphoamino acid analysis on the partially dephosphorylated proteins indicated that the decrease in phosphate was specific for phosphotyrosine. This supported the suggestion that tyrosine phosphatase activity was present in the 0.1M NaCl eluate. Experiments were repeated under slightly different experimental conditions in which instead of addition of EDTA to stop kinase activity, Sephadex G-25 was added to take up low molecular weight components of the kinase reaction. The 0.1M NaCl eluate, as before demonstrated phosphotyrosine phosphatase activity (fig.14). Further, phosphoamino acid analysis of partially of dephosphorylated proteins, indicated a decrease in phosphotyrosine and supported the specificity for phosphotyrosine (fig.15). In contrast to the above findings, when p-nitrophenyl phosphate (pNPP) was used as a substrate to test for PTPase activity, the equivalent amount of test sample did not hydrolyse pNPP. Hydrolysis was detected, however, when a 10 fold increased amount of CM-Sepharose bound protein was eluted with 0.1M NaCl. Thus, the apparent lack of pNPP hydrolysis was likely due to pNPP being a poorer substrate compared to endogenous ^{32}P-labelled plasma membrane proteins.

A synthetic phosphopeptide, YINAS, was also used as a substrate to test the

PTPase activity in 0.1M NaCl eluate. These studies indicated that the 0.1M NaCl eluate did not dephosphorylate the synthetic peptide, whereas when wheat germ agglutinin-purified proteins were added to phosphorylated-YINAS, it was dephosphorylated (fig.17). Since 0.1M NaCl eluate contains very small amount of pp180 (approximately 0.1ng), then there may have been insufficient pp180 to hydrolyze this substrate phosphate ester; that is, like pNPP, the peptide is a poor substrate.

As silver staining of SDS-PAGE gels of 0.1 M NaCl eluate showed lower molecular weight proteins, it remained to be determined if the PTPase activity was associated with pp180 or with the copurified proteins. Alternatively, if the latter proteins were degradation products of pp180, then the PTPase activity could be attributed solely to pp180. To investigate this further, a more pure pp180 was tested for PTPase activity. Application of 0.1 M NaCl CM-Sepharose chromatography eluate to antiphosphotyrosine agarose eliminated the lower molecular weight contaminants. The antibody purified proteins were then passed over wheat germ agglutinin-agarose to remove phenyl phosphate. This was necessary as phenyl phosphate is a PTPase inhibitor. ^{32}P-labelled wheat germ agglutinin-purified total glycoprotein was used as substrate in the PTPase assay. The purified pp180 did not show any PTPase activity; that is, the radiolabelled proteins were the same in the test lanes as they were in control lanes where sample buffer was the test sample (fig.18). In contrast, the flow through of the antiphosphotyrosine column did show PTPase activity (fig.18). Silver-stain of SDS-PAGE separated flow through fractions of the antiphosphotyrosine column showed several lower molecular weight polypeptides (fig.18). Silver-stain of the SDS-PAGE of

the protein that bound to the antibody column showed only pp180 (Fig.18). These findings indicate that pp180 is not a PTPase; but one or more of the lower molecular weight polypeptides which copurify with pp180 is. On the other hand, since non-phosphorylated pp180 may be present, but not detected in this affinity column flow through, one can not rule out that the PTPase activity is due to non-phosphorylated pp180. In this case, phosphorylation of pp180 would inhibit its PTPase activity and therefore pure pp180 would not demonstrate PTPase activity.

CHAPTER 5 FUTURE WORK

Insulin has its function by binding to its cell surface receptor. After insulin binds, the receptor is autophosphorylated and receptor tyrosine kinase activity increases. The increased tyrosine kinase activity can phosphorylate its substrate. These substrate could work as messengers to transfer the signal to the next messengers. pp180 has been identified as an insulin receptor substrate and it has been purified to homogeneity. In order to study further the function of pp180 in insulin signalling, obtaining the primary structure of pp180 is important. There are two ways to do this. First, purified pp180 can be accumulated to obtain enough amount to get partial peptide sequence. Based on the partial peptide sequence of pp180, oligonucleotides can be designed for pp180 gene cloning. Secondly, partially purified pp180 can be injected into rabbits to generate pp180-specific antibodies. This antibody can also be used to screen expression libraries to isolate pp180 cDNA. pp180-specific antibodies can also be used to immunoprecipitate pp180 and those proteins that bind to pp180. The coprecipitating proteins could be analyzed to see if they are immunologically related to presently known insulin signalling proteins, such as PI 3-kinase, IRS-1. The primary structure of pp180 and those proteins with which it co-immunoprecipitates could be analyzed for the presence of known motifs such as SH2/SH3 domains, kinase domains, phosphatase domains. GTP-binding domains, and others. Such studies should clarify the role of pp180 in insulin signal transduction. Another approach following pp180 gene cloning is to relate the structure of pp180 to its function in insulin stimulation. Based on the primary structure of pp180,

the synthetic peptides which include tyrosine phosphorylation sites can be designed. These different synthetic peptides can be tyrosine phosphorylated in the presence of tyrosine kinase and employed as insulin receptor substrate to determine the phosphorylation efficiency of different tyrosine site on pp180. In addition, the different tyrosine phosphorylation site on pp180 can be linked to the different cell response to insulin stimulation. To do this, different mutants which have point mutation on different tyrosine phosphorylation site can be made. Following insulin stimulation of cells transfected with mutant pp180, cell responses such as the rate of glucose transport, the activity of glycogen synthase can be measured. In this way the relationship between the phosphorylation site of pp180 and cell response may be connected.

Since preliminary results indicate that pp180 might have PTPase activity under certain conditions, this should be another area of future investigation. For investigation of PTPase activity of non-phosphorylated form of pp180, synthetic phospho- peptides should be used as substrate. These would be based on the peptide sequences surrounding the phosphotyrosines in the insulin receptor. The rationale is that pp180 is assumed to be a PTPase that functions to regulate insulin receptor tyrosine kinase activity. In addition to insulin receptor, other phosphorylated proteins could also be tested as substrates of pp180. One method (Mooney et al.,1992) is where rat liver cells are pre-incubated with ^{32}P-phosphate. The cells are permeabilized with digitonin. Insulin and Mn^{2+} are added to the cell with digitonin. After incubation, pp180 is added to the cell culture. Incubation is continued to allow dephosphorylation. The permeabilized cells are boiled to terminate dephosphorylation, and solubilized membrane and cytoplasmic

proteins are immunoprecipitated with antiphosphotyrosine. The tyrosine phosphorylated membrane and cytoplasmic proteins are analyzed by SDS-PAGE and autoradiography. Control can be made by adding buffer instead of pp180. This assay will determine which proteins are dephosphorylated by pp180 in situ.

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