OCCURRENCE AND STRUCTURE OF AN ACTIVATING ENZYME FOR AN S6 KINASE DETERMINED BY MONOCLONAL ANTIBODY ANALYSIS

DISSERTATION

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The production of monoclonal antibodies directed against the Activating Enzyme for an S6 kinase is described. Antibodies which specifically reacted with an Mr 116,000 and Mr 72,000 protein were obtained. Affinity chromatography of Activating Enzyme on Sepharose coupled to antibody specific for the Mr 116,000 protein yielded an Mr 116,000-72,000 protein complex with 1:1 stoichiometry. This protein complex contained Activating Enzyme activity and the major portion eluted from gel filtration chromatography with Mr 200,000. A smaller portion eluted at the void volume.

The S6 kinase can be activated in the presence of MgATP and the absence of exogenous Activating Enzyme, suggesting that an endogenous activator may be present. The Mr 72,000 protein was isolated as a single protein from S6 kinase preparations by affinity chromatography on Sepharose coupled to antibody specific for the Mr 72,000 protein. The purified protein contained Activating Enzyme activity. Removal of this protein from S6 kinase preparations resulted in a

dramatic reduction of MgATP activation of the S6 kinase. Full S6 kinase activity was restored by addition of the free Mr 72,000 protein. It is postulated that the Mr 72,000 protein is the catalytic subunit of the Mr 116,000-72,000 Activating Enzyme and that the Mr 116,000 protein is a regulatory subunit. Preliminary experiments demonstrated that the Mr 116,000 protein was phosphorylated by the insulin receptor kinase.

Evidence is presented for the association of an Mr 55,000 and Mr 95,000 protein with the S6 kinase. These proteins are phosphorylated in the presence of Activating Enzyme.

A sequence of regulatory events for insulin-stimulated phosphorylation of ribosomal protein S6 in cells is postulated as follows: insulin activates the receptor tyrosine kinase, which phosphorylates the Mr 116,000 subunit of Activating Enzyme. The Activating Enzyme then activates the S6 kinase by phosphorylation, and phosphorylation of the ribosomal protein S6 is promoted.

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LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
cAMP	Adenosine 3':5'-cyclic monophosphate
BSA	Bovine Serum Albumin
DEAE	Diethylaminoethyl
DMEM	Dulbecco's modification of Eagle's medium
DTE	Dithioerythritol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FCS	Fetal Calf Serum
[^{14}C]FSBA	Fluorosulfonyl benzoyladenosine, 5'-p-[adenine-8-^{14}C]-
GTP	Guanosine 5'-triphosphate
cGMP	Guanosine 3':5'-cyclic monophosphate
HBSS	Hank's Balanced Salt Solution
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid
IgG	Immunoglobulin G
i.p.	Intraperitoneal
MEM	Minimum Essential Medium
Mes	2-[N-Morpholino]ethanesulfonic Acid
Mr	Apparent molecular weight
PMSF	Phenylmethylsulfonyl Fluoride
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

TBS	Tris Buffered Saline: 20 mM Tris-Cl, pH 7.6, 0.5 M NaCl
Tris	Tris(Hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

Protein phosphotransferases (protein kinases) catalyze the transfer of the terminal phosphoryl group from ATP to a serine, threonine or tyrosine residue of the substrate proteins (18, 52). A divalent metal ion, usually magnesium or manganese, is required, as the actual nucleotide substrate is apparently the metal-ATP complex. The result of phosphorylation is a modulation of the substrate protein activity. In addition, protein kinases are themselves regulated in response to extracellular signals. Protein phosphorylation has been recognized as a major regulatory mechanism by which extracellular signals are translated to a change in metabolic activity in the cell. This regulatory sequence of events may be investigated on several levels.

Investigation of hormone-receptor interactions has led to the identification of a number of receptor proteins and elucidation of two major mechanisms for the first step of hormone signal transduction. The first is a rapid rise in the cytoplasmic concentration of a small ligand which can allosterically regulate a protein kinase. These small ligands have been termed second messengers and include cyclic AMP, calcium and diacylglycerol. The increase in

cytoplasmic concentration of the second messenger may be the result of synthesis, as in the case of cyclic AMP, or mobilization of intracellular or extracellular stores in the case of calcium. The rapid rise of the second messenger concentration is a direct result of receptor activation upon combination with ligand. The molecular basis by which receptor occupation is coupled with the generation of a second messenger has been described for cyclic AMP. Two distinct GTP-binding proteins, which are closely associated with the receptor in the plasma membrane, exert a stimulatory and inhibitory effect on adenyl cyclase, respectively. Receptor-hormone interactions which modulate cyclic AMP levels in the cell are communicated through this pair of proteins (39).

A second mechanism for the first step of hormone signal transduction has been proposed in response to observations first made with the epidermal growth factor receptor by S. Cohen and coworkers (20). The epidermal growth factor receptor contains an extracellular domain for ligand binding and an intracellular domain which is a protein kinase (20, 21, 30, 102). This protein kinase is activated upon ligand binding to the extracellular domain of the receptor and can phosphorylate exogenous protein substrates. The protein kinase is also unique in its strict specificity for tyrosine residues as the site of protein modification. Previously

described protein kinases, with the exception of some RNA tumor virus gene products (33, 47, 107), modified either serine or threonine residues. In the past five years, the receptors for platelet-derived growth factor (29, 45, 56), insulin (32, 49, 85) and colony-stimulating factor (84) have been isolated and demonstrated to contain a tyrosine-specific protein kinase domain.

Insulin binding stimulates autophosphorylation of its receptor on tyrosine, which results in the activation of the receptor kinase toward exogenous substrates (46, 50, 78, 90). The activation of other tyrosine-specific receptor kinases presumably occurs by a similar mechanism. The activation of a protein kinase in response to an extracellular signal is accomplished in the absence of a cytoplasmic second messenger. Consequently, some, and possibly all, of the effects of these hormones on metabolic and mitotic events may be mediated by a direct phosphorylation cascade.

Investigation of the sequence of biochemical events in hormone signal transduction can be focused on the protein kinase. A number of protein kinases have been purified and the mechanisms by which they may be activated delineated. The protein kinase may be allosterically activated by a second messenger generated in response to the hormone-receptor interaction. Cyclic AMP-dependent protein

kinase is the classic example of this type of mechanism (52). A variation of this mechanism is observed for myosin light chain kinase. The second messenger, calcium, combines with calmodulin prior to allosteric binding to the protein kinase (74). The second messenger for protein kinase C is diacylglycerol, but allosteric activation of the enzyme also requires phospholipid and calcium (51, 67). A protein kinase may also be activated by phosphorylation with another protein kinase. Phosphorylase kinase is phosphorylated by cyclic AMP-dependent protein kinase. The functional result of this phosphorylation is a decrease in the K_a for calcium, which is the allosteric activator for this enzyme (43). The tyrosine-specific receptor kinases are activated in response to ligand binding to the receptor.

A number of protein kinases have been isolated for which regulatory properties have not been demonstrated. This group includes the casein kinases (42, 99), glycogen synthase kinases (4, 83) and histone kinases (54, 86). The preparation of a protein kinase devoid of regulatory properties does not weaken the well-established concept that protein kinases are regulated enzymes. Several reasons may be cited for the difficulty of isolating a complex, regulated enzyme in its native conformation. A regulatory subunit may be separated or denatured during the purification, thus an activated form of the enzyme is

obtained. Alternatively, the regulatory domain of a single subunit protein kinase may be lost by proteolysis. The irreversible nature of protein kinase activation by proteolysis argues against the operation of this mechanism in vivo. However, limited proteolysis is a useful tool to obtain expression of activity from protein kinases isolated in their non-activated conformation. Loss of regulatory properties and concomitant activation by limited proteolysis has been demonstrated for cyclic AMP-dependent protein kinase (36), myosin light chain kinase (105), cyclic GMP-dependent protein kinase (48) and protein kinase C (95, 96). Two protein kinases have been isolated which can be activated by limited proteolysis with trypsin (93, 94), but no other mode of activation has been observed. Presumably, these enzymes are isolated with intact regulatory domains, but the appropriate effectors for activation have not been identified.

The purification of protein kinases has allowed the investigation of substrate specificity. Although a number of substrates may be utilized by a single protein kinase in vitro, the goal of these studies is the identification of physiologically relevent substrates and determination of functional changes of the target protein upon phosphorylation. Enzymes which regulate flux through hormonally sensitive pathways are prime candidates for

substrate studies with a purified protein kinase. Glycogen synthase is the flux-generating enzyme in glycogen formation and can be phosphorylated at multiple sites by different protein kinases (77). The best defined example is phosphorylation by cyclic AMP-dependent protein kinase, which results in decreased glycogen synthase activity (103). The flux-generating enzyme in fatty acid synthesis is acetyl CoA carboxylase which can also be phosphorylated at multiple sites (10, 13). The enzyme can be phosphorylated by cAMP-dependent protein kinase and this results in a decrease in activity (10). Insulin stimulates the phosphorylation of this enzyme in whole cells on a site distinct from that observed with cyclic AMP-dependent protein kinase. A cyclic nucleotide-independent protein kinase has been identified which phosphorylates the enzyme on the site observed in insulin-treated cells. This phosphorylation results in an activation of the acetyl CoA carboxylase (10-13, 26).

The cellular substrates for phosphorylation are not limited to enzymes of the major metabolic pathways. The regulatory light chains of the contractile protein myosin are phosphorylated by myosin light chain kinase. The functional result of this phosphorylation in smooth muscle is an increase in actin-activated hydrolysis of ATP which regulates contraction, and may also promote filament formation (3, 81).

Assignment of cellular substrates and a specific regulatory role to a protein kinase is a difficult task. For example, protein kinase C has been studied extensively. The activation mechanism, allosteric effectors and the second messenger (diacylglycerol) have been defined (68). Activation of the enzyme by tumor-promoting phorbol esters *in vitro* and *in vivo* has been demonstrated (16). The complete amino acid sequence has been predicted from the cDNA sequence (70). Various cellular substrates have been proposed including the regulatory light chains of myosin (41, 65) and the epidermal growth factor receptor (19). In each case a decrease in activity is observed. Yet, a consensus for the role of this protein kinase in cellular regulation and its appropriate cellular substrates *in vivo* has not been achieved.

The full sequence of regulatory events from the extracellular signal to cellular metabolic response has been delineated for few systems. The best established example is glycogen breakdown in response to epinephrine in muscle (89). The epinephrine-receptor interaction promotes adenyl cyclase activation through the stimulatory GTP-binding protein, resulting in a rapid rise in the cytoplasmic cyclic AMP concentration. Cyclic AMP-dependent protein kinase is activated, initiating a cascade of phosphorylation reactions. Cyclic AMP-dependent protein kinase activates

phosphorylase kinase by a phosphorylation, which in turn activates phosphorylase by a phosphorylation. The activated phosphorylase catalyzes the breakdown of glycogen to glucose 1-phosphate. Cyclic AMP-dependent protein kinase also phosphorylates glycogen synthase, decreasing the rate of glycogen synthesis and achieving coordinated regulation of the metabolic response.

Insulin promotes glucose uptake, glycogen synthesis, fatty acid synthesis and protein synthesis in various cell types. The net phosphate content of acetyl CoA carboxylase (committed step in fatty acid synthesis), ATP citrate lyase (regulated enzyme in fatty acid synthesis) and ribosomal protein S6 is observed to increase in insulin-treated cells (11, 12, 61, 6). Initially, the mechanism of insulin signal tranduction was proposed to be analogous to that observed for epinephrine. The epinephrine stimulated level of cyclic AMP is observed to decrease in insulin-treated cells. However, all of the effects of insulin could not be explained by decreased cyclic AMP levels and research was directed toward identification of the insulin second messenger (15, 37, 44, 82). Larner and coworkers (55) have isolated a peptide-like substance from insulin injected rats which they have termed an insulin mediator. This substance apparently directly inhibits cyclic AMP-dependent protein kinase by lowering its affinity for cyclic AMP. It also

activates glycogen synthase by activating the phosphoprotein phosphatase. Recent work by Saltiel et al. (80) has identified a glycolipid which may act as a second messenger in response to insulin. This glycolipid modulates the activity of the cyclic AMP phosphodiesterase and thus may mediate the effect of insulin on cellular cyclic AMP levels.

The identification of a tyrosine-specific protein kinase as an intracellular domain of the insulin receptor (32, 49, 85) has led to a reevaluation of the proposed models for insulin signal transduction. Insulin binding to the receptor stimulates autophosphorylation and activation of the receptor kinase (46, 78). An activated protein kinase is achieved in the absence of a second messenger molecule, leading to the proposal that at least some of the effects of insulin may be mediated by a direct phosphorylation cascade. However, cellular substrates, such as ribosomal protein S6, are phosphorylated on serine in insulin-treated cells (106). Therefore, at least one additional enzyme, a serine-specific protein kinase, must exist in the pathway between a tyrosine-specific receptor kinase and phosphorylation of serine residues on cellular substrates. Considerable research has been focused on the identification and characterization of this putative protein kinase.

Ribosomal protein S6 has been the most extensively

employed substrate to identify this putative protein kinase. S6 is phosphorylated on multiple sites in response to insulin, epidermal growth factor and Rous sarcoma virus treatment of whole cells (40, 60, 61, 88, 97). This phosphorylation apparently promotes increased polysome assembly and an increased rate of protein synthesis (14, 40, 97). The receptors for insulin and epidermal growth factor, and the src gene product of the virus, all contain a tyrosine-specific protein kinase. This suggests that a common pathway may mediate some of the effects of all the hormones that have receptors associated with a tyrosine-specific protein kinase.

Serum and tumor-promoting phorbol esters induce a pattern of S6 phosphorylation in whole cells (61, 88, 97) which is comparable to that observed in insulin-treated cells. The myriad components of serum make identification of the mechanism by which it stimulates cellular events difficult. Tumor-promoting phorbol esters are highly lipid-soluble molecules which bind and activate protein kinase C in the cell (16). The ability of protein kinase C to catalyze the phosphorylation of S6 has therefore been investigated. Protein kinase C was observed to catalyze incorporation of up to two moles of phosphate per mole of ribosomal subunits on S6 (57). However, S6 is phosphorylated on at least four distinct sites in cells

treated with insulin or phorbol ester (25, 56). These observations suggest that the phosphorylation of S6 observed in these cells is catalyzed by a protein kinase distinct from protein kinase C, but protein kinase C activation may be a step in the cascade.

The ability of the cyclic nucleotide-dependent protein kinases to phosphorylate S6 has also been investigated. Cyclic AMP-dependent protein kinase incorporates up to two moles of phosphate per mole of S6 and up to one mole is incorporated by cyclic GMP-dependent protein kinase (25, 31, 98). These phosphorylations occur on sites distinct from those observed in insulin-treated cells.

Several other protein kinases have been identified, which phosphorylate S6 with a pattern comparable to that observed in insulin-treated cells. These protein kinases have been termed S6 kinases. An S6 kinase has been purified to homogeneity from frog oocytes (34, 35). This enzyme was isolated by monitoring the purification with S6 as the protein substrate. The protein kinase can incorporate four to five moles of phosphate per mole of S6. Regulatory properties for the purified enzyme have not been demonstrated. Another protein kinase, initially identified as protease-activated kinase II (101), has been identifiied as an S6 kinase. The purified protein kinase phosphorylates S6 on five tryptic peptides which match the phosphopeptides

obtained from S6 isolated from insulin-treated cells (25, 58, 72, 73). This enzyme can be activated *in vitro* by limited trypsin digestion. A third enzyme, which was used for the studies in this dissertation, has been identified as an S6 kinase (23, 24, 28). This protein kinase was initially termed H4 protein kinase due to its *in vitro* substrate preference (22, 62). The protein kinase incorporates at least three moles of phosphate per mole of S6 (28).

The mechanism by which these S6 kinases are regulated in the cell has not been elucidated. An activated form of an S6 kinase can be identified from cells treated with insulin (17, 92), epidermal growth factor (69), fibroblast growth factor (71), the Rous sarcoma virus, serum and tumor-promoting phorbol esters (8, 92).

Extracts from epidermal growth factor-treated cells displayed a loss of S6 kinase activity in the absence of phosphatase inhibitors (69). The oocyte S6 kinase also required the presence of phosphatase inhibitors during the purification for maintenance of activity (35). These observations suggest that the activated state of the S6 kinase is dependent on maintenance of a phosphorylated site. The activation of S6 kinase in growth factor-treated cells may proceed by the direct phosphorylation of the S6 kinase.

A role for protein kinase C in the activation cascade

of the S6 kinase is suggested by results with tumor-promoting phorbol ester-treated cells. Tryptic peptide maps of S6 from cells treated with insulin or phorbol ester displayed identical patterns of phosphopeptides (100). The same pattern of phosphopeptides was obtained by in vitro phosphorylation of S6 with the protease-activated kinase II. However, insulin-mediated S6 phosphorylation was not impaired in protein kinase C deficient cells, though tumor-promoting phorbol ester stimulation of S6 phosphorylation was lost (7). The stimulation of S6 phosphorylation by fibroblast growth factor was also unimpaired in protein kinase C deficient cells (71). The receptor for fibroblast growth factor may be similar to other growth factor receptors, though its association with a tyrosine-specific protein kinase remains to be established.

Activation of an S6 kinase mediated by tyrosine-specific protein kinases or tumor-promoting phorbol ester apparently proceeds through a protein kinase C-independent and -dependent pathway, respectively. These two pathways may converge at the same S6 kinase. The terminal S6 kinase could be activated by either protein kinase C or by an activator from the tyrosine-specific protein kinase initiated pathway. Direct activation of the S6 kinases by a tyrosine-specific protein kinase has not

been reported. Thus, it is necessary to postulate one or more steps between the tyrosine kinase and activation of the S6 kinase.

The S6 kinase used for studies in this dissertation can be activated by preincubation with MgATP in the absence of a protein substrate, and the rate of that activation is enhanced by the addition of a factor termed Activating Enzyme (23, 24). The rate of MgATP activation of S6 kinase is dependent on the concentration of S6 kinase in the preincubation reaction. The doubling of S6 kinase concentration in the preincubation reaction results in a greater than doubling of the activation rate. This observed amplification suggests a bimolecular activation mechanism, possibly due to presence of endogenous Activating Enzyme.

The Activating Enzyme was initially proposed to be a MgATP-activated protease as the S6 kinase shifted to a lower Mr on gel filtration chromatography after activation (22). However, subsequent studies showed the inability of a number of protease inhibitors to inhibit the activation reaction (23, 24). The Mr shift of the activated S6 kinase may be due to the loss of a regulatory subunit or a conformational change.

The studies in this dissertation are aimed at the determination of the structure of Activating Enzyme, its interaction with the S6 kinase and potential interaction

with the insulin receptor kinase. Monoclonal antibodies were produced and employed as a major tool in these studies. The data obtained in these studies allowed the full sequence of events from insulin binding to its receptor to 40S ribosomal protein S6 phosphorylation to be postulated. A schematic representation of the postulated sequence of events is shown in Figure 1 at the end of Results. The structure and Mr of Activating Enzyme and S6 kinase as determined by SDS-PAGE and gel filtration chromatography analysis are shown in Table I. Proteins which are apparently derived from the subunits of Activating Enzyme by proteolysis are also listed as a guide to the reader.

CHAPTER II

EXPERIMENTAL PROCEDURES

Enzyme Purification

S6 kinase.--Human placentae were obtained from Caesarian deliveries and were transported to the laboratory in ice. The S6 kinase in the placental homogenate was purified by modifications of previously published procedures (22, 62). Blood and membranes were removed in an ice-cold bath of 0.15 M sodium chloride. The tissue pieces were homogenized (1:3 w/v) in ice-cold 10 mM Tris-Cl, pH 7.5, 5 mM EDTA, 30 mM 2-mercaptoethanol, 2 μM leupeptin, 0.2 mM PMSF and 25 mM benzamidine (Buffer A) in a blender. All subsequent steps of the purification were carried out at 4° C. The placental homogenate was centrifuged at 16,300 x g for 30 minutes and the supernatant was collected. The pellets were resuspended in half the original volume of Buffer A, centrifuged at 16,300 x g for 30 minutes and the supernatant was collected. The combined supernatants were centrifuged at 235,000 x g for 60 minutes and the post-ribosomal supernatant was collected. The conductivity of the sample was reduced to less than 2.2 mmhos by the addition of ice-cold distilled water (approximately 1:3

dilution). The sample was then incubated for one hour with approximately 400 ml DEAE cellulose equilibrated with Buffer B (Buffer A modified to contain only 6 mM benzamidine). Unbound proteins were removed by filtering the DEAE cellulose resin on Whatman 1 filter paper, pouring the resin into a 2.5 x 75 cm column and washing with 300 ml Buffer B. Elution of proteins was accomplished with an 1.8 l linear gradient. The initial buffer was Buffer B and the final buffer was Buffer A containing 0.7 M KCl.

Individual fractions were assayed, the active fractions pooled and subjected to ammonium sulfate precipitation at 60% (w/v) saturation. The sample was centrifuged for 15 minutes at 16,300 x g, the pellet resuspended in a minimal volume (5-10 ml/100 g tissue) of 10 mM MES, pH 6.8, 0.5 mM EDTA, 30 mM 2-mercaptoethanol (Buffer C) with 2 μM leupeptin, 0.1 mM PMSF and 25 mM benzamidine and then dialyzed against the same.

The sample was applied to a 40 ml bed (1.5 x 25 cm) of phosphocellulose equilibrated in Buffer C with the protease inhibitors. The column was washed with 50 ml of buffer and elution of proteins accomplished with a 240 ml linear gradient of Buffer C with protease inhibitors containing 0-1.0 M KCl. Active fractions were pooled, dialyzed against Buffer C, and applied to 10 ml CM-Sephadex resin equilibrated in Buffer C. The column was washed with 20 ml

Buffer C and proteins were eluted with Buffer C containing 0.3 M KCl. Eluted protein was pooled either on the basis of protein kinase activity or total protein as determined by Bradford assay (9).

The sample was dialyzed briefly against Buffer C and applied to a column of Sephacryl S200 (2.5 x 90 cm) equilibrated in 10 mM MES, pH 6.8, 1.0 mM EDTA, 1.0 mM DTE and 0.1 M KCl (Buffer D). Proteins were eluted with Buffer D; active fractions were pooled and dialyzed against Buffer C. The sample was applied to a column of 10 ml of DEAE cellulose equilibrated in Buffer C. The column was washed with 20 ml Buffer C and proteins eluted with Buffer C containing 0.12 M KCl. This pooled, concentrated enzyme was stored at -80° C in small aliquots. The specific activity of this enzyme preparation ranged from 2.2 to 7.5 nmol/min-mg after a ten minute MgATP activation reaction with 1 mg/ml histone H4 as the protein substrate.

S6 kinase prepared by this method was used for mouse immunization and as the antigen for all screening steps in the monoclonal antibody production. The S6 kinase used in other experiments was prepared by a modification of the above protocol (23, 24). Briefly, the protease inhibitors leupeptin, PMSF and benzamidine were present in all buffers. Upon completion of batch loading of the placental extract onto DEAE cellulose, the resin was batch washed twice before

the column was poured. Prior to Sephacryl S200 chromatography, the sample was dialyzed against Buffer D (with protease inhibitors) instead of Buffer C. These modifications allowed the preparation of a more homogeneous S6 kinase.

Activating Enzyme.--The Activating Enzyme was obtained from the same purification procedure as the S6 kinase. A peak of Activating Enzyme, which eluted at the void volume on gel filtration chromatography was completely resolved from S6 kinase activity. It was pooled separately from the S6 kinase and concentrated separately on DEAE cellulose. The pooled, concentrated Activating Enzyme was dialyzed against Buffer C containing 20% (v/v) glycerol and stored at -20° C in small aliquots.

S6 Kinase Assays

Phosphotransferase assays were performed by an adaptation of the method of Reimann et al. (76). The typical reaction mixture contained 20 mM MES, pH 6.8, 10 mM $MgCl_2$, approximately 100 μM [γ-^{32}P]ATP (50-200 dpm/pmol), 6 mM 2-mercaptoethanol, and 24 mM KCl in a total volume of 0.05 ml. Appropriate substrates and enzymes were added as noted in the individual experiments and phosphotransferase reactions were allowed to proceed for 10 minutes at 30° C. Modifications of this assay protocol are specified in the

individual experiments.

In activation assays, the enzymes were preincubated at 30° C for 10-20 minutes. The preincubation mixture contained 25 mM MES, pH 6.8, 12.5 mM $MgCl_2$, approximately 125 μM [γ-^{32}P]ATP (50-200 dpm/pmol), 7.5 mM 2-mercaptoethanol, 30 mM KCl and appropriate enzymes as specified in a total volume of 0.04 ml. The phosphotransferase assay was initiated by the addition of protein substrate (0.01 ml) and was carried out at 30° C for 10 minutes.

Histone phosphoprotein was quantitated by pipetting an aliquot of the reaction mixture onto a piece of Whatman ET 31 filter paper (6 x 15 mm) and immersing the filter paper in a beaker of ice-cold 10% (w/v) trichloroacetic acid. Papers were washed, dried and radioactivity determined by liquid scintillation counting. Histone H4 was prepared as previously described (22) from Histone type VIS (Sigma).

Phosphotransferase assays utilizing ribosomal 40S subunits (approximately 6 Absorbance units at 260 nm) as substrate contained 20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 0.1-0.2 mM [γ-^{32}P]ATP (100-600 dpm/pmol) and enzymes as specified. The phosphotransferase reaction was initiated by the addition of ribosomal 40S subunits and allowed to proceed for 10 minutes at 30° C. Activated assays were preincubated for 10-20 minutes prior to the addition of

ribosomal 40S subunits. Ribosomal proteins were separated by SDS-PAGE. Phosphorylation of S6 was quantitated by liquid scintillation counting of individual proteins sliced from dried gels or lasar scanning densitometric analysis of autoradiographs.

Full expression of S6 kinase activity was obtained by limited protease treatment of the assay sample immediately prior to the kinase assay. S6 kinase (0.2-2.0 mg/ml) or Activating Enzyme (0.2-1.0 mg/ml) was incubated with 0.5 volumes of a trypsin solution (12 µg/ml trypsin in 20 mM Tris-Cl, pH 8.0, 0.5 mM HCl, 2.2 mM 2-mercaptoethanol containing 4 mg/ml bovine serum albumin) for three minutes at 30° C. The reaction was stopped by the addition of 0.5 volumes inhibitor solution (24 µg/ml soybean trypsin inhibitor in 10 mM Tris-Cl, pH 7.5). This reaction mixture was used as the enzyme source for assays as described above. This method provided the best estimate of total S6 kinase specific activity.

Insulin receptor kinase (0.1 mg/ml) prepared from human placenta was incubated with 0.3 µM human insulin for one hour at room temperature immediately prior to the phosphotransferase assay. The insulin-insulin receptor kinase complex was then incubated for 5 minutes at 30° C with [γ-^{32}P]ATP (approximately 400 dpm/pmol) prior to addition of S6 kinase or Activating Enzyme as substrate.

Final insulin receptor concentration in the reaction was 10 µg/ml. The reaction was stopped by the addition of SDS-PAGE sample buffer; phosphoproteins were separated by SDS-PAGE and located by autoradiography. Phosphoprotein was quantitated by liquid scintillation counting of proteins sliced from the dried gel.

Immunoassay

The formation of antigen-antibody complexes was visualized and quantitated by a nitrocellulose-based enzyme-linked immunosorbent assay system (ELISA). The ELISA was performed with slight variations, dependent on the method of antigen application to the nitrocellulose.

Immunodot/ELISA.--Protein antigen, diluted in 20 mM Tris-Cl, pH 7.6, 0.5 M NaCl (TBS), was applied directly to nitrocellulose immobilized in the BioDot apparatus (BioRad). Excess protein binding sites on the nitrocellulose were blocked with TBS containing 1% (w/v) bovine serum albumin (globulin-free, Sigma). Primary antibody, diluted as indicated in individual experiments with TBS-1%BSA, was incubated with the nitrocellulose-bound antigen for two hours to overnight. A second, and in some cases third antibody, diluted in TBS-1%BSA was added sequentially for one hour to overnight. Prior to the addition of secondary and tertiary antibodies, the nitrocellulose was washed three

times with TBS containing 0.05% (v/v) Tween-20 (TBS-Tween). Upon completion of the antibody incubations, the nitrocellulose was washed twice with TBS-Tween and once with TBS alone. Antibody-antigen complexes were visualized by addition of horseradish peroxidase color development reagent containing 4-chloro-1-naphthol (BioRad) and hydrogen peroxide in TBS. All operations were carried out at room temperature, unless specified otherwise in individual experiments.

Screening of antibody-producing clones used peroxidase-linked goat anti-mouse antibody (BioRad) as the secondary antibody. A tertiary antibody was not employed in screening assays. All other immunodot/ELISA analyses employed a secondary and tertiary antibody: peroxidase-linked goat anti-mouse IgG Fraction and peroxidase-linked rabbit anti-goat IgG Fraction (Cappel), respectively.

Western Blot/ELISA.--Antigen proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose sheets in the Transblot apparatus (BioRad). Transfer was accomplished in 25 mM Tris/192 mM glycine, pH 8.3, with 20% (v/v) methanol at 150 mA for 12 hours. This method resulted in less than 20% transfer efficiency for some proteins. Up to 100% of the protein was transferred by the method of Szewczyk and Kozloff (91) in

which the buffer was 25 mM ethanolamine/glycine, pH 9.5, with 20% (v/v) methanol and transfer was conducted at 300 mA for 5 hours.

The ELISA reaction was conducted generally as described for immunodots with the modifications that excess protein binding sites on the nitrocellulose blot were blocked with TBS-Tween and all antibodies were diluted in TBS-Tween containing 0.1% (w/v) bovine serum albumin. Antibody dilutions and incubation times are noted in individual experiments.

Gel Electrophoresis

Polyacrylamide gels for slab gel electrophoresis were prepared using a modification of the Laemmli system (53) as described by Anderson et al. (5). The resolving gel was 10% (1.3% C), 12.5% (0.8% C) or 15% (0.6% C) acrylamide for use in the Bio-Rad Protean gel apparatus. The resolving gel was 10% or 12% (2.67% C) acrylamide for use in the Bio-Rad Protean II minigel system. Gels were fixed with methanol:acetic acid:water (4.5:1:4.5, v/v/v) and stained with 0.25% (w/v) Coomassie Brilliant Blue in methanol:acetic acid:water (5:1:5, v/v/v). Coomassie Blue destaining was carried out electrophoretically in methanol:acetic acid:water (5:1:5, v/v/v). Alternatively, gels were stained by the silver nitrate method (63). Gels were dried onto cellophane membrane backing (Bio-Rad) for lasar scanning

densitometry or onto Whatman 1 paper. Autoradiography of dried gels was carried out with Kodak BB5 film at -20° C. In some cases, the autoradiographic signal was enhanced with a 10X intensifying screen (Quanta II, Dupont) and exposure was at -80° C. Phosphoproteins were quantitated by liquid scintillation counting of individual (^{32}P)-labeled proteins sliced from dried gels.

Cell Lines and Maintenance

Basal medium in all tissue culture procedures was Dulbecco's modification of Eagle's medium (DMEM, with 4 mM glutamine, and 4.5 gm/l glucose) buffered with 3.7 gm/l $NaHCO_3$ and 4.29 gm/l HEPES, pH 7.4. This basal medium was supplemented with fetal calf serum (FCS) and other nutrients as indicated, immediately prior to use. The various supplements were kept as concentrated stock solutions at -20° C. All solutions for use in tissue culture were either purchased sterile or sterilized by filtration through a 0.22 micron mixed cellulose esters membrane (Millipore) in a negative pressure filtration apparatus (Gelman).

The myeloma cell line SP2/0-AG14 (87) is derived from the Balb/c mouse strain, displays 8-azaguanine resistance and does not secrete either heavy or light immunoglobulin chains. The cells were passaged twice per week by dilution (approximately 1:50) in basal medium supplemented with 15% (v/v) FCS and 2 mM glutamine. Established hybridoma cell

lines were passaged twice per week by dilution (approximately 1:50 - 1:100) in basal medium supplemented with 10% (v/v) FCS, 1.0 mM pyruvate, 4.0 mM glutamine and 50 µM 2-mercaptoethanol. Cells were incubated at 37° C in an atmosphere of 5% CO_2 and full humidity. Cell lines were maintained in the absence of antibiotics and routinely tested negative for mycoplasma contamination with the Mycoplasma T.C. Detection Kit (Gen-Probe, San Diego, CA.).

A stock of all cell lines was preserved in liquid nitrogen. Cells in log phase growth were pelleted at 500 x g for five minutes at room temperature, then resuspended at approximately 1 x 10^7 cells/ml in basal medium supplemented with 20% (v/v) FCS, 1.0 mM pyruvate and 4.0 mM glutamine. An equal volume of basal medium supplemented with 20% (v/v) FCS and 20% (v/v) dimethylsulfoxide was added dropwise to the cell suspension with constant, gentle agitation. The cell suspension (final concentrations: 5 x 10^6 cells/ml, 20% FCS and 10% dimethylsulfoxide) was aliquoted at 1 ml per cryotube (Nunc). The cryotubes were insulated in a styrofoam block and immediately placed at -20° C until frozen. The cryotubes were moved to -80° C overnight and then immersed in liquid nitrogen. A few hybridoma cell lines were not recovered from frozen with this protocol. The procedure was modified to include a final concentration of 40% (v/v) FCS, which proved satisfactory.

Immunization

Two immunized Balb/c mice were sacrificed for the fusion experiment. The immunization schedule for each mouse differed and will be described separately.

Mouse #1 (6 weeks old) received subcutaneous injection of 0.4 mg S6 kinase preparation emulsified in an equal volume of Freund's complete adjuvant (0.4 ml, 3 injection sites) on day 1. An intraperitoneal (i.p.) booster injection of 0.26 mg S6 kinase preparation emulsified in an equal volume of Freund's incomplete adjuvant (0.4 ml) was administered on day 22. A second booster injection (i.p.) of 0.8 mg S6 kinase preparation in Hank's Balanced Salt Solution (HBSS, with Ca^{2+} and Mg^{2+}, 0.6 ml) was administered on day 32. A final booster injection (i.p.) of 0.8 mg S6 kinase preparation in HBSS (0.6 ml) was administered on day 97, which was three days prior to sacrifice.

Mouse #2 (5 months old) received subcutaneous injection of 0.4 mg S6 kinase preparation emulsified in an equal volume of Freund's complete adjuvant (0.6 ml, 3 sites) on day 1. A booster injection (i.p.) of 0.4 mg S6 kinase preparation emulsified in an equal volume of Freund's incomplete adjuvant (0.6 ml) was administered on day 21. A final booster injection (i.p.) of 0.8 mg S6 kinase preparation in HBSS (0.6 ml) was administered on day 31, which was three days prior to sacrifice.

Serum was obtained from a sample of tail blood removed from each mouse four days prior to the final booster injection. The presence of serum antibodies was determined in the immunodot and Western Blot ELISA. Blood was collected from the inferior vena cava at the time of sacrifice, allowed to coagulate, and serum from each mouse stored at -20° C for further use.

Monoclonal Antibody Production

Antibody-secreting hybridoma cell lines were established by a modification of the method of Galfre and Milstein (38). The modified protocol employed is described. All medias were warmed to 37° C, but operations were carried out at room temperature in a laminar flow hood. Spleens from the two immunized mice were removed aseptically and cells flushed from the organ by the repeated infusion of basal medium containing 100 units/ml penicillin and 100 µg/ml streptomycin (Media A) through a 21 gauge needle. The cell suspension was passed through sterile gauze to remove tissue clumps, cells pelleted and resuspended at 1×10^7 cells/ml. A total of 3.4×10^8 nucleated cells were obtained from the combined spleens. The cell suspension was maintained at 37° C in a water bath until use.

The SP2/0-AG14 myeloma cells were passaged daily for four days prior to the fusion experiment to maintain the cells in vigorous log phase growth. On the day of fusion

the cells were determined to be greater than 95% viable by the Trypan Blue exclusion test. Cells were pelleted, washed once with Media A and resuspended at 1 x 10^7 cells/ml. A total of 2.1 x 10^8 cells were prepared and kept at 37° C in a water bath until use.

The spleen and myeloma cells were pelleted at 500 x g for 5 minutes, combined and resuspended in a total of 50 ml Media A and pelleted again. Fusion was initiated by the addition of 1 ml 50% (v/v) polyethylene glycol 1540 with 7.5% (v/v) dimethylsulfoxide in Media A to the combined cell pellet. Addition of the fusion agent was accompanied by gentle stirring of the cell pellet. The reaction mixture was diluted slowly after two minutes with 2 ml Media A, followed by 47 ml Media A supplemented with 20% (v/v) FCS. Cells were pelleted and resuspended in Media A supplemented with 20% (v/v) FCS, 4 mM glutamine, 1 mM pyruvate, 1% (v/v) MEM non-essential amino acids, 0.2 unit/ml insulin, 50 µM 2-mercaptoethanol, 100 µM hypoxanthine, 16 µM thymidine (Media B) and 0.4 µM aminopterin and plated at 5 x 10^5 cells/well into 96 well tissue culture plates. Spleen and myeloma cells not subjected to the fusion reaction were seeded separately at 2 x 10^5 cells/well in Media B containing 0.4 µM aminopterin as controls for the rate of non-hybrid cell death. Tissue culture plates for fusion and cloning steps were seeded with 5 x 10^3 peritoneal

macrophages per well in Media B with aminopterin, as a feeder layer one day prior to introduction of hybrid cells (27).

Cells obtained from selected fusion wells were subjected to two cloning steps in Media B. Fusion wells containing cells which displayed exceptionally vigorous growth were diluted in Media B and seeded in a 96 well plate at 5 cells per well for the first cloning step. In all other cases, cells from a single fusion well were serially diluted 11 times to 0.25 cells/well in a 96 well plate with an 8 channel multipipette (Titertek). This method provided a set of heavily seeded wells which were allowed to overgrow for antibody assay and lightly seeded wells which produced single colonies for further cloning. Antibody positive plates were examined microscopically to identify wells containing a single colony for the second cloning step, which was performed by the serial dilution method. Single colonies from antibody positive plates in the second cloning step were established in mass culture in the absence of feeder layer cells. The presence of antibody in the cell culture media was detected by the immunodot/ELISA method.

Radioimmunoprecipitation Assays

Antigen was radioactively labeled in an autophosphorylation reaction. S6 kinase preparation (0.17 mg) was incubated at 30° C with 20 mM MES, pH 6.8,

7.5 mM 2-mercaptoethanol, 30 mM KCl, 0.12 mM EDTA, 10 mM $MgCl_2$ and 0.3 mM [γ-^{32}P]ATP (6600 dpm/pmol) in a total volume of 0.4 ml for 20 minutes. Excess [γ-^{32}P]ATP was removed by three dialysis steps (50 ml each) against 20 mM MES, pH 6.8, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.12 M KCl in the presence of approximately 20 gm of activated charcoal. The specific radioactivity of the ^{32}P-labeled-S6 kinase antigen was 5.6 x 10^3 dpm/μg.

The S6 kinase preparation in the presence of the Activating Enzyme preparation was autophosphorylated in a similar manner. S6 kinase preparation (0.17 mg) and Activating Enzyme preparation (0.08 mg) were incubated together at 30° C with 14 mM MES, pH 6.8, 15 mM 2-mercaptoethanol, 60 mM KCl, 0.25 mM EDTA, 7 mM $MgCl_2$ and 0.2 mM [γ-^{32}P]ATP (2800 dpm/pmol) in a total volume of 0.4 ml for 20 minutes. The specific radioactivity of the ^{32}P-labeled-AE-S6 kinase antigen was 1.8 x 10^3 dpm/μg.

Conditioned medium from hybridoma cultures (1 ml) was harvested at 7-9 days of culture, adjusted to approximately pH 7.6 with Tris buffer and incubated with 4-7 μl radiolabeled antigen for 1.5 hours at room temperature. Antigen-antibody complexes were precipitated by the addition of 0.2 ml Protein A crude cell suspension (10% w/v, Sigma) for 15 minutes. Cells were pelleted at 12,000 x g for 4 minutes, washed twice with 50 mM Tris-Cl, pH 8.3, 0.6 M

NaCl, 0.5% Triton-X 100 then resuspended in 0.1 ml hot SDS-PAGE sample buffer (modified to contain 4% SDS). The samples were boiled for 10 minutes, cellular debris was removed by centrifugation at 12,000 x g for 4 minutes, and the immune complex proteins were separated by 12.5% SDS-PAGE. Radiolabeled, precipitated antigens were detected by autoradiography.

Ascites Tumors

Established hybridoma cell lines were injected intraperitoneally at 2-5 x 10^6 cells in 0.5 ml basal medium per mouse. Mice were primed by intraperitoneal injection of 0.5 ml pristane (2,6,10,14-tetramethylpentadecane) 3-5 days prior to introduction of cells from tissue culture. Mice were sacrificed after 2-4 weeks, ascites fluid collected asceptically, cells removed by centrifugation and fluid stored in small aliquots at -80° C. Cells obtained from the ascites fluid were reinjected at 2-5 x 10^6 cells in 0.5 ml basal medium per mouse without prior pristane injection. Ascites fluid typically contained 40 mg/ml soluble protein, 30-50% of which was antibody, based on estimates from SDS-PAGE stained with Coomassie Blue. The major contaminating protein appeared to be albumin.

Purification of Ascites Fluid

Antibodies were partially purified from mouse ascites

fluid by chromatography on DEAE AffiGel Blue (BioRad). The ascites fluid was dialyzed against 10 mM Tris-Cl, pH 8.0, and applied to a column of DEAE AffiGel Blue equilibrated in 10 mM Tris-Cl, pH 8.0, at a ratio of 1 ml ascites fluid per 5 ml resin. Elution of IgG was accomplished with a 100 ml linear gradient of 10 mM Tris-Cl, pH 8.0, containing 0 to 0.5 M NaCl. Protein elution was monitored spectrophotometrically at 280 nm. The first eluted protein peak occurred at 40-80 mM NaCl and contained the majority of IgG as determined by SDS-PAGE analysis.

Antibody Affinity Chromatography

Monoclonal antibody obtained from DEAE AffiGel Blue chromatography of ascites fluid was coupled to CNBr-activated Sepharose 4B in a ratio of 5 mg antibody protein per ml of swollen resin. Resin was swollen in 1 mM HCl on a scintered glass filter and rinsed briefly with 0.2 M $NaHCO_3$, pH 8.5, containing 0.5 M NaCl (coupling buffer). Antibody protein was dialyzed against coupling buffer and combined with the resin. Coupling was allowed to proceed for 16 hours at 4° C with end-over-end mixing. Excess coupling groups on the resin were blocked with 0.2 M glycine, pH 8.0, for 16 hours at 4° C or 2 hours at room temperature. Non-covalently bound protein was removed by three wash cycles of coupling buffer alternated with 0.1 M acetate, pH 4, containing 0.5 M NaCl. Resins were

equilibrated and stored in TBS-Tween with 0.005% (w/v) Thimerisol.

Affinity chromatography of either Activating Enzyme or S6 kinase was performed in TBS-Tween. The sample was dialyzed against TBS-Tween containing 15 mM 2-mercaptoethanol and applied to the antibody-Sepharose. The column was washed with 10 bed volumes of TBS-Tween and bound protein eluted with 0.1 M glycine-Cl, pH 2.2. Fractions (0.5 ml) containing unbound proteins were raised to 15 mM 2-mercaptoethanol by the addition of 10% (v/v) 150 mM 2-mercaptoethanol. Eluted protein fractions were neutralized by the addition of 10% (v/v) 1.0 M Tris, pH 10.5 containing 150 mM 2-mercaptoethanol.

Other Methods

Coomassie Blue dye-binding to specific proteins and antibody-antigen complexes on Western Blot or immunodot analyses were quantitated by lasar scanning densitometry on an LKB Model 2202 densitometer. Data was analyzed by the LKB GELSCAN computer program. [γ-^{32}P]ATP was prepared by the method of Walseth and Johnson (104). The 40S subunit of rabbit reticulocyte ribosomes was generously provided by B. Hardesty and G. Kramer at the University of Texas at Austin. Insulin receptor was generously provided by Richard A. Roth at Stanford University School of Medicine. Myeloma cell line SP2/0-AG14 was generously provided by E. Hansen at

the University of Texas Health Science Center at Dallas. Protein was determined by the method of Bradford (9). Total protein on nitrocellulose was visualized by staining with 0.1% (w/v) Amido Black in 7% acetic acid. Destaining was accomplished in 7% acetic acid. The Mr of proteins separated by gel electrophoresis was estimated from standard curves constructed from the migration of BioRad Mr standard proteins: myosin, 200,000; β-galactosidase, 116,200; phosphorylase B, 92,500; albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; and soybean trypsin inhibitor, 21,500. The Mr of proteins separated by gel filtration chromatography was estimated from a standard curve constructed from the elution volumes of the following standard proteins (BioRad): thyroglobulin, void volume; gamma globulin, 158,000; albumin, 66,200; ovalbumin, 45,000; and myoglobin, 17,000.

Materials

DMEM, HBSS, penicillin-streptomycin, bovine insulin, sodium pyruvate and MEM nonessential amino acids were purchased from GIBCO. Fetal calf serum was from Flow Laboratories. Globulin-free albumin, CNBr-activated Sepharose 4B, cell culture grade glutamine, HEPES, Trypan Blue stain (0.4%) and Freund's adjuvants were from Sigma Chemical Corp. Polyethylene glycol 1540 was from Polysciences, Inc. DEAE AffiGel Blue, gel electrophoresis

reagents, Bradford reagent, standard proteins for Mr determination, equipment for immunodot and Western Blot analyses, nitrocellulose, EIA purity Tween-20, HRP color development reagent and peroxidase-linked goat anti-mouse IgG for screening of hybrid clones were purchased from BioRad. Peroxidase-linked goat anti-mouse IgG fraction and peroxidase-linked rabbit anti-goat IgG fraction for Western Blot and immunodot analyses of gel filtration chromatography were purchased from Cappel. Tissue culture plasticware was from Corning Glass Works.

CHAPTER III

RESULTS

Enzyme Purification and Characterization

A protein kinase which utilized both the ribosomal protein S6 and histone H4 as substrate *in vitro* was purified from human placenta. This enzyme has been termed S6 kinase as ribosomal protein S6 is the putative substrate *in vivo*. The enzyme was purified by a modification of the procedure employed to isolate the murine lymphosarcoma enzyme (22, 62). The procedure is detailed in Experimental Procedures. Briefly, the placental tissue was homogenized, and the post-ribosomal supernatant obtained by ultracentrifugation was subjected to six purification steps: DEAE cellulose chromatography, ammonium sulfate precipitation, phosphocellulose chromatography, concentration on CM-Sephadex, Sephacryl S200 chromatography, and concentration on DEAE cellulose.

Histone H4 activity and recovery at each of these steps is outlined in Table II. All activity determinations were made after a 10 minute preincubation with MgATP. The percent yield was greater than 100% and increased after ammonium sulfate precipitation, phosphocellulose chromatography and CM-Sephadex chromatography. The S6

kinase is a regulated enzyme and the observed activity during purification may be influenced by several factors. The removal of an endogenous inhibitor to the S6 kinase or an inhibitor to an enzyme which activates the S6 kinase would account for the increase in observed activity. Alternatively, increased activity could be due to an activation of the S6 kinase by the loss of a regulatory domain during the purification. The fold purification and percent yield drops from 114 and 450, to 75 and 185, respectively, after Sephacryl S200 chromatography. This observed decrease in activity may represent the separation of an activator of the S6 kinase at this step.

The S6 kinase preparation is activated by preincubation with MgATP prior to addition of the protein substrate. Activation of the enzyme by MgATP preincubation was observed at every step after ammonium sulfate precipitation. The fold activation of the enzyme reached 6.8 after CM-Sephadex chromatography, which also represented the highest percent yield of the enzyme. The fold activation dropped to 4.7 after Sephacryl S200 chromatography, which would be consistent with the separation of an activator at this step. The final enzyme preparation had a specific activity of 6.7 nmol/min-mg and represented a 95% recovery from the DEAE cellulose chromatography.

The S6 kinase purification was improved by the addition

of protease inhibitors to all buffers throughout the purification. Batch washes at the first DEAE cellulose chromatography were also added. The enzyme prepared in Table II and enzyme prepared by the modified protocol were analyzed by 12.5% SDS-PAGE as shown in Figure 2. The major proteins in both preparations were Mr 72,000 and Mr 55,000; however, the former sample displays a large number of additional proteins. Since the major modification of the purification procedure entailed more extensive use of protease inhibitors, many of these proteins may be the products of proteolysis. The specific activity of the MgATP-activated S6 kinase preparation and the fold MgATP activation of the enzyme prepared by either protocol was similar.

The effect of MgATP activation of the S6 kinase preparation on gel filtration chromatography elution behavior is examined in Figure 3. The unactivated enzyme eluted from gel filtration chromatography with Mr 57,000 (288 ml elution volume). A second peak of protein kinase activity containing approximately 17% of the activity in the major peak was observed to elute with an Mr of 24,000 (345 ml elution volume). MgATP activation of the enzyme prior to gel filtration chromatography resulted in a qualitatively similar elution pattern. However, the peak of protein kinase activity eluted with Mr 24,000 contained

approximately 34% of the activity observed in the major peak. Previous work with the S6 kinase preparation established that the unactivated enzyme eluted from gel filtration chromatography with Mr 82,000. MgATP activation of the enzyme prior to gel filtration chromatography resulted in a shift in enzyme elution to Mr 44,000 (23, 24). The elution of the S6 kinase from gel filtration chromatography with a smaller Mr than previously observed is consistent with the apparent proteolysis observed in Figure 2. These observations were addressed by the extensive use of protease inhibitors in the modified purification protocol.

The S6 kinase preparation from human placenta catalyzed phosphorylation of the 40S ribosomal protein S6 as well as histone H4. Enzyme was prepared by the modified purification protocol and the Sephacryl S200 gel filtration chromatography step was assayed with either mixed histone or 40S ribosomal subunits as the protein substrate (Figure 4). A single peak of protein kinase activity was observed with each substrate. The activity peaks were entirely coincident and eluted with Mr 82,000. The possibility existed that two protein kinases coeluted.

To test definitively the utilization of both ribosomal protein S6 and histone H4 by a single protein kinase, a competition experiment was designed. The S6 kinase

preparation was incubated with a nonsaturating concentration of 40S ribosomal subunits in the presence of increasing concentrations of histone H4 (Figure 5). Histone H4 inhibited S6 phosphorylation, indicating that a single protein kinase utilized both substrates. Confirmation of the usage of these two substrates by a single S6 kinase allowed histone to be used as the routine substrate for assay. Histone H4 is a convenient substrate for routine use as it can be used in the assay of Reimann et al., saturating concentrations are possible, and it is used at a low rate by other protein kinases (23, 24). Ribosomes are less convenient as a routine substrate as it is not possible to achieve saturating concentrations and proteins must be separated electrophoretically prior to quantitation of phosphoprotein. However, ribosomes were employed periodically to confirm results obtained with histone H4.

The phosphorylation of individual proteins in the S6 kinase preparation by MgATP-activation was examined. This analysis allowed identification of proteins which were candidates for subunits of the S6 kinase, as many protein kinases catalyze autophosphorylation. The phosphorylation pattern upon addition of Activating Enzyme to the MgATP preincubation was also examined. This allowed identification of proteins in the S6 kinase preparation which could be potential substrates for phosphorylation by

Activating Enzyme.

MgATP activation of the S6 kinase preparation resulted in the phosphorylation, with approximately equal intensity, of two proteins with Mr 56,000 and Mr 38,000 (Figure 6). These two proteins were minor components (< 5% total protein) of the S6 kinase preparation as determined by visual inspection of the Coomassie Blue stained gel. The major protein in the S6 kinase preparation (Mr 54,000; approximately 70% total protein) was also phosphorylated, but with approximately 25% of the intensity displayed by the Mr 56,000 and Mr 38,000 phosphoproteins. Therefore, the stoichiometry of phosphorylation was considerably less for the Mr 54,000 protein than that observed for the Mr 56,000 and Mr 38,000 proteins.

The phosphorylation of S6 kinase proteins upon addition of Activating Enzyme to the MgATP preincubation was also examined. Two new phosphoproteins, with Mr 116,000 and Mr 95,000, were labelled with approximately equal intensity. The Mr 116,000 protein was present in the Activating Enzyme preparation. The phosphorylation of the Mr 56,000 and Mr 54,000 proteins in the S6 kinase preparation was enhanced 2 to 3 fold in the presence of Activating Enzyme. The Activating Enzyme alone did not display autophosphorylation. These observations were consistent with the activation of S6 kinase by Activating Enzyme occurring via a phosphorylation

reaction.

Each major phosphoprotein observed when Activating Enzyme and S6 kinase preparations were preincubated with MgATP was quantitated as a function of increased S6 kinase activity (Table III). The greatest increase in phosphorylation occurred on the Mr 116,000 protein (240% of control). This protein was present in the Activating Enzyme preparation and may be phosphorylated by the S6 kinase preparation. The remaining proteins displayed an increase in phosphorylation of 26 to 54% in the presence of Activating Enzyme. The S6 kinase activity was 471% of that observed in the absence of the Activating Enzyme. The increase in the phosphorylation of proteins in the S6 kinase preparation was less than directly proportional to the increase in S6 kinase activity. This observation is consistent with signal amplification in a cascade type of activation.

Activating Enzyme, MgATP-activatable S6 kinase and trypsin-activatable S6 kinase eluted from gel filtration chromatography with different buffer volumes as shown in Figure 7. The peak of S6 kinase activity observed after MgATP-activation of the fractions was assymetrical with the major portion of the activity eluting with an Mr 85,000. Additional S6 kinase activity was observed when the fractions were preincubated briefly with trypsin prior to

the MgATP-activation reaction. This activity results from an activation by proteolysis (24, 58). The peak of S6 kinase activity requiring trypsin treatment for expression was determined by subtraction of the MgATP-dependent activity from the total activity observed after trypsin-activation. The trypsin-dependent enzyme represented approximately 50% of the total S6 kinase activity.

The elution of Activating Enzyme activity was obtained by preincubating individual fraction samples with an aliquot of S6 kinase from the MgATP-activatable S6 kinase peak for ten minutes. The activation of the S6 kinase above that observed with MgATP alone was used as the measure of Activating Enzyme activity. Two peaks of Activating Enzyme were eluted from the gel filtration chromatography. The first peak eluted at the void volume and was completely separated from the S6 kinase activity. This peak was pooled, concentrated on DEAE cellulose and employed as the exogenous Activating Enzyme in further experiments. A second activity peak eluted with an Mr 200,000, although this peak could not be fully defined due to overlap with S6 kinase activity. In most cases, the full range of S6 kinase activity was pooled and concentrated on DEAE cellulose as the last purification step. Extrapolation of the second Activating Enzyme activity peak strongly suggests that a

portion of this activity was pooled with the S6 kinase preparation.

Monoclonal antibodies were produced against proteins of the S6 kinase preparation. The antibodies provided the tool by which regulatory components of the S6 kinase preparation could be monitored and identified. This approach was chosen in preference to additional purification of the enzyme.

Reactivity of Mouse Immune Serum

Balb/c mice were immunized with S6 kinase prepared by the unmodified purification protocol as described in Experimental Procedures. The success of the fusion experiment was dependent upon obtaining a strong and specific immune response in the mouse. Prior to the final antigen injection, a blood sample was obtained from the tail of each mouse. The presence and extent of the serum immune response was determined by immunodot analysis with 1 µg of the S6 kinase preparation as antigen, various dilutions of serum (10 dilutions ranging from 1:400 to 1:100,000) as primary antibody and peroxidase-linked goat anti-mouse serum (BioRad) diluted 1:4500 as the second antibody. Incubation times were three and one hours, respectively. Immunoreactivity was detectable by visual inspection to a dilution of 1:3500 with mouse #1 serum and to 1:5900 with mouse #2 serum. Serum from a non-immunized mouse was employed as a negative control. Immunoreactivity was not

observed against the S6 kinase preparation at any nonimmune serum dilution.

Western Blot analysis of the S6 kinase preparation used for immunization was performed with the mouse immune serum. This technique confirmed that the immunoreactivity observed in the immunodot analysis was directed against specific proteins present in the S6 kinase preparation. Results are shown in Figure 8. The pattern of immunoreactive proteins differed with serum from either mouse and the reaction with mouse #2 serum was stronger. The major antigens detected with mouse #1 serum were a strong doublet with Mr 80,000-83,000 and a single antigen with Mr 66,000. Serum from mouse #2 detected the Mr 80,000-83,000 doublet with at least 10 fold greater intensity. Mouse #2 serum detected four proteins with Mr 60,000-72,000 which included the Mr 66,000 protein detected by mouse #1 serum. An Mr 48,000 and 37,000 protein were also detected by the mouse #2 serum. Immunoreactivity was not observed with serum from the non-immunized mouse. The strength and specificity of the serum immune response were excellent in both the immunodot and Western Blot analysis and were considered quite sufficient to proceed with the fusion experiment.

Immunodot analysis was repeated after the final antigen injection to quantitate the strength of the immune response at the time of fusion. Blood was collected from the

inferior vena cava of each immunized mouse at the time of sacrifice. Analysis was performed with 160 ng of the S6 kinase preparation as antigen, various dilutions of serum as primary antibody and peroxidase-linked goat anti-mouse (BioRad) diluted 1:4500 as secondary antibody. Incubation times were four and one hours, respectively. Immunoreactivity was detectable by visual inspection to a dilution of 1:1600 with mouse #1 and to 1:3200 with mouse #2 serum. Compared with the previous serum titre determination, the final serum sample detected antigen at five fold less concentration, with only a doubling of serum concentration. Non-immune serum tested negative. These data demonstrate the increased sensitivity of the mouse immune serum detection of antigen, which was expected subsequent to the final booster injection.

The ability of the polyclonal immune serum to crossreact with Activating Enzyme, mouse lymphosarcoma S6 kinase and protein kinase C preparations was tested as a preliminary step in the identification of antigens to which monoclonal antibodies could be obtained. Western Blot analysis of human placenta Activating Enzyme with the immune serum revealed an antigen pattern qualitatively similar to that observed with the S6 kinase preparation. A strong doublet and a single band of lower Mr were observed (data not shown). However, the immune serum failed to react with

S6 kinase prepared from mouse lymphosarcoma (22) in Western Blot analysis. The immune serum also failed to react with protein kinase C prepared from mouse lymphosarcoma (59) in Western Blot analysis. This result was consistent with the absence of protein kinase C activity in the S6 kinase preparation as previously reported (23, 24). These data raise the possibility that antibody specific for the Activating Enzyme was obtained.

The effect of the mouse immune serum on S6 kinase activity and activation was tested. Immune serum was added to the phosphotransferase and the MgATP-activation assays of S6 kinase. Neither inhibition nor activation of the S6 kinase activity was observed (Figure 9), indicating that antibody specific for the active site of the enzymes was not obtained.

Monoclonal Antibody Production

The fusion reaction was conducted with spleenocytes from the immune mice and the SP2/0-AG14 myeloma cell line. It is critical to the success of the experiment that the myeloma cells be in vigorous log phase growth and free from contamination at the time of fusion. The growth parameters for the SP2/0-AG14 cell line were determined as shown in Figure 10. The population doubling time was 12 hours in both the standard maintenance media and Media B (HT media to be used in cloning). Log phase growth was observed below a

concentration of 1 x 10^6 cells/ml. Addition of aminopterin to Media B resulted in 100% cell death within five days (data not shown). The growth of these cells is severely affected by the presence of mycoplasma contamination, but the growth parameters observed were consistent with healthy cells as previously reported (87). The cells were free from other contamination by microscopic inspection.

A total of 162 antibody-producing monoclonal cell lines were established from the fusion experiment. Fusion and cloning steps were performed as described in Experimental Procedures. A flow chart delineating the number of clones recovered at each step of the procedure is shown in Figure 11. A total of 245 wells from 960 seeded (25%) were positive for hybridoma growth. Colonies of hybridoma cells were visible in the fusion wells within seven days. Death of nonhybridized myeloma cells was complete by day 7, however nonhybridized spleen cell viability was greater than 70% by the trypan blue exclusion test. Cell conditioned medium from all fusion wells as well as from spleen cell controls tested positive, with equal intensity, for antibody by immunodot analysis. Conditioned medium from myeloma cell controls tested negative. The uniform intensity of the response suggested the presence of antibody secreted by viable spleen cells present in all fusion wells. Therefore, fusion wells were selected for cloning which displayed

vigorous growth and apparently contained few, preferably one, hybrid colony. The high antibody positive background was not encountered in the cloning steps.

The 133 fusion wells selected as described above were subjected to the first cloning step between 12 and 32 days after the fusion reaction. Each fusion well contained between 2 x 10^3 and 7 x 10^5 hybrid cells at the time of cloning. Hybrid cell viability ranged from 25 to 75%. Each of these 133 fusion wells was seeded into a 96 well plate for the first cloning step. Immunodot assay detected 49 antibody-positive plates of the original 133. These antibody-positive plates were inspected microscopically, and two to three wells, containing single colonies, were chosen from each for the second cloning step.

Selected Clone I wells (n = 102) were subjected to the second cloning step between 12 and 34 days after the first cloning step. Clone I wells averaged between 2 x 10^4 and 5 x 10^5 hybrid cells at the time of the second cloning step. Hybrid cell viability was generally greater than 70%. Immunodot assay detected 73 antibody-positive plates of the 102 Clone II plates. These antibody-positive plates were inspected microscopically, and two to three wells, containing single colonies, were chosen from each for establishment in mass culture.

Selected Clone II wells (n = 215) were transferred to mass culture between 9 and 28 days after the second cloning step. A total of 162 cell lines survived after ten passages and were considered to be established. Established cell lines initially displayed an average population doubling time of 17 hours. Within three months of continuous culture the population doubling time of greater than 90% of the hybrid cell lines decreased to 12 hours and displayed greater than 95% viability under normal maintenance conditions. All cell lines which originated from a single fusion well were defined as a family. Cell lines in a family have a high probability of producing identical antibody.

Identification of Immunoreactive Proteins

Activating Enzyme, MgATP-activatable S6 kinase and trypsin-dependent S6 kinase activities were resolved by Sephacryl S200 gel filtration chromatrography (Figure 7). Analysis of proteins from individual gel filtration chromatography fractions by SDS-PAGE, immunodot and Western Blot was employed to obtain the elution patterns for individual proteins. Comparison of the activity and individual protein elution patterns allowed identification of proteins associated with Activating Enzyme and both forms of the S6 kinase.

SDS-PAGE analyses.--Individual gel filtration fractions were subjected to SDS-PAGE analysis in order to identify proteins associated with S6 kinase activity. An Mr 95,000 and 55,000 protein were major proteins in the SDS-PAGE analysis. These two proteins were also observed to be phosphorylated in Figure 6, with the Mr 95,000 phosphoprotein appearing upon activation with Activating Enzyme. These two proteins are therefore good candidates for components of the S6 kinase. The distribution of these two proteins was quantitated by laser scanning densitometry of the stained gel (Figure 12). Comparison with Figure 7 shows that the elution of the Mr 95,000 protein is entirely coincident with the S6 kinase dependent on trypsin for expression. The Mr 55,000 protein eluted as two peaks. The first corresponded to the trypsin-dependent S6 kinase which eluted with Mr 158,000. The second peak eluted in the area of S6 kinase which was activatable by MgATP without trypsin and eluted with Mr 80,000. The total distribution of the Mr 55,000 protein completely encompassed all S6 kinase activity. These were the only major proteins observed which eluted from gel filtration chromatography coincident with the trypsin-dependent and all S6 kinase activity, respectively.

Analysis with mouse immune serum.--The polyclonal mouse immune serum was employed to identify the proteins in gel filtration chromatography fractions to which monoclonal antibodies might have been obtained. In addition, comparison of the antigen elution pattern with the enzyme activity allowed tentative assignment of these antigens as components of the enzyme. Western Blot analysis of individual gel filtration chromatography fractions with mouse immune serum is shown in Figure 13. The two principal antigens which were identified by the ELISA reaction had an Mr 72,000 and 116,000 as determined by comparison with SDS-PAGE molecular weight standard proteins.

The ELISA reaction was quantitated by lasar scanning densitometry of individual lanes. Three peaks of ELISA reactivity with the Mr 116,000 protein were observed (Figure 14). Based on the elution volumes of the gel filtration chromatography, these peaks eluted at the void volume and with Mr 200,000 and Mr 85,000. A comparable SDS-PAGE gel of the gel filtration fractions was stained with Coomassie Blue and the distribution of the Mr 116,000 protein quantitated by laser scanning densitometry (Figure 14). The Mr 116,000 protein was observed to elute in two peaks which were coincident with the occurrence of the ELISA reactive protein eluted from gel filtration chromatography at the void volume and with Mr 200,000. No Coomassie Blue stained Mr 116,000

protein was detected by the densitometer in the fractions which contained the third peak of immunoreactive protein (elution Mr 85,000), although a small amount of protein was visible in the stained gel.

ELISA reactivity with the Mr 72,000 protein occurred in a symmetrical peak with the maximum reactivity in the gel filtration fraction number 64. This peak eluted from gel filtration chromatography with Mr 90,000 (Figure 15). Two peaks of Mr 72,000 protein were observed by lasar scanning densitometry of the Coomassie Blue stained SDS-PAGE of gel filtration fractions (Figure 15). The first peak occurred in gel filtration fraction number 59 and eluted with an Mr of 200,000. This peak was preceded by a shoulder at gel filtration fraction 50 which eluted at the void volume. This asymmetrical peak of Mr 72,000 protein was qualitatively similar to the distribution of the Mr 116,000 protein detected on the Coomassie Blue stained gel and was not detected by the immune serum. The second peak of Mr 72,000 protein observed on the Coomassie Blue stained gel was coincident with the immunoreactive Mr 72,000 protein.

Comparison of the Mr 116,000 and 72,000 protein distribution with Figure 7 shows that the first eluted peak of immunoreactive Mr 116,000 protein was coincident with Activating Enzyme activity eluted at the void volume. The second eluted peak of immunoreactive Mr 116,000 protein was

coincident with Activating Enzyme activity eluted with Mr 200,000. This pattern also corresponded to the distribution of nonimmunoreactive Mr 72,000 protein. The distribution of immunoreactive Mr 72,000 protein was coincident with the MgATP-dependent S6 kinase activity.

Minor ELISA reactivity was observed with two additional proteins with Mr 110,000 and 62,000 on the Western Blot of gel filtration fractions with mouse immune serum (Figure 13). The pattern observed for the Mr 110,000 protein precisely followed the Mr 116,000 protein distribution, suggesting that these proteins may be related. The Mr 62,000 protein eluted as a broad peak in gel filtration fractions 52 to 64. An immunoreactive protein which corresponded to the distribution of total S6 kinase activity or trypsin-dependent S6 kinase activity was not observed.

Analyses with monoclonal antibodies.--Immunodot analysis of proteins from individual gel filtration fractions was performed with monoclonal antibody from different cell line families. ELISA reactivity was quantitated by laser scanning densitometry. Comparison of the immunoreactivity pattern obtained by immunodot analysis with the elution pattern for specific antigens obtained by Western Blot analysis with serum (Figure 13) allowed classification of the monoclonal antibodies by antigen recognition. Monoclonal antibodies specific for either the

Mr 116,000 or Mr 72,000 antigen were then chosen for further study.

Antigen distribution on gel filtration chromatography for three monoclonal antibodies (05-E11C, 18-F9D and 05-J12C) is shown in Figure 16. All three antibodies detected an antigen in a broad, asymmetrical peak (fractions 48-66) which was qualitatively comparable to the elution pattern of the Mr 116,000 protein (Figure 14). Figure 17 shows two monoclonal antibodies (23-H10D and 05-Y11B) which detected antigen in a symmetrical peak which was qualitatively comparable to the elution pattern of the immunoreactive Mr 72,000 protein (Figure 15). Results of the additional 34 monoclonal antibodies tested are summarized in Table IV. Only two patterns of antigen distribution were observed, suggesting that all the monoclonal antibodies tested were specific for either the Mr 116,000 or immunoreactive Mr 72,000 protein.

Three monoclonal antibodies were chosen for further study: 1) the 18-F9D monoclonal antibody, which had immunodot reactivity corresponding to the elution pattern of the Mr 116,000 antigen, 2) the 23-H10D monoclonal antibody, which had immunodot reactivity corresponding to the elution pattern of the Mr 72,000 antigen, and 3) the 05-Y11B monoclonal antibody, which had immunodot reactivity corresponding to the Mr 72,000 antigen. The 05-Y11B

monoclonal antibody was chosen in addition to the 23-H10D monoclonal antibody, because the immunodot pattern for these two was slightly different (Figure 17).

Definitive identification of the antigen for each of these three monoclonal antibodies was made by Western Blot analysis of gel filtration fractions. The 18-F9D monoclonal antibody reactivity in immunodot analysis (Figure 16) corresponded to the elution pattern for the Mr 116,000 antigen and also exhibited a positive ELISA in Western Blot analysis. The Western Blot of gel filtration fraction number 58, representing the peak immunoreactivity for the 18-F9D antibody, was laser scanned, stained for total protein with Amido Black and scanned again (Figure 18). The major immunoreactive protein was Mr 116,000 with minor reactivity of an Mr 110,000 protein, consistent with the immune serum Western Blot. No other protein was detected by ELISA, demonstrating the monospecificity of the 18-F9D monoclonal antibody.

A similar analysis was conducted with the 23-H10D monoclonal antibody, which in immunodot analysis (Figure 17) corresponded to the elution pattern for the immunoreactive Mr 72,000 antigen. A positive ELISA was obtained in Western Blot analysis of gel filtration fractions and identification of the antigen was performed as with the 18-F9D monoclonal antibody (Figure 18). A single immunoreactive protein of Mr

72,000 was observed, demonstrating the monospecificity of the 23-H10D monoclonal antibody.

Western Blot analysis of gel filtration fractions with the 05-Y11B monoclonal antibody was also performed. Immunodot analysis with this monoclonal antibody gave a immunoreactivity pattern which corresponded to the elution pattern of the immunoreactive Mr 72,000 protein (Figure 17). Western Blot analysis with gel filtration fractions yielded an extremely strong reaction with an Mr 65,000 protein between fractions number 61 to 71 (Figure 19). This protein eluted from gel filtration chromatography as a symmetrical peak with Mr 80,000. The elution pattern of this Mr 65,000 protein was comparable to that previously observed for the Mr 72,000 protein in Western Blot analysis with mouse immune serum (Figure 13).

The relationship between the Mr 65,000 protein detected by the 05-Y11B monoclonal antibody and the Mr 72,000 protein detected by the mouse immune serum in a previous experiment was investigated. Gel filtration fractions were subjected to Western Blot analysis with mouse immune serum. This experiment was similar to the one previously described in Figure 13 with two exceptions. First, the gel filtration fractions employed were the same as used for the 05-Y11B monoclonal antibody analysis. This assured that the same set of antigens was present for both the Western Blot with

05-Y11B monoclonal antibody and the one with mouse immune serum. Second, the mouse immune serum utilized was a 4:1 mix of serum from mouse #1 and mouse #2, respectively. The immunoreactivity of each serum was somewhat different as shown in Figure 8. Serum from mouse #2 detected four antigens between Mr 60,000-72,000, while mouse #1 serum only detected the antigen with Mr 66,000 in this group. Also, mouse #2 serum displayed a 10 fold greater reactivity with the Mr 80,000-83,000 antigen doublet than mouse #1 serum.

The Western Blot of gel filtration fractions with mouse immune serum (4:1 ratio of mouse #1 to mouse #2 serum) is shown in Figure 20. The Mr 65,000 antigen was detected between fractions number 63 to 71. The peak of this antigen eluted from gel filtration chromatography with Mr 80,000. This is apparently the same antigen detected by the Mr 05-Y11B monoclonal antibody. An Mr 56,000 antigen was detected with immune serum which eluted as a broad peak between fractions number 55 to 65. The Mr 116,000 or Mr 110,000 antigens were not detected. The Mr 66,000 antigen was apparently the same antigen detected by the Mr 05-Y11B monoclonal antibody. The Mr 56,000 antigen is comparable with the Mr 62,000 antigen detected in the previous Western Blot with mouse immune serum (Figure 13). The absence of the Mr 116,000 antigen, which was present in Figure 13, may be due to the difference in serum immunoreactivity in the

two experiments.

The Mr 65,000 antigen may represent a proteolytic product of the Mr 72,000 protein. A Western Blot of gel filtration fractions, comparable to the one used for analysis of the 05-Y11B monoclonal antibody, was stained with Amido Black for total protein. The major proteins generally observed in the MgATP-activatable S6 kinase area of the gel filtration chromatography are Mr 72,000 and Mr 55,000. The Amido Black stain of the Western Blot showed a triplet of proteins with Mr 64,000-70,000 in place of the Mr 72,000 protein usually observed. These observations suggest that the Mr 65,000 protein is derived from the Mr 72,000 protein, possibly by proteolysis.

The immunoreactivity of the 05-Y11B monoclonal antibody by immunodot analysis was ten fold less than that observed with the 23-H10D antibody (Figure 17). However, the immunoreactivity of the 05-Y11B monoclonal antibody with the Mr 65,000 protein in this Western Blot analysis was the strongest reaction observed for any monoclonal antibody tested in Western Blot analysis. These observations suggest that the 05-Y11B monoclonal antibody preferentially reacts with a specific proteolytic product of the Mr 72,000 antigen.

Monoclonal Antibody Affinity Chromatography

To investigate the composition and activity of the native proteins which reacted with the monoclonal antibodies, affinity resins were constructed with selected monoclonal antibodies. Ascites fluid was prepared as described in Experimental Procedures and partially purified by DEAE Affigel Blue chromatography (Figure 21). The pooled IgG fractions (#25-29) constituted 39% of the ascites fluid protein.

Efficiency of the DEAE Affigel Blue chromatography was evaluated by 12.5% SDS-PAGE (Figure 22). Six major proteins with Mr 85,000, 68,000, 55,000, 27,000, 25,000 and 17,000 were observed to be present in the ascites fluid prior to DEAE Affigel Blue chromatography. Based on the known Mr for major serum proteins, the Mr 55,000 and 27,000 proteins were identified as the IgG heavy and light chains, respectively. The Mr 85,000 protein was identified as transferrin and the Mr 68,000 protein as albumin. The DEAE Affigel Blue chromatography efficiently removed all non-IgG proteins with the exception of transferrin. Transferrin exhibits a similar affinity for the resin as IgG. Ascites fluid from monoclonal cell lines 18-F9D and 23-H10D was purified and coupled to CNBr-activated Sepharose as described in Experimental Procedures.

23-H10D-Sepharose chromatography.--The 23-H10D monoclonal antibody monospecifically recognized the immunoreactive Mr 72,000 protein in Western Blot analysis of gel filtration chromatography fractions. The elution pattern for this protein corresponded to the MgATP-activatable S6 kinase. This protein was isolated by affinity chromatography in order to investigate its potential role in the activity or regulation of the S6 kinase.

Pooled, concentrated S6 kinase preparation (0.95 mg) was chromatographed on 23-H10D-Sepharose and fractions monitored spectrophotometrically for protein at 280 nm (Figure 23). Approximately 20% of the total protein was bound to the resin. The fractions were also assayed for S6 kinase or Activating Enzyme activity. The S6 kinase activity was observed exclusively in the unbound fractions. The bound protein fractions displayed Activating Enzyme activity. Addition of 1.9 µg of an exogenous S6 kinase preparation to the bound fractions resulted in a 3.6 fold increase in the observed S6 kinase activity.

The initial S6 kinase preparation and 23-H10D-Sepharose unbound and bound protein samples were analyzed by SDS-PAGE (Figure 24). The resin specifically bound the Mr 72,000 protein, which had been previously determined to carry the antigenic site for the 23-H10D monoclonal antibody. The Mr

65,000 protein, which was previously implicated as a proteolytic product of the Mr 72,000, was also observed as less than 10% of the total bound protein. The major proteins present in the initial S6 kinase preparation were the Mr 72,000 and 55,000 proteins. The unbound S6 kinase preparation was depleted in the Mr 72,000 protein. The protein was not completely removed, because the binding capacity of the 23-H10D-Sepharose was exceeded.

The role of this Mr 72,000 protein in the regulation of S6 kinase was investigated by examining the activity of the initial S6 kinase preparation and the S6 kinase preparation depleted in Mr 72,000 protein. The specific activity was determined for the S6 kinase preparation initially applied to the resin and the S6 kinase preparation recovered in the unbound portion of the chromatography. These two S6 kinase samples were also tested for their ability to be activated by MgATP alone or in conjuction with the bound protein fraction. Total S6 kinase activity was obtained by MgATP-activation after limited trypsin digestion. Results are shown in Table V.

The specific activity of both samples was comparable in the absence of MgATP-activation. Upon MgATP-activation, the enzyme depleted in the Mr 72,000 protein (unbound S6 kinase) displayed 42% less activity compared to the unchromatographed S6 kinase preparation. Addition of the

bound protein to the MgATP preincubation or limited trypsin treatment prior to the MgATP preincubation restored full activity to the unbound enzyme. The bound protein fraction was completely devoid of S6 kinase activity and no activity was observed after limited trypsin digestion. These data suggest that the unbound enzyme retained 100% of the S6 kinase activity, but was deficient in a component required for activation. This component was the Mr 72,000 protein.

18-F9D-Sepharose chromatography.--The 18-F9D monoclonal antibody monospecifically recognized the Mr 116,000 protein in Western Blot analysis of gel filtration chromatography fractions. The elution pattern for this protein corresponded to the Activating Enzyme activity. The potential role for this protein in the activity or regulation of the Activating Enzyme was investigated.

Pooled, concentrated Activating Enzyme (0.2 mg), which eluted from gel filtration chromatography at the void volume, was chromatographed on 18-F9D-Sepharose. The fractions were monitored spectrophotometrically for protein at 280 nm (Figure 25). Approximately 50% of the total protein was bound to the resin. The fractions were assayed for Activating Enzyme activity by addition of 2.8 µg of an exogenous S6 kinase preparation to the MgATP preincubation. The bound protein fractions increased the observed activity of the S6 kinase preparation two fold. The unbound protein

fractions displayed no S6 kinase activating activity. Neither the initial Activating Enzyme, 18-F9D-Sepharose unbound nor bound protein displayed S6 kinase activity. Trypsin treatment did not generate S6 kinase activity in these three samples.

Protein bound to the 18-F9D-Sepharose resin was analyzed by SDS-PAGE (Figure 26). Two proteins with Mr 110,000 and 72,000 were observed. The Mr 110,000 was observed previously with the 18-F9D monoclonal antibody in Western Blot analysis along with the Mr 116,000 protein, from which it is apparently derived. The 18-F9D monoclonal antibody did not detect the Mr 72,000 protein in Western Blot analysis of gel filtration chromatography fractions. Nine separate chromatographies on the 18-F9D-Sepharose consistently yielded these two bound proteins. The Mr of the larger protein was either 110,000 or 116,000. The smaller protein had an Mr of 72,000 each time. The apparent stoichiometry of this protein complex was 1:1 in all experiments. The monoclonal antibody 05-E11C was also coupled to CNBr-activated Sepharose and chromatography of Activating Enzyme yielded the same complex (data not shown). These data strongly support the conclusion that the Mr 116,000 and 72,000 proteins are distinct, dissimilar subunits which are associated in the native state.

The free Mr 72,000 protein isolated on

23-H10D-Sepharose is strikingly similar to the Mr 72,000 protein which is part of the complex isolated on 18-F9D-Sepharose. The Mr 72,000 protein associated with the Activating Enzyme and the Mr 72,000 protein associated with MgATP-activatable S6 kinase preparation are indistiguishable on 10% or 12.5% SDS-PAGE analysis of gel filtration fractions. Also, both the free and complexed Mr 72,000 protein display Activating Enzyme activity.

An additional piece of evidence for the hypothesis that there is a single Mr 72,000 protein is the pattern of endogenous proteolysis observed for this protein. It was concluded from work presented with the 05-Y11B monoclonal antibody that the Mr 65,000 protein recognized by this antibody was a proteolytic product of the free Mr 72,000 protein. In fact, Amido Black analysis of the nitrocellulose blot revealed a triplet of proteins with Mr 60,000-70,000 in place of the free Mr 72,000 protein. Similar endogenous proteolysis has been observed for the Mr 72,000 protein in the protein complex isolated on 18-F9D-Sepharose.

An Activating Enzyme preparation purified by the modified protocol was observed to contain an Mr 60,000 protein. This Activating Enzyme was purified on 18-F9D-Sepharose to determine the relationship of the Mr 60,000 protein to the Mr 72,000 subunit. The protein

complex isolated on 18-F9D-Sepharose contained the Mr 116,000 protein and the Mr 60,000 protein (Figure 27). The Mr 60,000 protein was present instead of the Mr 72,000 protein previously described. The two proteins displayed a 1:1 stoichiometry in SDS-PAGE analysis of the 18-F9D-Sepharose bound protein fraction. SDS-PAGE analysis of the Activating Enzyme prior to 18-F9D-Sepharose chromatography showed the Mr 116,000 and Mr 60,000 proteins as major components of the preparation.

The ability of the initial Activating Enzyme and the bound Mr 116,000-60,000 protein complex to activate S6 kinase was tested. Results are shown in Table VI. The initial Activating Enzyme enhanced the activation of the S6 kinase in a concentration dependent manner. A six fold activation of the S6 kinase was observed with 1.3 µg of the initial Activating Enzyme. The bound Mr 116,000-60,000 protein complex also enhanced the activation rate of the S6 kinase. A 3.6 fold activation of the S6 kinase was observed with 0.7 µg bound protein. It is concluded that the bound protein is the Activating Enzyme previously observed to be bound to the 18-F9D-Sepharose. The Mr 60,000 protein is apparently a proteolytic product of the complexed Mr 72,000 protein. The similar pattern of endogenous proteolysis observed for both the free and complexed Mr 72,000 protein is additional evidence that these proteins are the same.

To investigate the occurrence and native form of the Activating Enzyme on gel filtration chromatography, individual fractions were subjected to 18-F9D-Sepharose chromatography. Fractions selected from the gel filtration chromatography shown in Figure 7 were as follows: A) Activating Enzyme, fraction 49 (eluted at void volume); B) Activating Enzyme, fraction 57 (eluted with Mr 200,000); C) trypsin-activatable S6 kinase, fractions 62-63; D) MgATP-activatable S6 kinase, fractions 68-69. The protein which bound to 18-F9D-Sepharose from each sample was analyzed by 12.5% SDS-PAGE as shown in Figure 28. In all cases, the Mr 116,000-72,000 protein complex was bound, though the quantity of the complex obtained varied. Quantitatively, the greatest recovery of the complex (50 µg) was obtained from fraction 57, which eluted from gel filtration chromatography with Mr 200,000 and contained the highest peak of Activating Enzyme activity. The total recovery of the complex from each sample was as follows: A) 2 µg; B) 50 µg; C) 20 µg; D) 2 µg. The occurrence of the complex was qualitatively and quantitatively comparable with the distribution of the Mr 116,000 protein as analyzed by immunodot and SDS-PAGE of gel filtration fractions.

Activating Enzyme activity was observed with the bound protein complex. Addition of the Mr 116,000-72,000 protein complex isolated from sample B to S6 kinase resulted in a

three fold increase in the activation rate (Table VII). The same complex isolated from sample A, C or D did not activate the S6 kinase, probably due to the low concentration of the complex. The Mr 116,000-72,000 protein complex was devoid of histone phosphotransferase activity with mixed histone and preincubation with MgATP or limited trypsin digestion did not generate activity.

Phosphorylation by Insulin Receptor Kinase

The possible utilization of Activating Enzyme as a substrate for the insulin receptor kinase was investigated. Activating Enzyme was isolated from gel filtration fraction number 49 (eluted at void volume) or 57 (eluted with Mr 200,000; Figure 7) by chromatography on 18-F9D-Sepharose. The antibody-purified Activating Enzyme was added as the protein substrate to insulin receptor kinase as described in Experimental Procedures. Results are shown in Figure 29.

The phosphorylation of the Mr 116,000 protein was practically undetectable in the absence of the insulin receptor kinase. Addition of the insulin receptor kinase resulted in the incorporation of up to 0.26 pmol phosphate into the Mr 116,000 protein (background of insulin receptor autophosphorylation subtracted from total). Comparable results were obtained with both antibody-purified Activating Enzyme samples. The Mr 116,000 protein was the only Activating Enzyme protein observed to be phosphorylated in

the presence of insulin receptor. Receptor autophosphorylation was observed on the Mr 95,000 β-subunit.

The stoichiometry of the Mr 116,000 protein phosphorylation catalyzed by insulin receptor kinase was estimated. The concentration of the Mr 116,000-72,000 protein complex recovered from 18-F9D-Sepharose chromatography was estimated spectrophotometrically at 280 nm. The total moles of Activating Enzyme was calculated using an Mr of 200,000. Quantitation of the phosphate incorporated into the Mr 116,000 protein was determined by liquid scintillation counting of the protein sliced from the dried gel. Based on these assumptions, the stoichiometry of phosphorylation was estimated to be 10 and 4% for the Activating Enzyme isolated from gel filtration fraction number 49 and 57, respectively. Similar results were obtained with concentrated Activating Enzyme which was not antibody purified (R. A. Masaracchia, unpublished results).

Characterization of Monoclonal Antibodies

Two techniques were employed to gain additional information on the specificity of monoclonal antibodies from different families. The potential recognition of each antibody family for either the Mr 116,000 or Mr 72,000 antigen was obtained by immunodot analysis of gel fitration chromatography fractions as shown in Table IV. In addition,

a member of each family was tested for its ability to react with antigen in Western Blot analysis and to precipitate autophosphorylated antigen. These two techniques are useful for isolation and identification of specific antigen from heterogeneous mixtures, such as cell extracts. Monoclonal antibodies which would be appropriate for such studies were identified. The radioimmunoprecipitation assays also allowed observation of complex formation between Activating Enzyme and S6 kinase.

Western Blot analysis.--The S6 kinase preparation used for immunization was subjected to Western Blot analysis with monoclonal antibody from cell lines representing 38 different families (Table VIII). Mouse immune serum was used as a positive control. Four monoclonal antibodies (23-H10D, 05-U11C, 05-W10A and 23-P11C) detected a single antigen which migrated as a broad band with Mr 63,000-68,000. The 05-Y11B monoclonal antibody detected a slightly smaller antigen with Mr 60,000-63,000 and numerous smaller antigens suggesting reactivity with proteolytic fragments. Thirteen monoclonal antibodies displayed nonspecific, minor reactivity with all available antigens. This result suggested that although a high concentration of antibody was present, an appropriate antigen was not available. Twenty monoclonal antibodies tested negative in this procedure. These values are consistent with the

observation of others that monoclonal antibodies often fail to react in Western Blot analysis, probably due to denaturation of the epitope.

The antigen for the 23-H10D antibody has been previously demonstrated to reside on an Mr 72,000 protein. The Mr 72,000 protein is also the predicted antigen for the 05-U11C monoclonal antibody based on immunodot analysis with gel filtration chromatography fractions (Table IV). In order to confirm these antigen assignments, all monoclonal antibodies which gave a positive reaction with the immunizing S6 kinase preparation were tested with S6 kinase prepared by the modified protocol. This also allowed the relationship between antigens in the two S6 kinase preparations to be determined.

Monoclonal antibodies which recognized a specific antigen by Western Blot analysis with S6 kinase prepared by the modified protocol are shown in Figure 30. The 23-H10D and 05-U11C monoclonal antibodies detected a single antigen with Mr 72,000, as predicted. These results confirmed the antigenic relationship between the Mr 72,000 protein and the Mr 63,000-68,000 protein in the two S6 kinase preparations. The Mr 63,000-68,000 was presumably derived from the Mr 72,000 protein by proteolysis. The 05-Y11B monoclonal antibody displayed major reactivity with an Mr 64,000-66,000 and Mr 48,000 antigen. This result confirmed the preference

of the 05-Y11B antibody for proteolytic products of the Mr 72,000 protein as previously described in Figure 19. The 20-H10B monoclonal antibody detected an Mr 56,000-59,000 antigen which may be the third antigen detected by mouse immune serum in Western Blot analysis of gel filtration chromatography fractions (Figure 13). This possibility was consistent with the fraction range of ELISA reactivity for this antibody observed in immunodot analysis of gel filtration chromatography fractions (Table IV).

The results of all monoclonal antibodies tested in Western Blot analysis of S6 kinase prepared by the modified protocol are summarized in Table IX. The results were qualitatively similar to those obtained in Table VIII with the exception of of the 23-P11C monoclonal antibody. The 23-P11C monoclonal antibody detected an antigen with Mr 63,000-68,000 in the Western Blot analysis of the S6 kinase preparation used for immunization (Table VIII). The cause for the shift in the antigen to an Mr 40,000 protein with S6 kinase prepared by the modified protocol is not clear. Additional information has not been obtained for this monoclonal antibody as it failed to react specifically in immunodot analysis of gel filtration chromatography fractions (Table IV).

Radioimmunoprecipitation Assays.--The proteins associated with Activating Enzyme have been identified as Mr

116,000 and Mr 72,000. The proteins associated with the S6 kinase have been identified as Mr 95,000 and Mr 55,000. The association of these proteins as complexes in solution was investigated. The technique employed was radioimmunoprecipitation of antigenic proteins with monoclonal antibody and Protein A crosslinked in Staphylococcus aureus cells. Immunoprecipitated proteins were subjected to SDS-PAGE analysis and radioactively labelled antigens located by autoradiography. This technique also allowed the identification of monoclonal antibodies which could be bound to Protein A.

The S6 kinase preparation or a combination of the S6 kinase preparation and Activating Enzyme was radioactively labelled in an autophosphorylation reaction. This method of labelling the antigen has the advantage of specifically labelling all of the proteins of interest with the exception of the Mr 72,000 protein. Some nonphosphorylated antigens, including the Mr 72,000 protein, were obscured on stained gels by the high concentration of Staphylococcus aureus proteins, which migrated with Mr 10,000-80,000. However, the presence of the Mr 95,000 protein could be observed on the Coomassie Blue stained gel.

Monoclonal antibodies from 31 cell lines representing 20 different families were tested for their ability to immunoprecipitate phosphorylated antigens in the S6 kinase

preparation or a mixture of the S6 kinase and Activating Enzyme preparations. Results are summarized in Table X. Three monoclonal antibodies (20-A7D, 20-K8B and 23-I9D) precipitated the Mr 95,000, 55,000 and 38,000 proteins as a complex. These data support the hypothesis that the Mr 55,000 and 95,000 proteins are subunits of the S6 kinase. The role of the Mr 38,000 protein has not been elucidated. The quantity of this protein is very small in the S6 kinase preparation.

Addition of Activating Enzyme to the S6 kinase preparation resulted in the inclusion of the Mr 116,000 protein in the complex precipitated by the 20-A7D and 20-K8B monoclonal antibodies. It is possible that the Mr 72,000 protein was also included in this complex; however, it is not a phosphoprotein and was obscured on the Coomassie Blue stained gel by the Staphylococcus aureus proteins. The 23-I9D monoclonal antibody failed to precipitate antigen upon addition of Activating Enzyme.

The precipitation pattern observed with these three antibodies is evidence for the interaction of S6 kinase and Activating Enzyme in solution. The antigenic site for the 23-I9D monoclonal antibody was apparently masked by the addition of Activating Enzyme to the complex. The 20-H10B monoclonal antibody precipitated the complex only in the presence of the Activating Enzyme, suggesting that its

antigenic site resided on an Activating Enzyme protein. Eight monoclonal antibodies precipitated the Mr 116,000 protein from the S6 kinase\Activating Enzyme mix and 14 monoclonal antibodies tested negative by this procedure. A total of 13 monoclonal antibodies representing 10 families which bound to Protein A and reacted specifically with a component of the enzyme system were identified.

Occurrence of Activating Enzyme During Purification

Production of monoclonal antibodies against the Activating Enzyme provided the tool by which the chromatographic behavior of this enzyme could be determined. The activity assay for Activating Enzyme is based on the increased expression of activity of the exogenous S6 kinase preparation. The presence of protein kinases in the Activating Enzyme sample interfered with determination of Activating Enzyme activity in two ways. First, the increased activity of exogenous S6 kinase by activation with Activating Enzyme may be small compared to the endogenous protein kinase activity background. Second, an unidentified protein kinase in the Activating Enzyme sample may also modulate the exogenous S6 kinase activity. The activity assay was used to obtain the chromatographic behavior of the Activating Enzyme on gel filtration, in which a portion of the enzyme is completely separated from the S6 kinase. However, the presence of multiple protein kinase activities

has precluded determination of the behavior of the Activating Enzyme in other chromatographic steps of the purification with the activity assay. The monospecificity of the 18-F9D monoclonal antibody allowed elution profiles for the Activating Enzyme to be obtained in the presence of other protein kinases.

The proteins eluted in phosphocellulose chromatography fractions were subjected to immunodot analysis with the 18-F9D monoclonal antibody. The elution profile for the Activating Enzyme is shown in Figure 31. The 18-F9D monoclonal antibody detected three peaks of antigen. The first contained approximately half of the immunoreactive protein and did not bind to the phosphocellulose resin. The remainder of the immunoreactive protein eluted from the resin in two peaks at approximateley 120 mM and 270 mM KCl. The second bound peak overlapped the S6 kinase activity and was pooled with the S6 kinase preparation for further purification.

The S6 kinase activity pooled from phosphocellulose chromatography was concentrated by batch elution from CM-Sephadex. Proteins from the CM-Sephadex chromatography fractions were subjected to immunodot analysis with the 18-F9D monoclonal antibody. Approximately equal amounts of immunoreactive protein were observed in the bound and unbound CM-Sephadex fractions (data not shown). The bound

protein was further purified on gel filtration chromatography. The Activating Enzyme from gel filtration chromatography has been determined to consist of an Mr 116,000-72,000 protein complex.

The structure of the immunoreactive protein which failed to bind to the CM-Sephadex resin was investigated. The CM-Sephadex unbound fractions were pooled and portions subjected to antibody affinity chromatography on 18-F9D-, 23-H10D- and 05-E11C-Sepharose, respectively. The 18-F9D and 05-E11C monoclonal antibodies specifically recognize the Mr 116,000 protein. Chromatography of Activating Enzyme on 18-F9D- or 05-E11C-Sepharose has yielded the Mr 116,000-72,000 Activating Enzyme complex. The 23-H10D monoclonal antibody specifically recognizes the Mr 72,000 protein. Chromatography of the S6 kinase preparation on the 23-H10D-Sepharose has yielded the Mr 72,000 protein with Activating Enzyme activity.

Chromatography of CM-Sephadex unbound proteins on 18-F9D- or 05-E11C-Sepharose yielded protein complexes comparable to those obtained previously (Figure 32). The 18-F9D-Sepharose purified complex was Mr 116,000, 110,000 and 65,000. The 05-E11C-Sepharose bound protein was Mr 116,000, 110,000, 72,000 and 65,000. Previous results have suggested that the Mr 116,000 and Mr 72,000 proteins are antigenicly related to the Mr 110,000 and Mr 65,000

proteins, respectively, possibly by proteolysis. Chromatography of CM-Sephadex unbound proteins on 23-H10D-Sepharose yielded a single protein with Mr 72,000. This is consistent with previous results. These results suggest that the elution of the Activating Enzyme as various forms is not due to the presence or absence of various subunits.

TABLE I

PROTEINS ASSOCIATED WITH S6 KINASE
AND ACTIVATING ENZYME

A summary of the Mr of Activating Enzyme and S6 kinase as determined by Sephacryl S200 gel filtration chromatography and SDS-PAGE analysis is shown. SDS-PAGE determined Mr of proteins proposed to be derived from the subunits of Activating Enzyme by endogenous proteolysis are also shown.

	Mr		
Enzyme	SDS-PAGE	Gel Filtration	Proteolysis
Activating Enzyme holoenzyme	116,000 72,000	≧ 350,000 and 200,000	- 60,000
Activating Enzyme regulatory subunit	116,000	-	110,000 83,000 80,000
Activating Enzyme catalytic subunit	72,000	90,000	70,000 66,000 63,000 60,000
S6 kinase holoenzyme	95,000 55,000	158,000	- -
S6 kinase catalytic subunit	55,000	82,000	-

TABLE II

PURIFICATION OF S6 KINASE FROM HUMAN PLACENTA

S6 kinase was prepared from 334 g of human placenta as described in Experimental Procedures. A sample of the enzyme preparation after each purification step was assayed for protein kinase activity with a 10 minute preincubation in the presence of 100 μM [γ-^{32}P]ATP (50 dpm/pmol) and 12 mM $MgCl_2$. Phosphotransferase assays were initiated with 1 mg/ml histone H4 for 1, 2, 4, 6, 8, 12, 16 and 20 minutes. Activity was calculated from the slope of pmol P_i transferred vs time.

Purification Step	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min-mg)	Percent Yield	Fold Purification	Fold MgATP Activation
DEAE Cellulose	2,157	73.4	0.03	100	1	0.9
$(NH_4)_2SO_4$ Precipitation	1,081	133.0	0.12	181	3.6	1.1
Phosphocellulose	304	313.2	1.03	427	30.3	2.2
CM-Sephadex	85	330.4	3.88	450	114.2	6.8
Sephacryl S200	53	135.9	2.55	185	75	4.7
DEAE Cellulose	10.4	69.6	6.7	95	197	4.2

TABLE III

PHOSPHOPROTEINS ASSOCIATED WITH S6 KINASE AND ACTIVATING ENZYME

Trypsin-activatable S6 kinase (10.5 µg) from gel filtration chromatography (fraction 61) was incubated with varying amounts of Activating Enzyme which eluted from gel filtration chromatography at the void volume (fraction 51) for 30 minutes at 30° C with 6 mM $MgCl_2$ and 103 µM [γ-^{32}P]ATP (439 dpm/pmol) in a volume of 90 µl. The phosphotransferase reaction was intiated by addition of 10 µl histone H4 (50 µg) and allowed to proceed for 10 minutes. An aliquot (20 µl) of the assay mix was used for quantitation of protein kinase activity as described in Experimental Procedures. The remainder of the assay mix was subjected to 12.5% SDS-PAGE and phosphoproteins quantitated as described in Experimental Procedures.

Activating Enzyme	^{32}P-Protein pmol/band (% Control)				S6 Kinase Activity % Control
	116K	95K	62K	55K	
0.0 µg	25 (100)	50 (100)	95 (100)	68 (100)	100
2.4 µg	34 (136)	49 (102)	115 (121)	65 (96)	-
4.8 µg	37 (148)	41 (82)	110 (116)	70 (103)	188
7.2 µg	54 (216)	45 (90)	100 (105)	73 (107)	242
9.6 µg	70 (300)	58 (116)	105 (110)	92 (135)	354
12.0 µg	60 (240)	66 (132)	120 (126)	105 (154)	471

TABLE IV

SCREEN OF MONOCLONAL ANTIBODIES BY IMMUNODOT ANALYSIS WITH GEL FILTRATION FRACTIONS

Aliquots (100 µl) of alternate gel filtration fractions 50-78 (Figure 6) were used as antigen in the immunodot assay with various hybridoma cell line conditioned mediums as the primary antibody as described in Experimental Procedures. Incubation times were 17 hours for the primary antibody and 1 hour each for the secondary and tertiary antibodies (both diluted 1:4000). ELISA reactivity was quantitated by laser scanning densitometry.

Antibody	Positive ELISA Reaction	
	Fraction Range	Peak Fraction
05-AA10A	50-68	58
05-C10H	50-68	58
20-G9E	50-64	58
05-W10A	50-76	56
05-J12C	50-64	56
20-V8E	50-64	56
05-A10F	50-62	58
20-H10B	50-66	56
05-K12E	50-66	56
05-I11C	50-68	58
05-E11C	50-68	56
05-W10A	50-76	56
05-U11C	66-70	68
21 Clones	Negative	

TABLE V

EFFECT OF THE 23-H10D MONOCLONAL ANTIBODY ANTIGEN ON THE MgATP-DEPENDENT ACTIVATION OF S6 KINASE

S6 kinase (0.95 mg) was chromatographed on 23-H10D-Sepharose as described in Figure 23. The S6 kinase activity of the initial enzyme preparation and the enzyme which did not bind to the resin was determined in the presence and absence of MgATP activation, trypsin activation and reconstitution with bound protein. Preincubation reactions were for 10 minutes with 92 μM [γ-^{32}P]ATP (69 dpm/pmol) and phosphotransferase assays were conducted with 2 mg/ml mixed histone. The bound protein (1.1 μg per assay) was added to the preincubation reaction as indicated. Trypsin activation was performed as described in Experimental Procedures. Enzyme protein in each assay was as follows: 6.6 μg unbound S6 kinase; 9.5 μg initial S6 kinase in reactions A and B; 1.9 μg initial S6 kinase in reactions C - E.

Reaction	Enzyme	Protein Kinase Activity (pmol/min-mg)	
		Initial	Unbound
A	S6 kinase	1167	1250
B	S6 kinase + MgATP	4103	2396
C	S6 kinase + MgATP	847	-
D	S6 kinase + MgATP +Bound protein	3036	4509
E	S6 kinase + Trypsin + MgATP	4280	4700

TABLE VI

ACTIVITY OF ACTIVATING ENZYME
WITH Mr 116,000-60,000

Activating Enzyme (1.4 mg) was chromatographed on 18-F9D-Sepharose as described in Figure 27. The activation of exogenous S6 kinase (2 µg per assay) by the initial Activating Enzyme preparation and bound protein fraction was determined in a MgATP-activation assay. Preincubation reactions were for 20 minutes with 90 µM [γ-^{32}P]ATP (20 dpm/pmol) and phosphotransferase assays were conducted for 10 minutes with 2 mg/ml mixed histone.

Enzyme Preincubation	Protein Kinase Activity (pmol/min-ml)
S6 kinase	76
Activating Enzyme (6.7 µg)	233
S6 kinase + Activating Enzyme (6.7 µg)	1568
S6 kinase + Activating Enzyme (1.3 µg)	473
Bound protein (0.7 µg)	0.5
S6 kinase + Bound protein (0.7 µg)	278

TABLE VII

ACTIVATION OF S6 KINASE BY 18-F9D-SEPHAROSE PURIFIED ACTIVATING ENZYME FROM GEL FILTRATION FRACTIONS

Activating Enzyme was obtained from chromatography of individual gel filtration fractions on 18-F9D-Sepharose as described in Figure 28 (A: fraction 49; B: fraction 57; C: fraction 62-63; D: fraction 68-69). The Activating Enzyme bound to the resin was assayed for activation of S6 kinase as described in Experimental Procedures with a 10 minute preincubation in the presence of 100 μM [γ-^{32}P]ATP (70 dpm/pmol). Phosphotransferase assays were initiated by the addition of 2 mg/ml mixed histone. Trypsin activation was performed as described in Experimental Procedures. Preincubation reactions contained 2 μg S6 kinase when indicated and Activating Enzyme when indicated as follows: A, 0.03 μg; B, 1.5 μg; C, 0.3 μg and D, 0.03 μg.

Enzyme Preincubation	Protein Kinase Activity (nmol/min-ml)			
	A	B	C	D
S6 kinase	1.8	2.6	2.3	3.5
Trypsin-activated S6 kinase	11.7	11.7	15.7	5.7
S6 kinase + Activating Enzyme	2.7	7.6	2.7	2.8
Activating Enzyme	<0.1	<0.1	<0.1	0.1
Trypsin-activated Activating Enzyme	<0.1	<0.1	0.4	0.1

TABLE VIII

SCREEN OF MONOCLONAL ANTIBODIES BY WESTERN BLOT ANALYSIS

S6 kinase used for mouse immunization was subjected to 12.5% SDS-PAGE, transferred electrophoretically to nitrocellulose in ethanolamine-glycine-methanol buffer and strips (approximately 25 µg protein per strip) incubated with various hybridoma cell line conditioned mediums as the primary antibody. Incubation times were 4 hours for the primary antibody, 12 hours for secondary antibody (diluted 1:4000) at 4° C and 2 hours for tertiary antibody (diluted 1:4000) at room temperature. ELISA reactive antigens were visualized as described in Experimental Procedures. Immune serum was a 1:1 mix of serum from mouse #1 and #2.

Antibody	Mr of Major ELISA reactive antigens
Immune Serum 1/300	83K, 78K, 76K, 68K, 62K
23-H10D	63-68K
05-U11C	63-68K
05-W10A	63K
05-Y11B	60-63K and multiple smaller antigens
23-P11C	63-68K
13 Clones	nonspecific reaction
20 Clones	Negative

TABLE IX

RESCREEN OF MONOCLONAL ANTIBODIES
BY WESTERN BLOT ANALYSIS

Antibodies which showed any immunoreactivity in Table VIII were the primary antibody in a second Western Blot analysis. The procedure was the same as described in Table VIII except S6 kinase prepared by the modified purification protocol was used as antigen.

Antibody	Mr of Major ELISA reactive antigens
Immune Serum 1/200	100K, 72K, 65K
23-H10D	72K
05-U11C	72K
05-W10A	NT
05-Y11B	64-66K, 48K, multiple smaller antigens
23-P11C	40K
20-H10B	59-62K
8 Clones	nonspecific reaction
3 Clones	Negative

NT: Not Tested

TABLE X

IMMUNOPRECIPITATION COMPLEXES

Phosphorylated S6 kinase (^{32}P-S6k, 1.7 µg, 5.6 x 10^3 dpm/µg) or S6 kinase and Activating Enzyme phosphorylated together (^{32}P-S6k-AE, 5 µg, 1.8 x 10^3 dpm/µg) were incubated with various hybridoma cell line conditioned mediums and antigen-antibody complexes precipitated with Protein A crude cell suspension as described in Experimental Procedures. Antigens were separated by 12.5% SDS-PAGE and visualized by Coomassie Blue and autoradiography on BB5 film with a Quanta II intensifying screen.

Antibody	Mr of Precipitated Proteins	
	^{32}P-S6k	^{32}P-S6k-AE
20-A7D	95K, 55K, 38K	116K, 95K, 55K, 38K
20-K8B	95K, 55K, 38K	116K, 95K, 55K, 38K
20-H10B	Negative	116K, 95K, 55K, 38K
23-H10D	95K	116K, 95K, 55K, 38K
23-I9D	95K, 55K, 38K	Negative
23-BB19E	NT	116K
23-F10C	NT	116K
23-AA18H	NT	116K
23-X8C	NT	116K
23-I8G	NT	116K
23-L9F	NT	116K
23-R11F	NT	116K
05-U11C	NT	116K
2 Clones	Negative	Negative
4 Clones	Negative	NT
12 Clones	NT	Negative

NT: Not Tested

Figure 1. Schematic representation of the roles of Activating Enzyme and S6 kinase in insulin-stimulated 40S ribosomal protein S6 phosphorylation. The mechanism by which insulin stimulates 40S ribosomal protein S6 phosphorylation in cells is postulated to be mediated by a phosphorylation cascade consisting of three protein kinases. The insulin receptor contains an intracellular protein kinase domain, which is activated by insulin binding to the extracellular domain of the receptor. The activated insulin receptor kinase catalyzes the phosphorylation of the Mr 116,000 regulatory subunit of Activating Enzyme on a tyrosine residue. This results in the release of the Mr 72,000 catalytic subunit of the Activating Enzyme. The Activating Enzyme catalyzes the phosphorylation of the S6 kinase on a serine residue, resulting in the activation of the Mr 55,000 catalytic subunit of the S6 kinase. The activated S6 kinase catalyzes the phosphorylation of 40S ribosomal subunit on up to five serine residues of the S6 protein. The functional result of 40S ribosomal S6 phosphorylation is the formation of polysomes and an increased rate of protein synthesis.

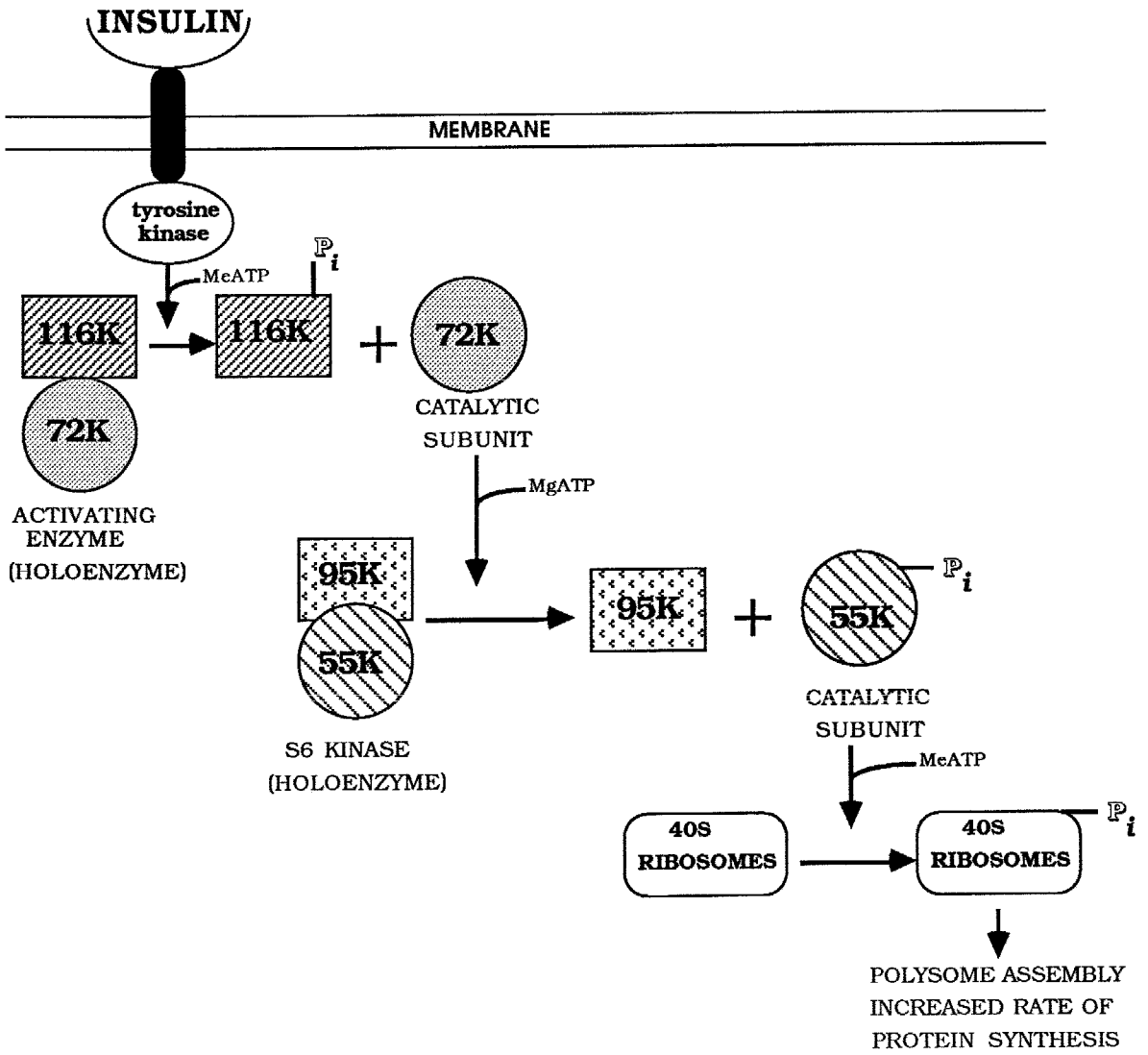

INSULIN
MEMBRANE
tyrosine kinase
MeATP
P_i
116K
116K
72K
72K
CATALYTIC SUBUNIT
ACTIVATING ENZYME (HOLOENZYME)
MgATP
95K
55K
95K
55K
P_i
S6 KINASE (HOLOENZYME)
CATALYTIC SUBUNIT
MeATP
40S RIBOSOMES
40S RIBOSOMES
P_i
POLYSOME ASSEMBLY
INCREASED RATE OF
PROTEIN SYNTHESIS

Figure 2. SDS-PAGE analysis of S6 kinase from human placenta. Lane B contains 40 µg of S6 kinase prepared from 334 g placenta as described in Table II. This enzyme preparation was used for immunization and antibody screening. Lane C contains 30 µg of S6 kinase prepared from 268 g placenta by the modification of the purification procedure described in Experimental Procedures. Lanes A and D contain low and high molecular weight standard proteins (BioRad), respectively. The resolving gel was 12.5% acrylamide and proteins stained with Coomassie Blue.

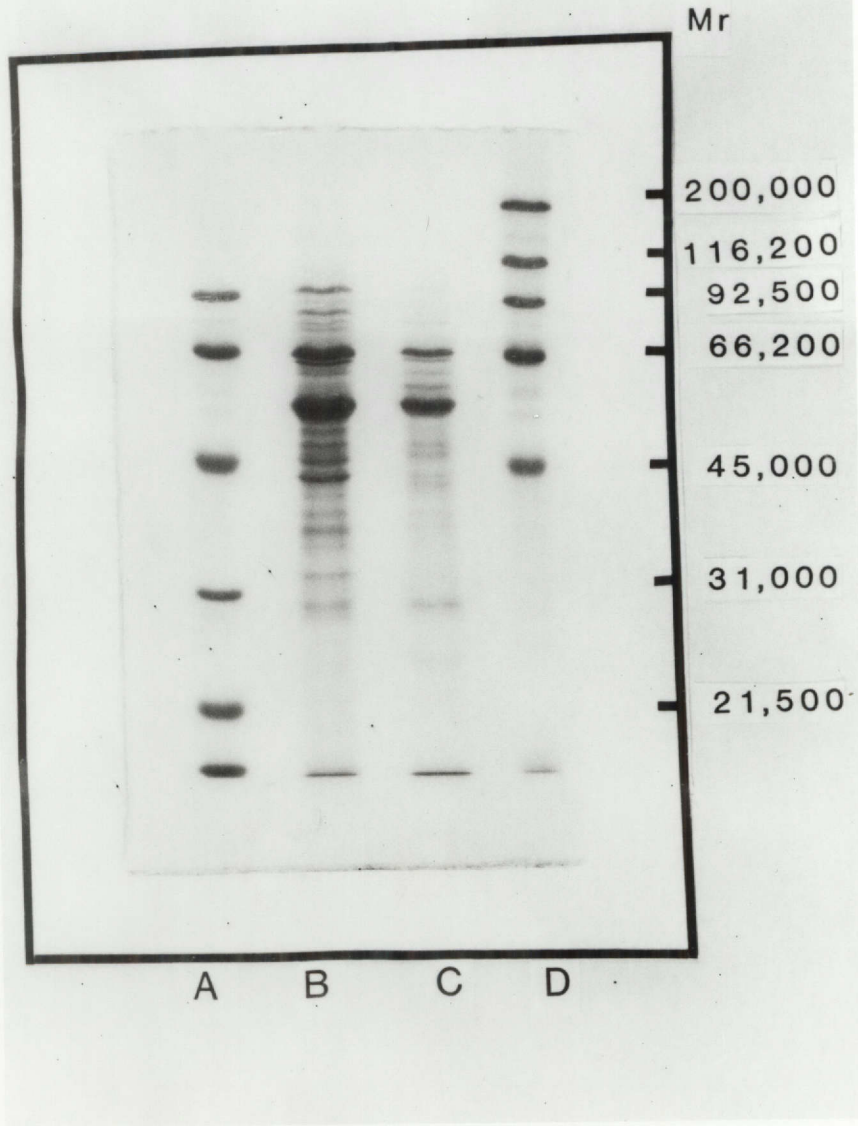

Mr
200,000
116,200
92,500
66,200
45,000
31,000
21,500
A
B
C
D

Figure 3. Effect of MgATP activation on elution of S6 kinase from Sephacryl S200 gel filtration chromatography. The S6 kinase was prepared as described in Experimental Procedures, without the modifications, through CM-Sephadex chromatography. The sample was then incubated for 30 minutes at room temperature with 0.2 mM ATP and 10 mM $MgCl_2$ and chromatographed on Sephacryl S200 (-O-). Individual fractions (3 ml) were assayed (without activation) for protein kinase activity as described in Experimental Procedures with 2 mg/ml mixed histone as substrate. The sample chromatographed without prior MgATP activation (-●-) was assayed (without activation) for 20 minutes with mixed histone as substrate. Elution of BioRad molecular weight standard proteins is noted.

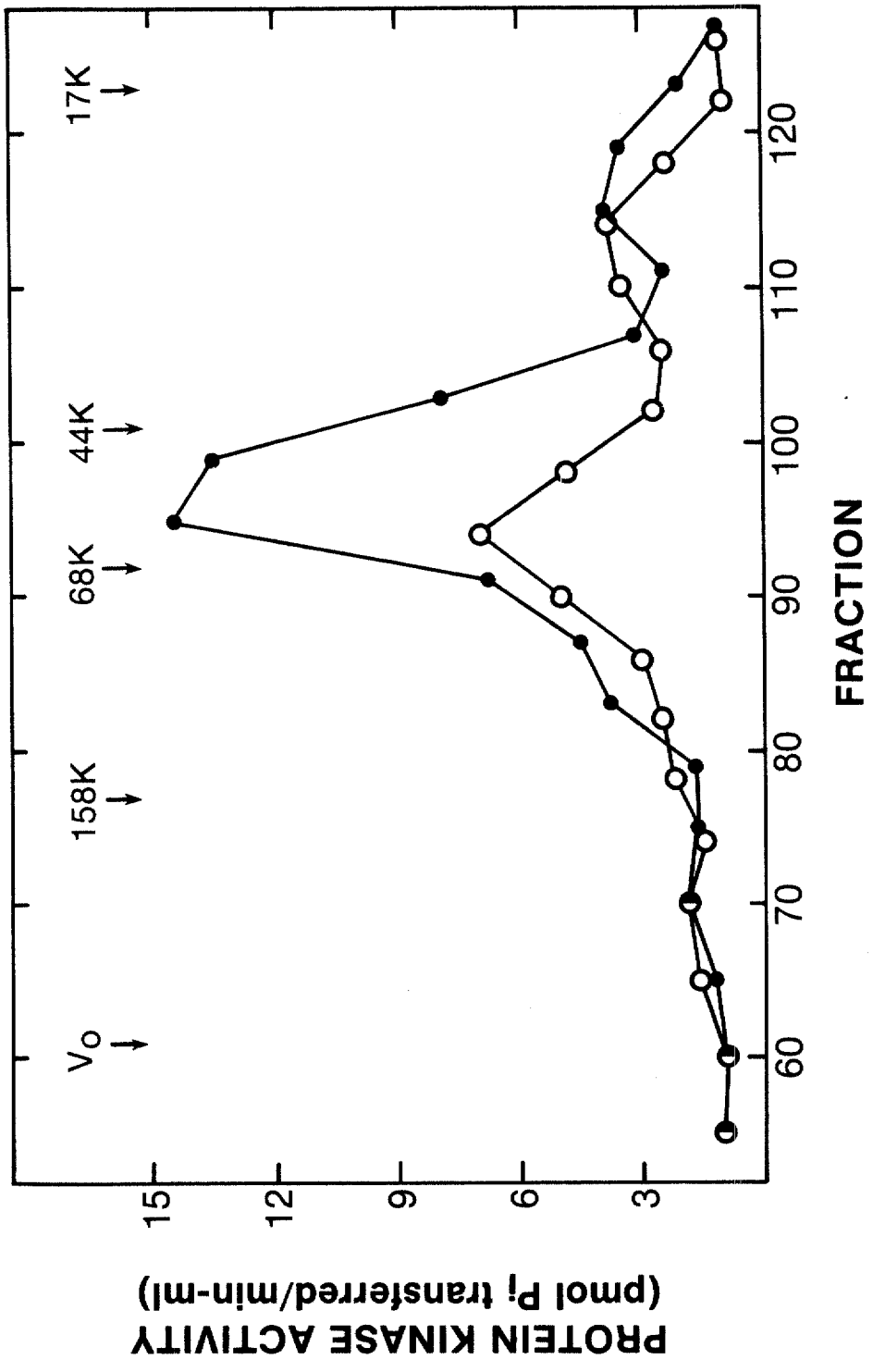

PROTEIN KINASE ACTIVITY
(pmol Pi transferred/min-ml)
15
12
9
6
3
Vo
158K
68K
44K
17K
60
70
80
90
100
110
120
FRACTION

Figure 4. Substrate specificity of human placenta S6 kinase observed on Sephacryl S200 chromatography. Enzyme from 268 g of placenta was prepared by the modified purification protocol described in Experimental Procedures. The Sephacryl S200 fractions (3.8 ml) were assayed for protein kinase activity with 2 mg/ml mixed histone (-●-) or 7.1 µg/reaction 40S ribosomal subunits (0.8 absorbance units at 260 nm; -O-). Samples were preincubated for 10 minutes with 120 µM [γ-^{32}P]ATP and 12 mM $MgCl_2$. Histone phosphorylation was quantitated by the method of Reimann et al. (76). Ribosomal proteins were separated by 12.5% SDS-PAGE and S6 phosphorylation quantitated by laser scanning densitometry of the autoradiograph.

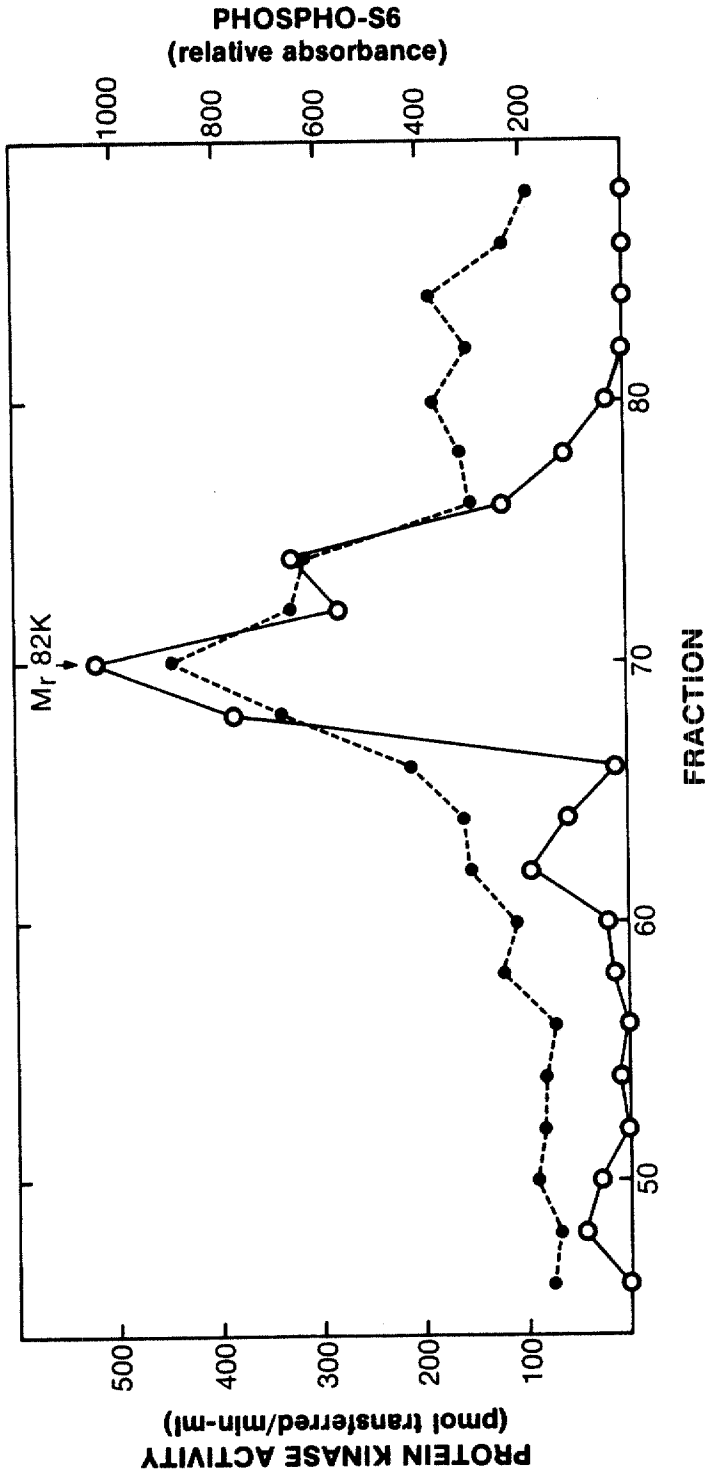

PHOSPHO-S6
(relative absorbance)
1000
800
600
400
200
M_r 82K
50
60
70
80
FRACTION
500
400
300
200
100
PROTEIN KINASE ACTIVITY
(pmol transferred/min-ml)

Figure 5. Competition of S6 kinase-catalyzed S6 phosphorylation by histone H4. The S6 kinase was trypsin-activated as described in Experimental Procedures. The activated S6 kinase (5 µg) was preincubated with 12 mM $MgCl_2$, 0.33 mM [γ-^{32}P]ATP (300 dpm/pmol) and 2.0 mM β-glycerophosphate for 10 minutes as described in Experimental Procedures. The phosphotransferase reaction was initiated by the addition of a nonsaturating concentration of 40S ribosomal subunits (7.1 µg/reaction, 0.8 absorbance units at 260 nm) in the presence or absence of histone H4 ranging from 0.1 to 10 x K_m (0.02-2.0 mg/ml). Reactions were stopped by the addition of SDS-PAGE sample buffer. Proteins were separated by 15% SDS-PAGE and S6 phosphorylation quantitated by liquid scintillation counting.

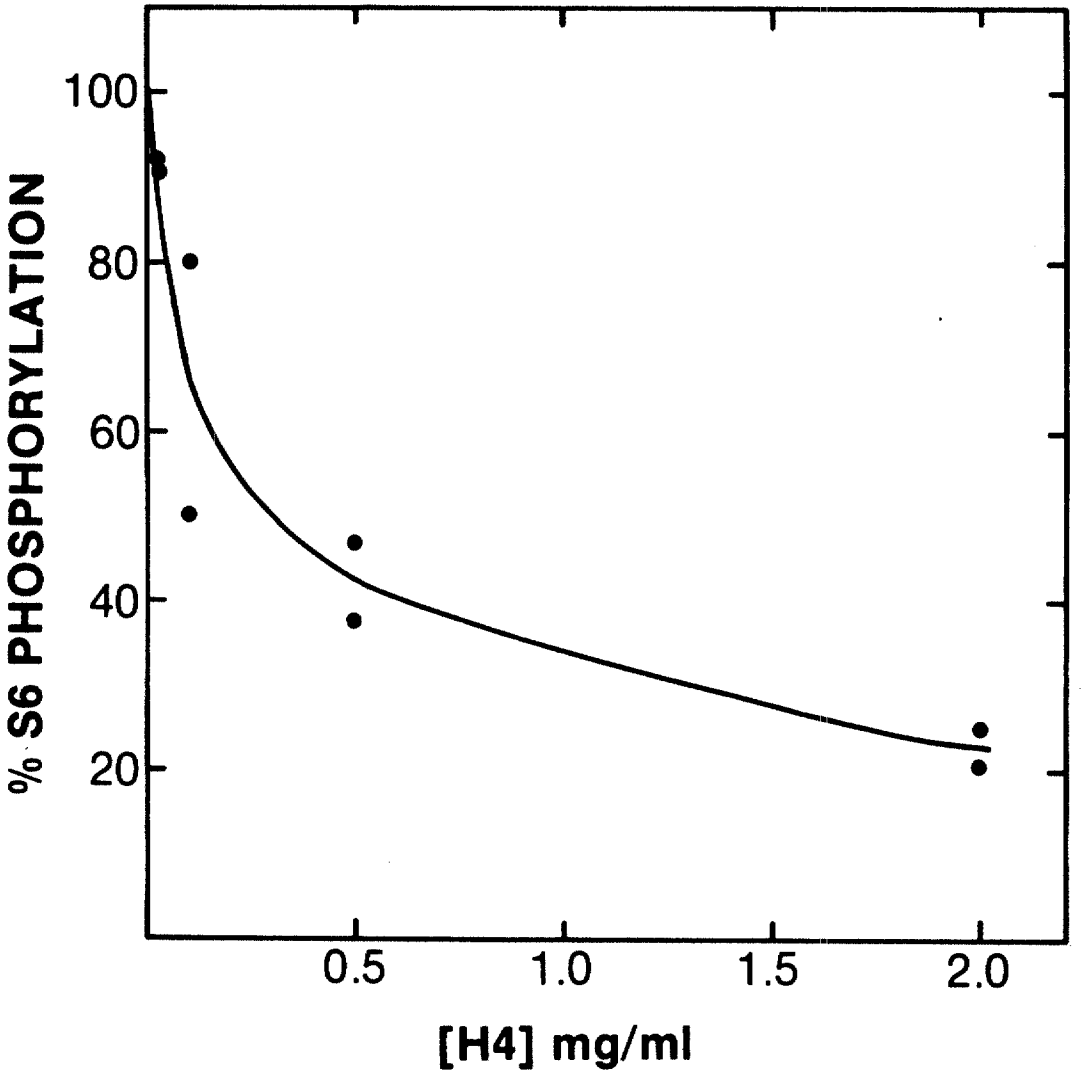

% S6 PHOSPHORYLATION
100
80
60
40
20
0.5
1.0
1.5
2.0
[H4] mg/ml

Figure 6. Phosphoproteins in the S6 kinase and Activating Enzyme preparations analyzed by SDS-PAGE. The enzymes were prepared by the modified protocol described in Experimental Procedures. S6 kinase (A; 28 µg), Activating Enzyme (C; 13.4 µg) or both enzymes together (B) were incubated in a total volume of 100 µl with 10 mM $MgCl_2$ and 100 µM [γ-^{32}P]ATP (150 dpm/pmol) for 10 minutes at 30° C. The reaction was terminated by the addition of SDS-PAGE buffer and proteins separated on 12.5% SDS-PAGE. The gel was stained with Coomassie Blue, dried and subjected to autoradiography for 16 hours at -20° C. The Mr of phosphoproteins in the autoradiograph shown were estimated by comparison with BioRad molecular weight standard proteins on the stained gel.

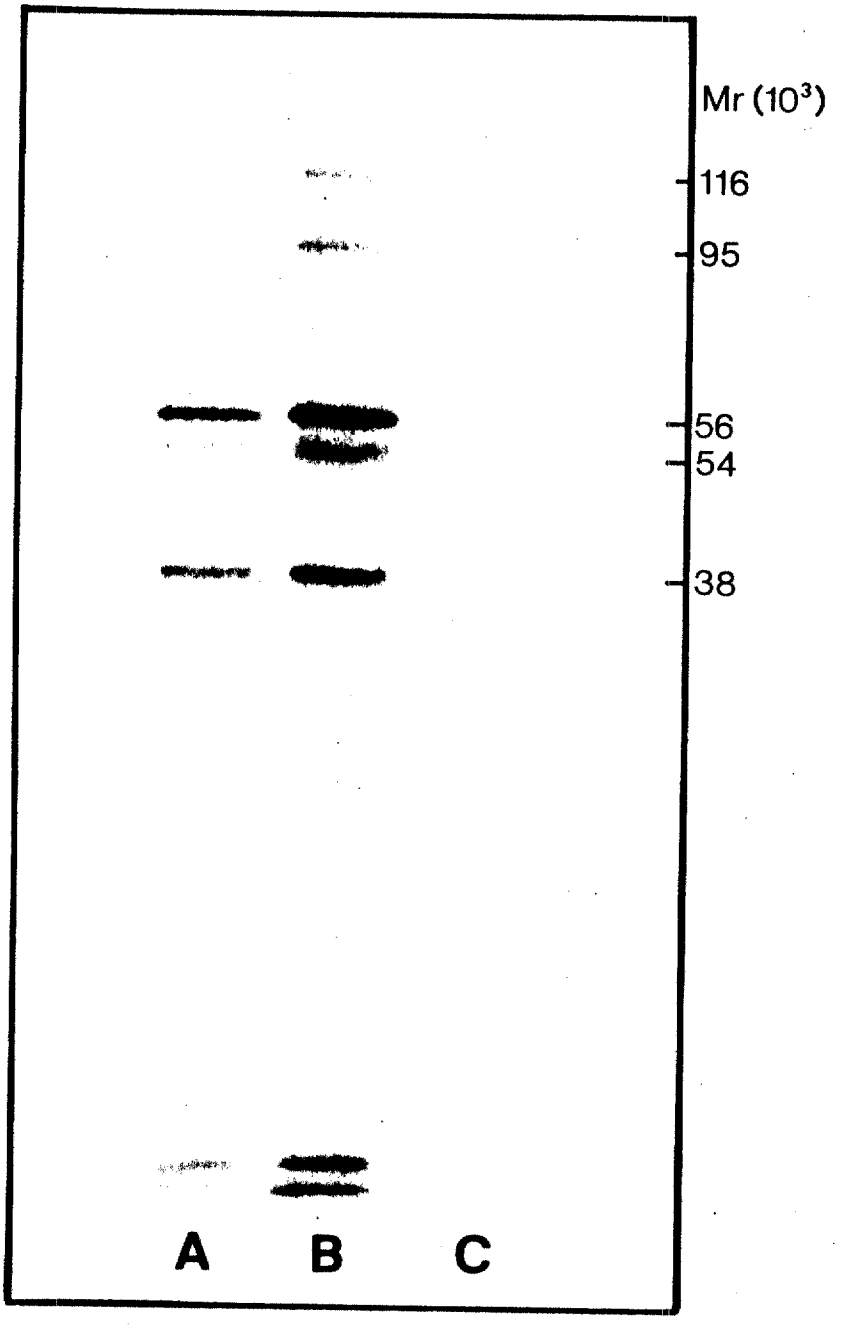

Mr (10^3)
116
95
56
54
38
A
B
C

Figure 7. Sephacryl S200 gel filtration chromatography of Activating Enzyme and S6 kinase from human placenta. The enzymes were prepared from 322 g of human placenta by the modified purification protocol described in Experimental Procedures. Individual fractions (3.8 ml) were assayed by three methods with 2 mg/ml mixed histone as the protein substrate. All phosphotransferase assays were preceded by a 10 minute preincubation step with 110 μM [γ-^{32}P]ATP (83 dpm/pmol) and 12 mM $MgCl_2$. The MgATP-activatable S6 kinase was detected with the standard protein kinase phosphotransferase assay (-O-). The trypsin-activatable S6 kinase was detected by limited trypsin treatment of the individual fractions as described in Experimental Procedures prior to the standard assay (-●-). The Activating Enzyme was detected by addition of 5 μl of fraction 69 to the preincubation mixture for fractions 46-60 (-■-). The shaded area represents the net amount of S6 kinase activity requiring trypsin for expression. The elution volumes of standard proteins (BioRad) were used to estimate Mr.

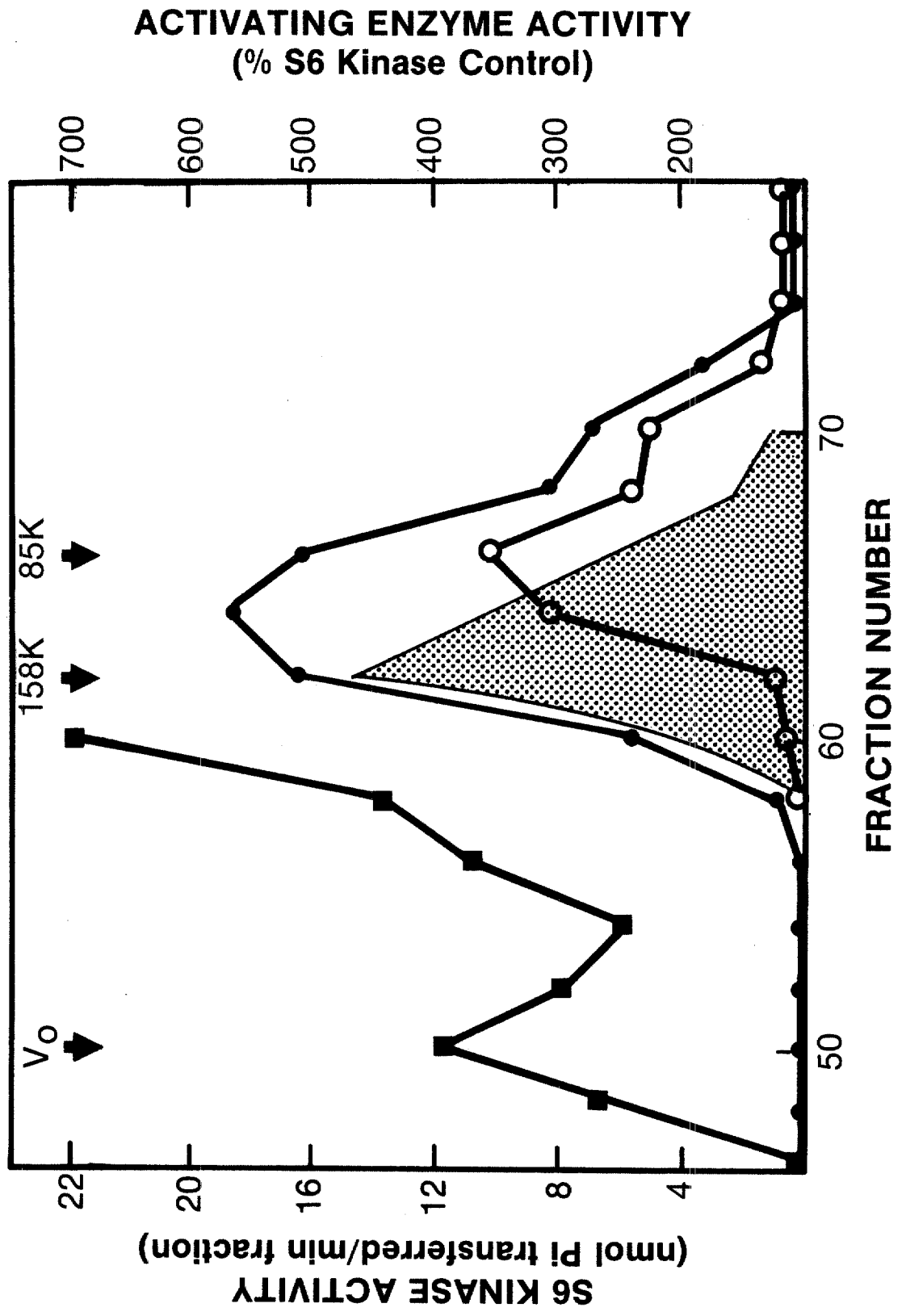

ACTIVATING ENZYME ACTIVITY
(% S6 Kinase Control)
700
600
500
400
300
200
158K
85K
V_O
22
20
16
12
8
4
50
60
70
FRACTION NUMBER
S6 KINASE ACTIVITY
(nmol Pi transferred/min fraction)

Figure 8. Western Blot analysis of S6 kinase associated proteins with mouse immune serum. The S6 kinase preparation used for immunization was electrophoresed on 12.5% SDS-PAGE (approximately 25 µg per lane) and proteins were electrophoretically transferred to nitrocellulose in Tris-glycine-methanol buffer. Nitrocellulose strips were incubated for 4 hours with serum from mouse #1 (A; diluted 1:440) or from mouse #2 (B; diluted 1:370). The second antibody was peroxidase-linked goat anti-mouse (BioRad) diluted 1:4500 and the incubation was one hour. The antigen Mr is estimated by comparison with BioRad molecular weight standard proteins on a comparable stained gel.

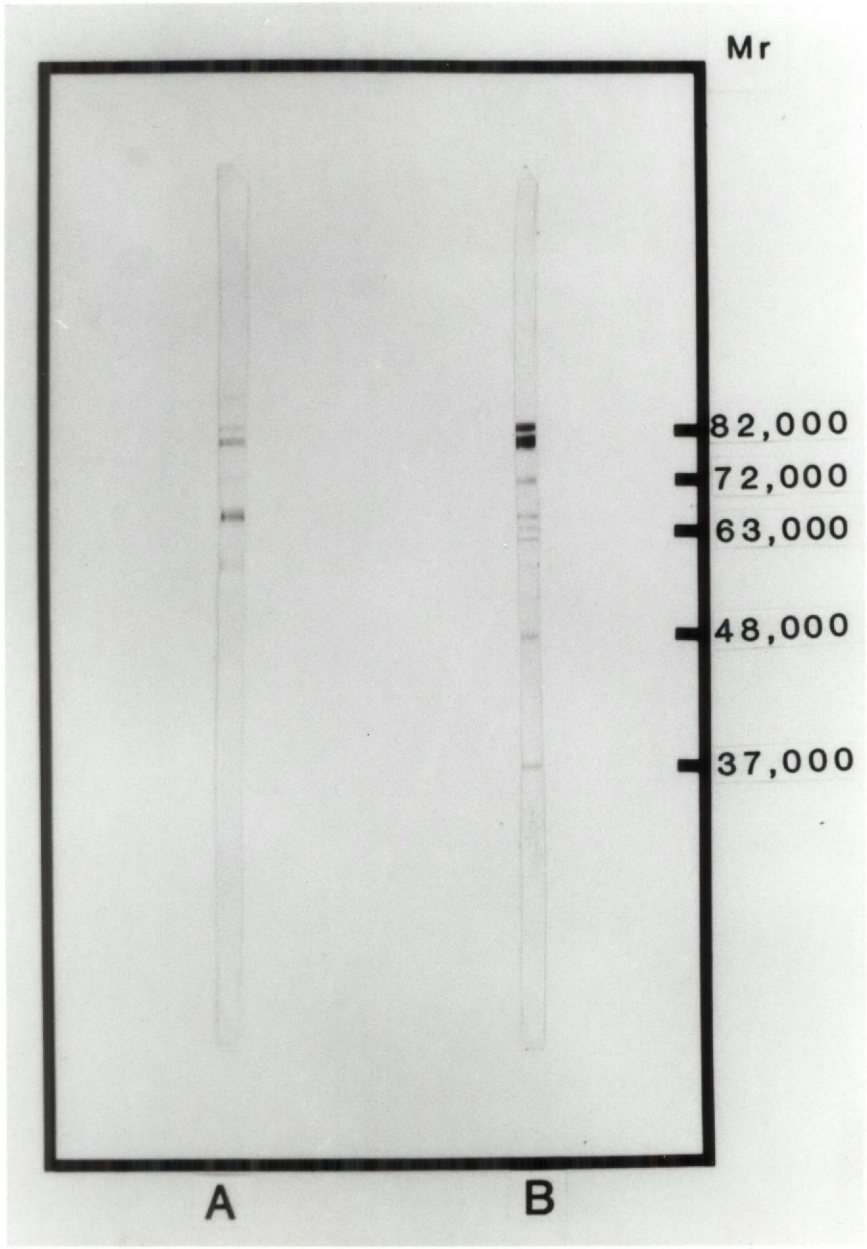

Mr
82,000
72,000
63,000
48,000
37,000
A
B

Figure 9. Effect of mouse immune serum on S6 kinase activity. Mouse immune serum was diluted as indicated in TBS-1%BSA and included in the protein kinase assay at 10% of the final assay volume. The assay contained 2.1 µg S6 kinase, 100 µM [γ-^{32}P]ATP (40 dpm/pmol) and 1 mg/ml histone H4. S6 kinase was assayed without (A) and with (B) a 20 minute preincubation prior to the 10 minute phosphotransferase reaction. Serum was present in both the preincubation and phosphotransferase reactions. Range of duplicate assay samples are marked.

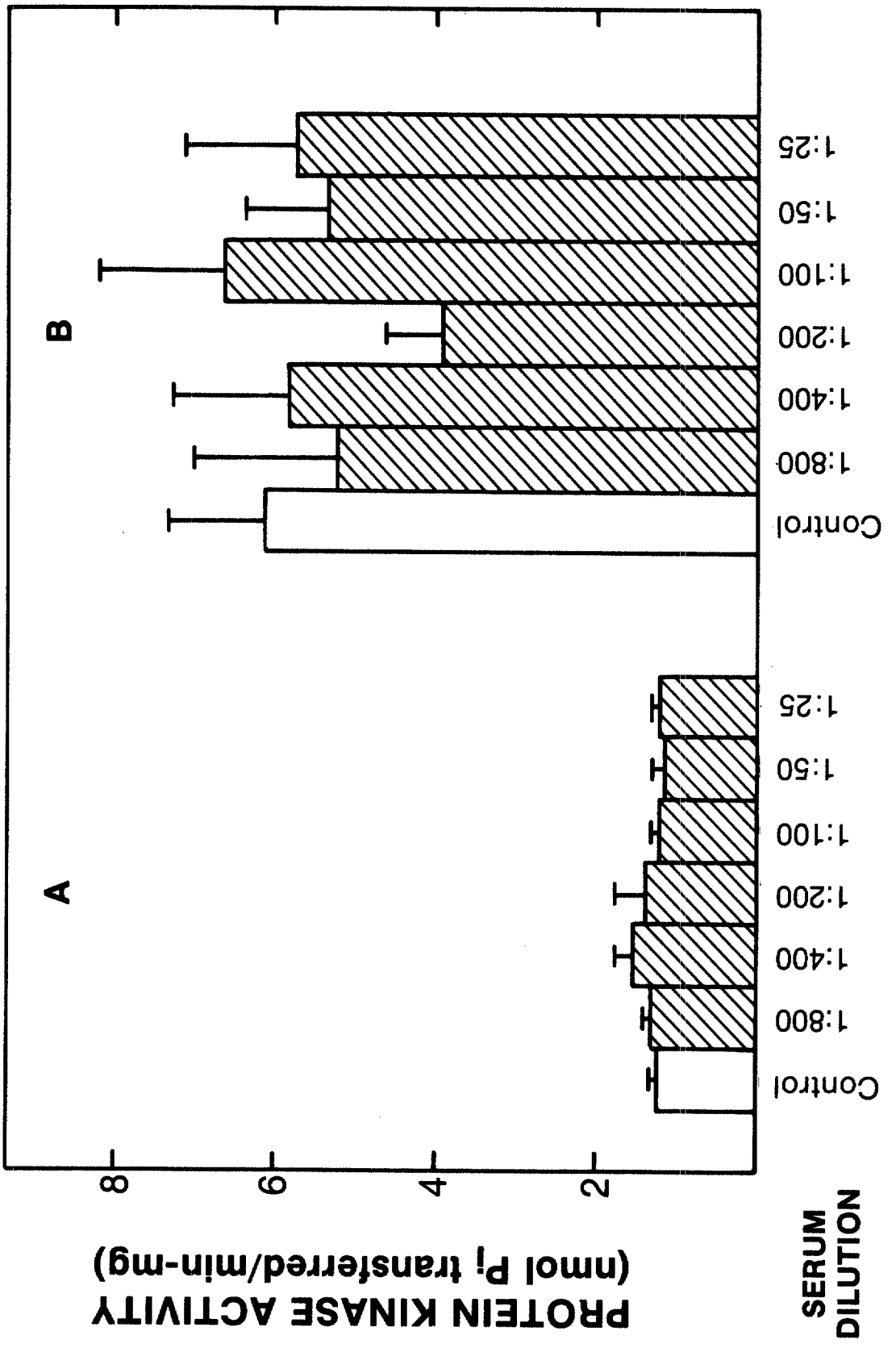

PROTEIN KINASE ACTIVITY
(nmol Pi transferred/min-mg)
2
4
6
8
A
B
Control
1:800
1:400
1:200
1:100
1:50
1:25
SERUM DILUTION

Figure 10. Growth of SP2/0-AG14 myeloma cells. Cells were seeded at 1 x 10^4 cells/ml in a 24 well plate in basal media supplemented with 15% FCS and 2 mM glutamine (-O-) or Media B (-●-) as defined in Experimental Procedures. At specified times the cell concentration in duplicate wells was determined with a hemocytometer. Cell viability was determined by the Trypan Blue exclusion test and was greater than 95% at all points.

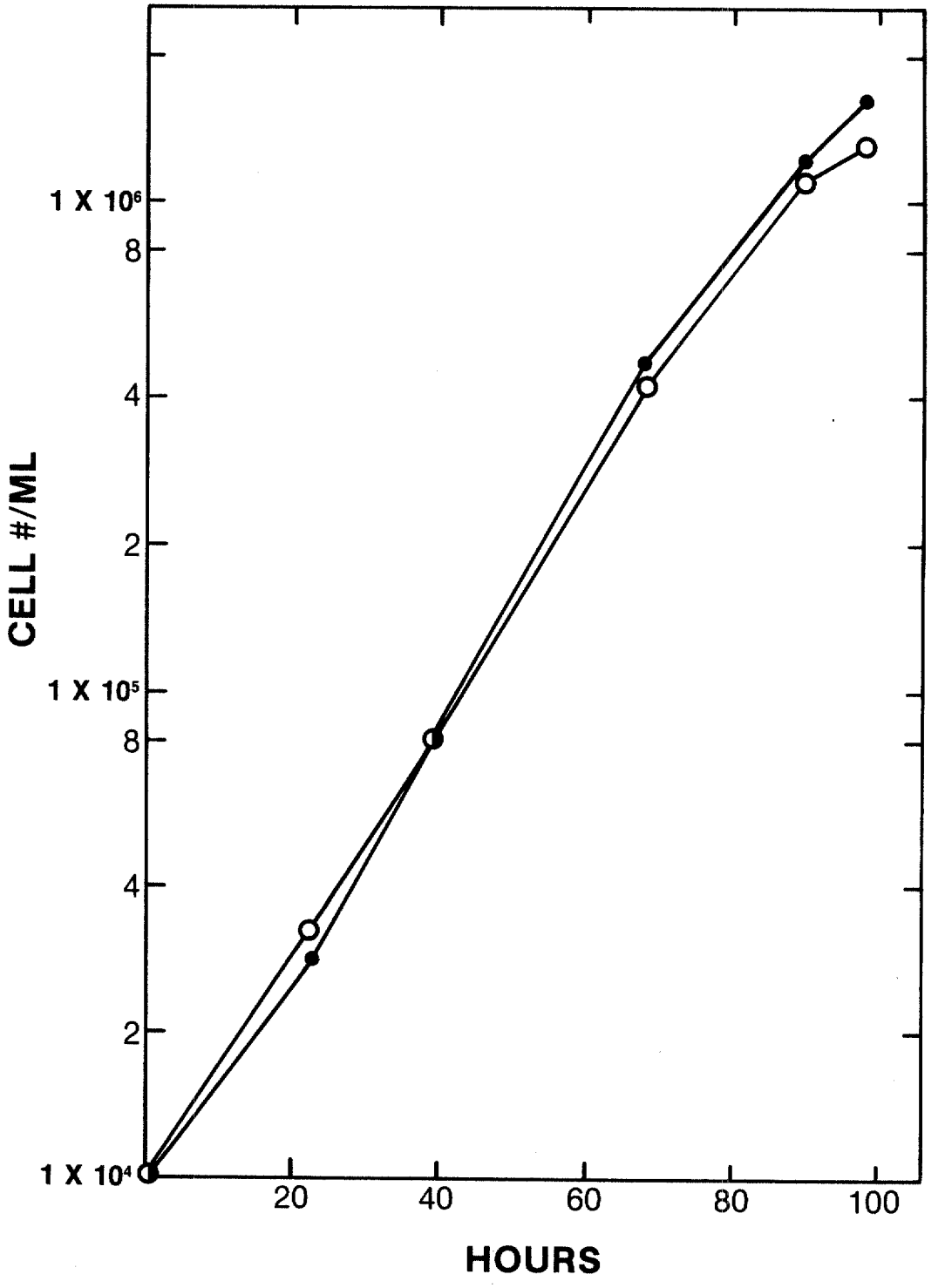

CELL #/ML
1 X 10⁶
8
4
2
1 X 10⁵
8
4
2
1 X 10⁴
20
40
60
80
100
HOURS

Figure 11. Establishment of antibody-secreting monoclonal cell lines. A total of 162 cell lines were established from the fusion experiment as described in Experimental Procedures. The recovery of antibody-secreting cell lines at each step was as follows: clone I, 31% representing 49 families; clone II, 72% representing 41 families; establishment in mass culture, 73% representing 38 families.

MONOCLONAL ANTIBODY PRODUCTION

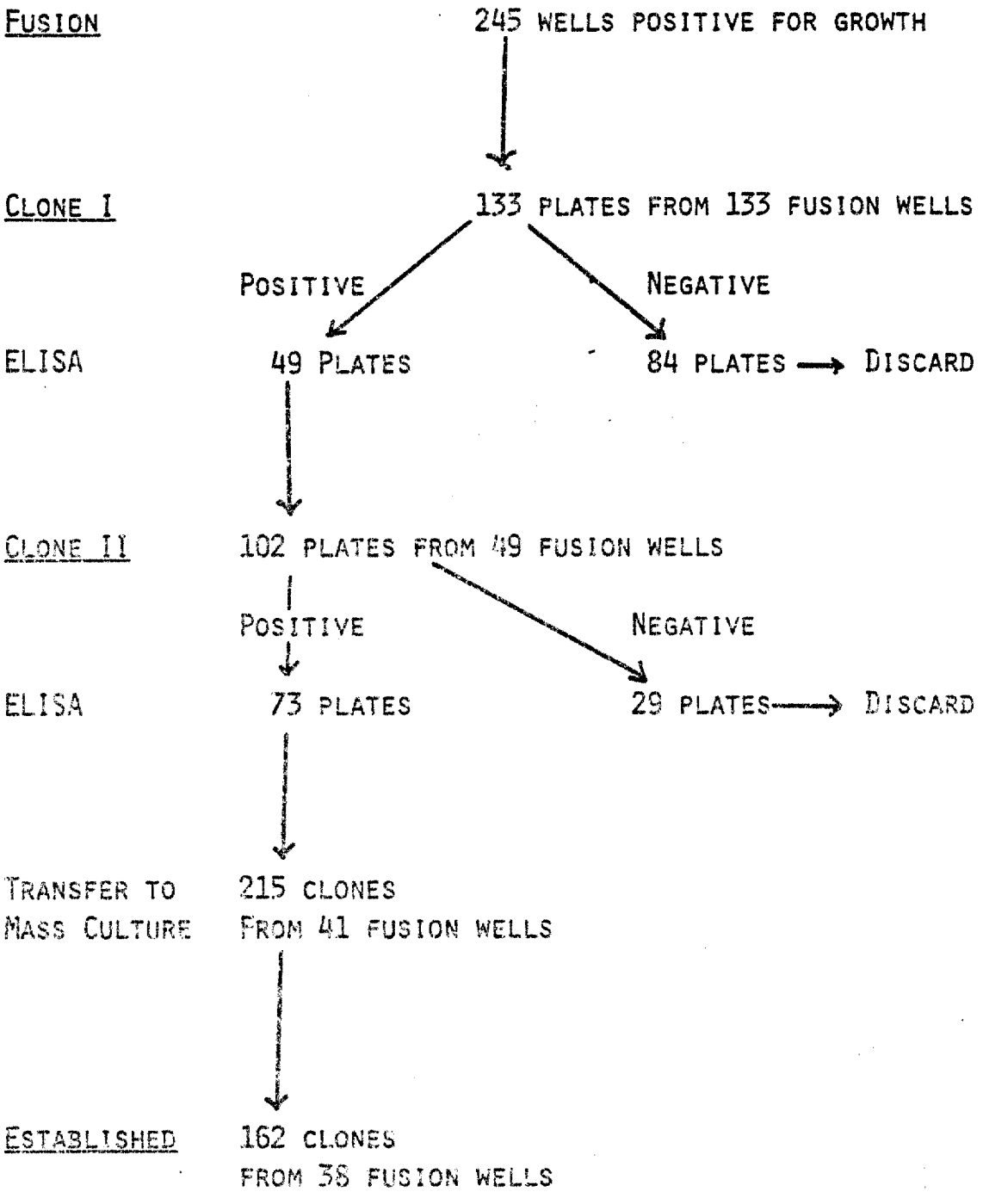


Figure 12. Occurrence of proteins coincident with S6 kinase activity on gel filtration chromatography. Proteins from individual gel filtration fractions (25 µl aliquots) as shown in Figure 7 were separated by 10% SDS-PAGE and stained with Coomassie Blue. Lasar scanning densitometry was used to quantitate the occurrence of the Mr 95,000 (-O-) and Mr 55,000 (-●-) proteins.

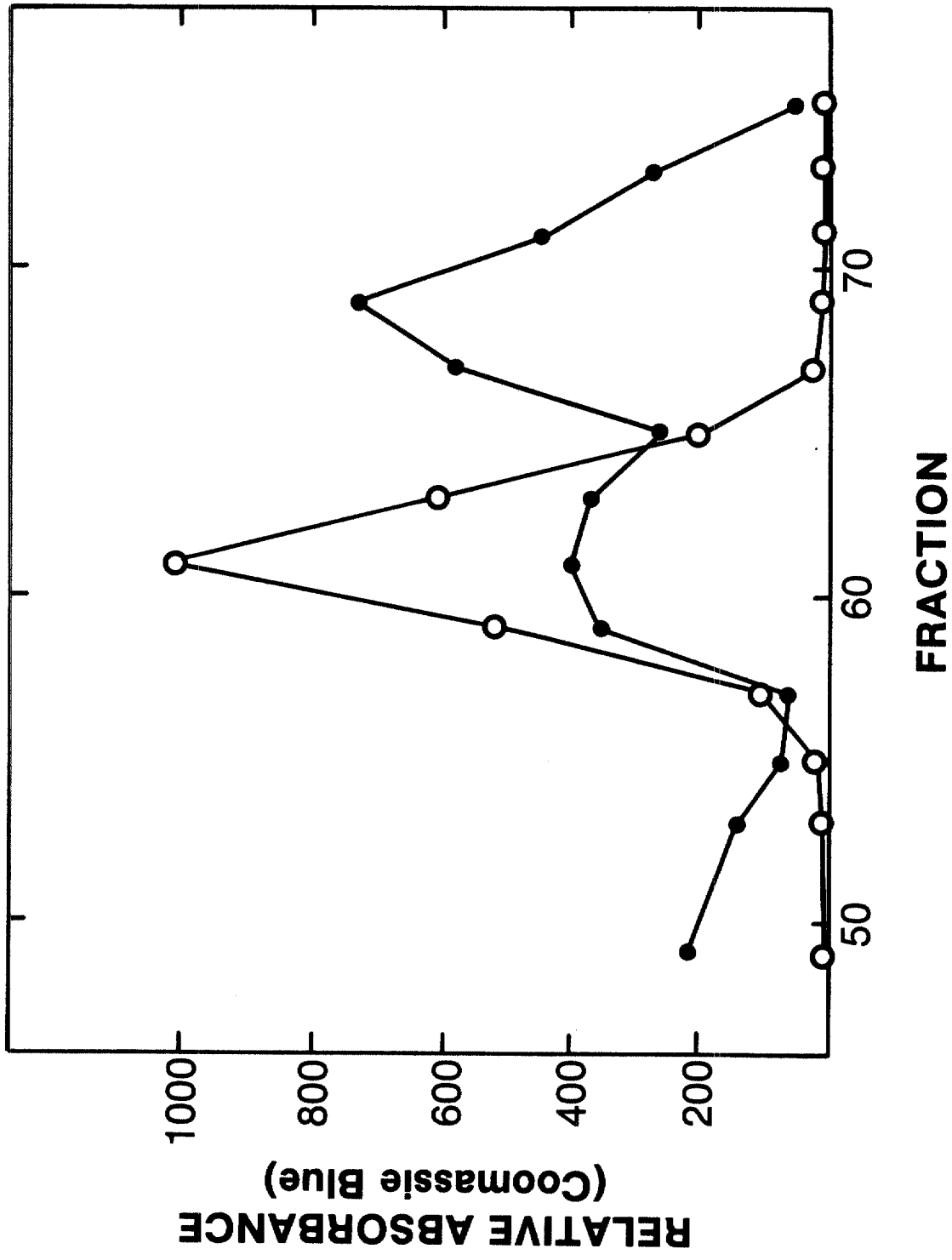

RELATIVE ABSORBANCE
(Coomassie Blue)
1000
800
600
400
200
50
60
70
FRACTION

Figure 13. Western Blot analysis of Sephacryl S200 gel filtration chromatography fractions with mouse immune serum. Proteins from individual fractions of the gel filtration chromatography (50 µl aliquots) were separated by 12.5% SDS-PAGE and transferred electrophoretically to nitrocellulose in Tris-glycine-methanol buffer. The ELISA was performed with a mix (1:1) of mouse immune serum from mouse #1 and #2 diluted 1:1000, peroxidase-linked goat anti-mouse IgG diluted 1:4000 and peroxidase-linked rabbit anti-goat IgG diluted 1:4000. The incubation times were 4 hours, 2 hours and 2 hours, respectively. Antigen Mr was estimated by comparison with BioRad molecular weight standard proteins from the gel.

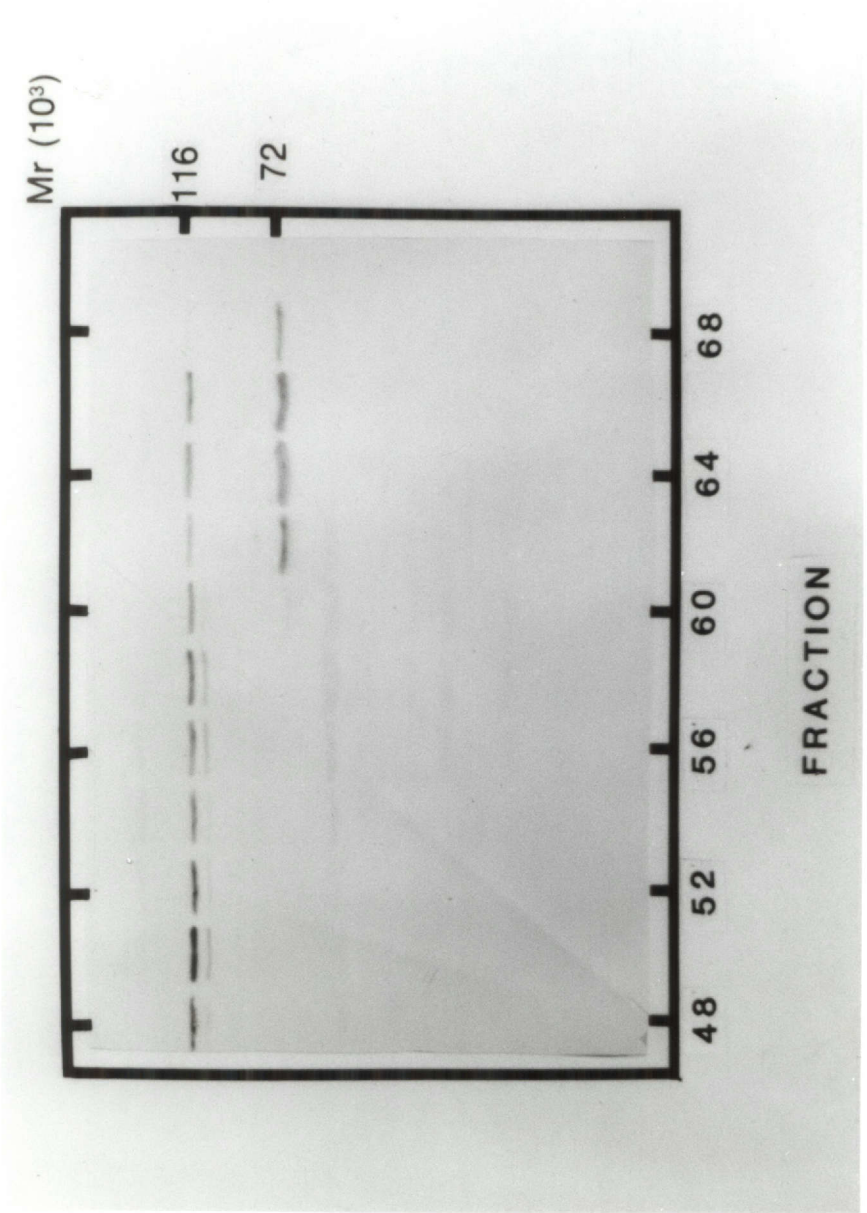

Mr (10³)
116
72
48
52
56
60
64
68
FRACTION

Figure 14. Quantitation of the Mr 116,000 protein. The occurrence of the immunoreactive Mr 116,000 protein (-O-) in gel filtration chromatography fractions shown in Figure 13 and the Mr 116,000 protein detected on a Coomassie Blue stained duplicate gel (-●-) were quantitated by laser scanning densitometry.

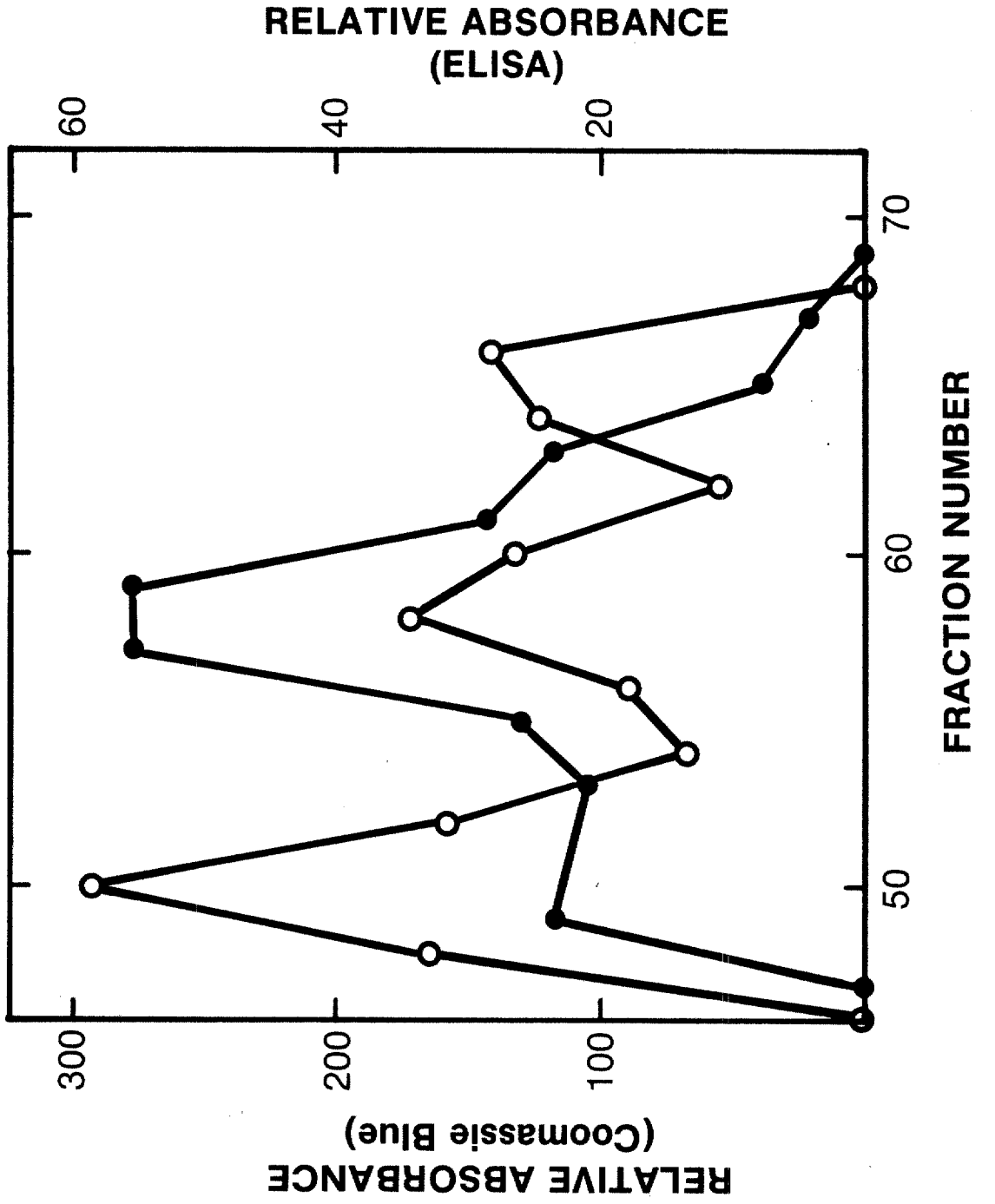

RELATIVE ABSORBANCE
(ELISA)
60
40
20
70
60
50
FRACTION NUMBER
300
200
100
RELATIVE ABSORBANCE
(Coomassie Blue)

Figure 15. Quantitation of the Mr 72,000 protein. The occurrence of the immunoreactive Mr 72,000 protein (-O-) in gel filtration chromatography fractions shown in Figure 13 and the Mr 72,000 protein detected on a Coomassie Blue stained duplicate gel (-●-) were quantitated by laser scanning densitometry.

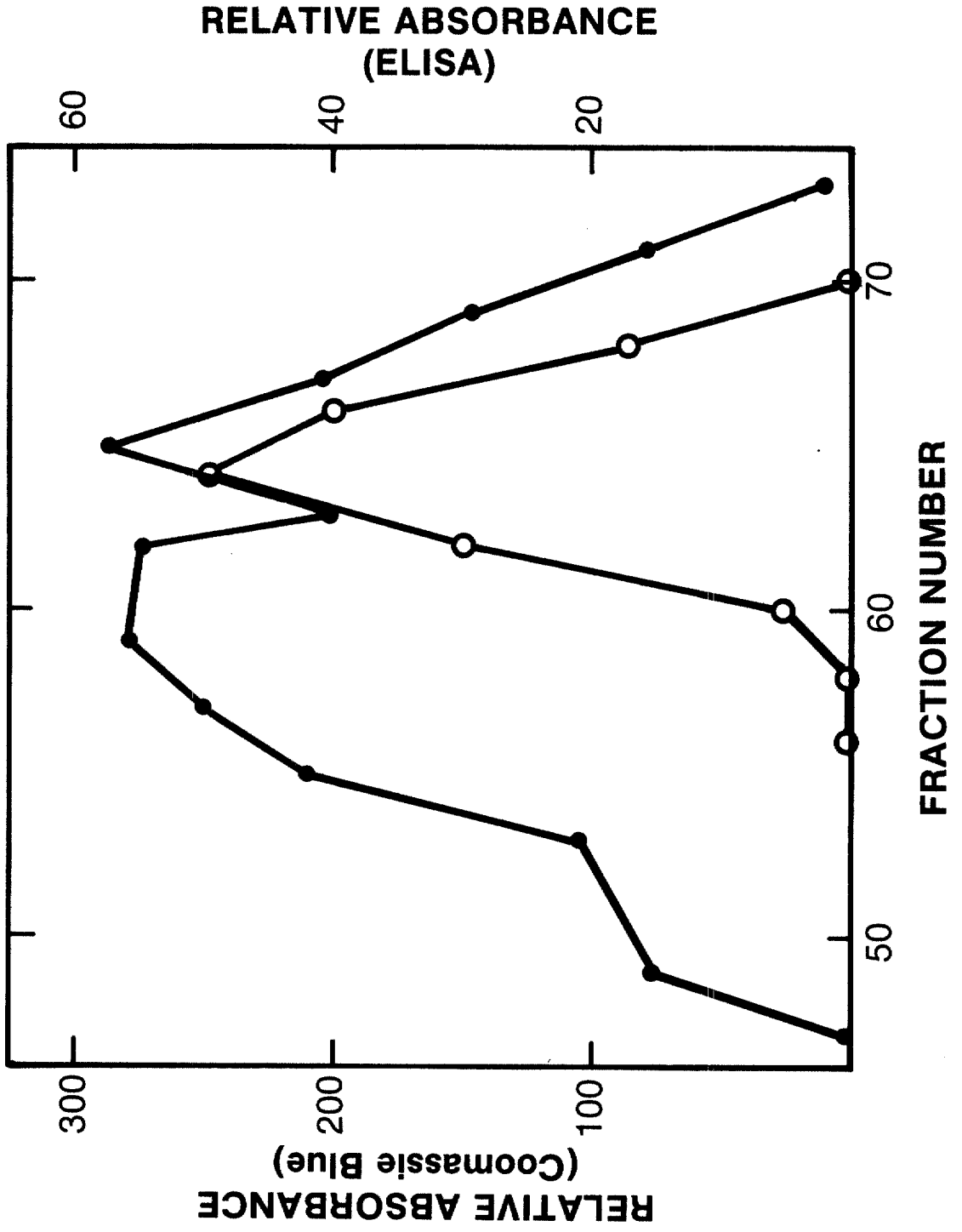

RELATIVE ABSORBANCE
(ELISA)
60
40
20
70
60
50
FRACTION NUMBER
300
200
100
RELATIVE ABSORBANCE
(Coomassie Blue)

Figure 16. Immunodot analysis of gel filtration chromatography fractions with monoclonal antibody. Proteins from individual gel filtration fractions (20 µl aliquots, ≦ 20 µg) as shown in Figure 7 were applied directly to nitrocellulose in the BioDot apparatus (BioRad). The primary antibody in the ELISA was 18-F9D ascites fluid diluted 1:4000 (-O-), 05-E11C ascites fluid diluted 1:4000 (-●-) or 05-J12C cell conditioned medium diluted 1:1 (-■-). Second and third antibodies were peroxidase-linked goat anti-mouse IgG and peroxidase-linked rabbit anti-goat IgG. Antibody incubation times were 4 hours, 4 hours and 1 hour, respectively. Reactions were quantitated by laser scanning densitometry.

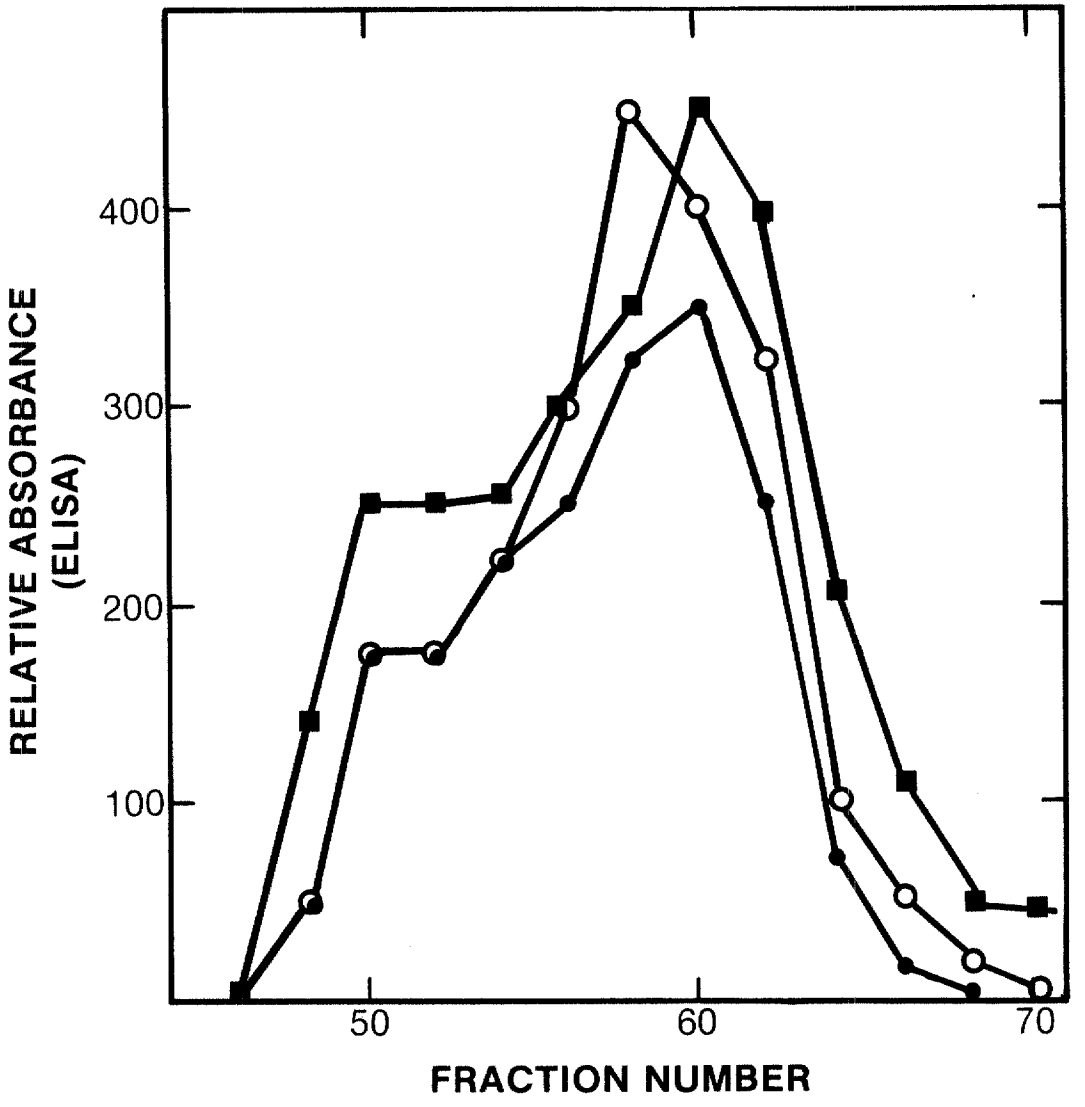

RELATIVE ABSORBANCE
(ELISA)
400
300
200
100
50
60
70
FRACTION NUMBER

Figure 17. Immunodot analysis of gel filtration chromatography fractions with monoclonal antibody. Immunodot analysis was performed as described in Figure 16, except the primary antibody was 23-H10D ascites fluid diluted 1:4000 (-O-) or 05-Y11B ascites fluid diluted 1:4000 (-●-).

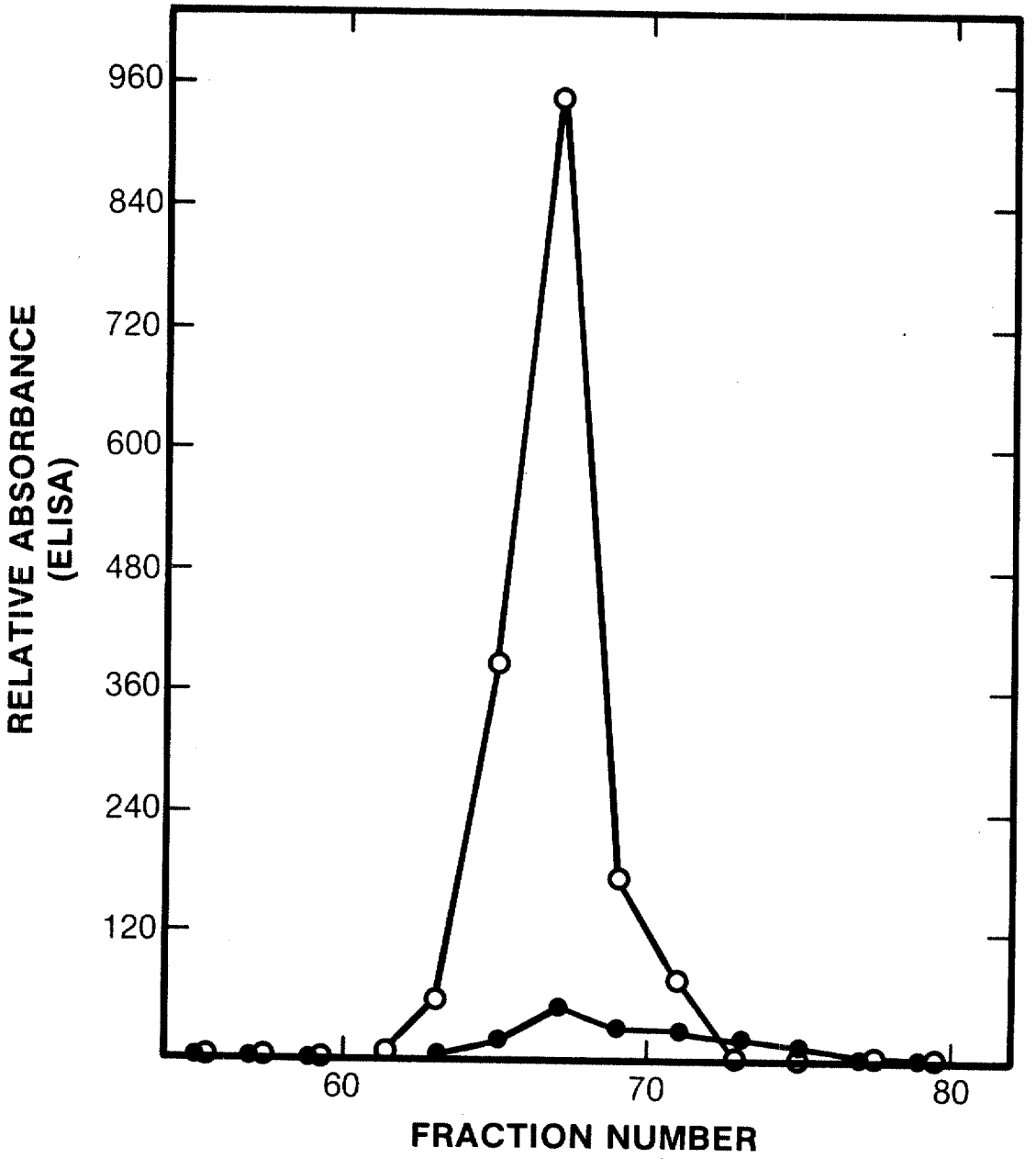

960
840
720
600
480
360
240
120
RELATIVE ABSORBANCE
(ELISA)
60
70
80
FRACTION NUMBER

Figure 18. Identification of the antigen for monoclonal antibodies by Western Blot analysis. Proteins from individual gel filtration fractions (50 µl aliquots) as shown in Figure 7 were separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose in Tris-glycine-methanol buffer. The ELISA was performed with 18-F9D ascites fluid diluted 1:1000 (A) or 23-H10D ascites fluid diluted 1:1000 (B) as the primary antibody. The second and third antibody were as described in Experimental Procedures. The occurrence of antibody-antigen complex in a single lane (---), which contained the most intense ELISA reaction was measured by lasar scanning densitometry. The blot was then stained with Amido Black and the lane scanned for total protein (——). Lanes containing BioRad molecular weight standard proteins were also stained with Amido Black and their location shown.

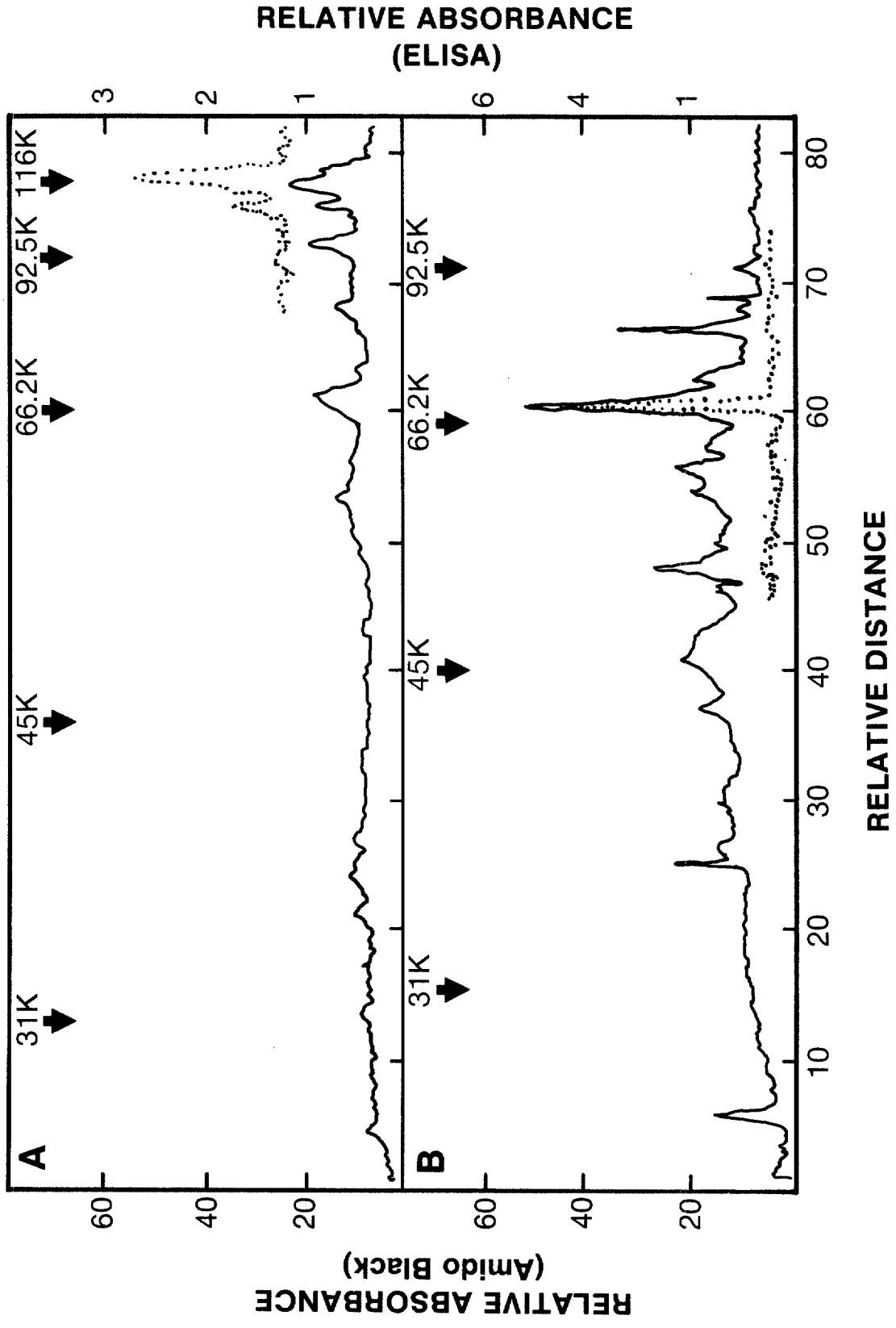

RELATIVE ABSORBANCE
(ELISA)
A
B
31K
45K
66.2K
92.5K
116K
RELATIVE ABSORBANCE
(Amido Black)
RELATIVE DISTANCE

Figure 19. Western Blot analysis of gel filtration chromatography fractions with 05-Y11B monoclonal antibody. Proteins from individual gel filtration fractions (50 µl aliquots) as shown in Figure 7 were separated by 10% SDS-PAGE and transferred electrophorectically to nitrocellulose in ethanolamine-glycine-methanol buffer. The ELISA was performed with 05-Y11B ascites fluid diluted 1:1000, peroxidase-linked goat anti-mouse IgG diluted 1:4000 and peroxidase-linked rabbit anti-goat IgG diluted 1:4000. Antibody incubation times were 7, 1.5 and 1.5 hours, respectively. Lanes containing BioRad molecular weight standard proteins were stained with Amido Black.

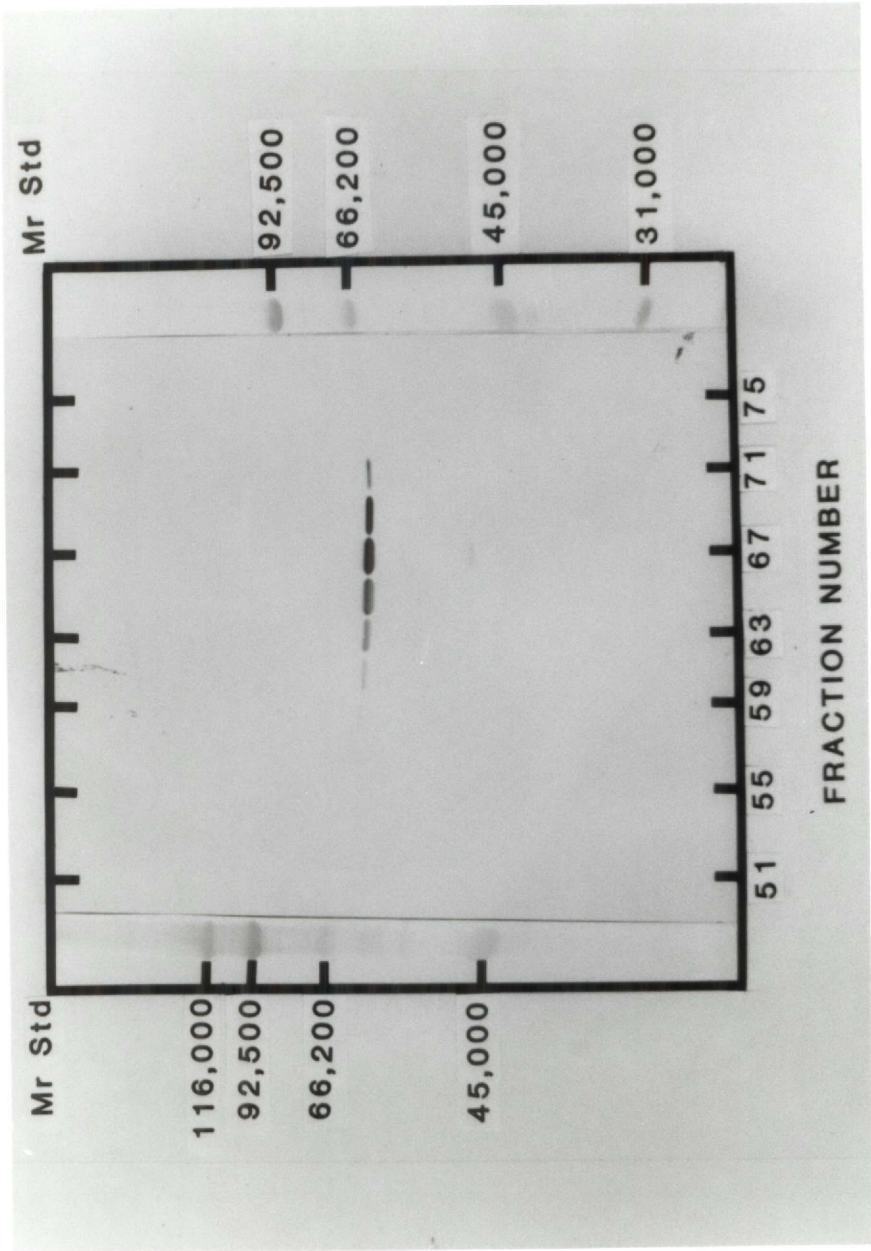

Mr Std
116,000
92,500
66,200
45,000
Mr Std
92,500
66,200
45,000
31,000
51
55
59
63
67
71
75
FRACTION NUMBER

Figure 20. Western Blot analysis of gel filtration chromatography fractions with mouse immune serum. The experiment was performed exactly as described in Figure 19 except the primary antibody was a mix (4:1) of immune serum from mouse #1 and #2 diluted 1:1000.

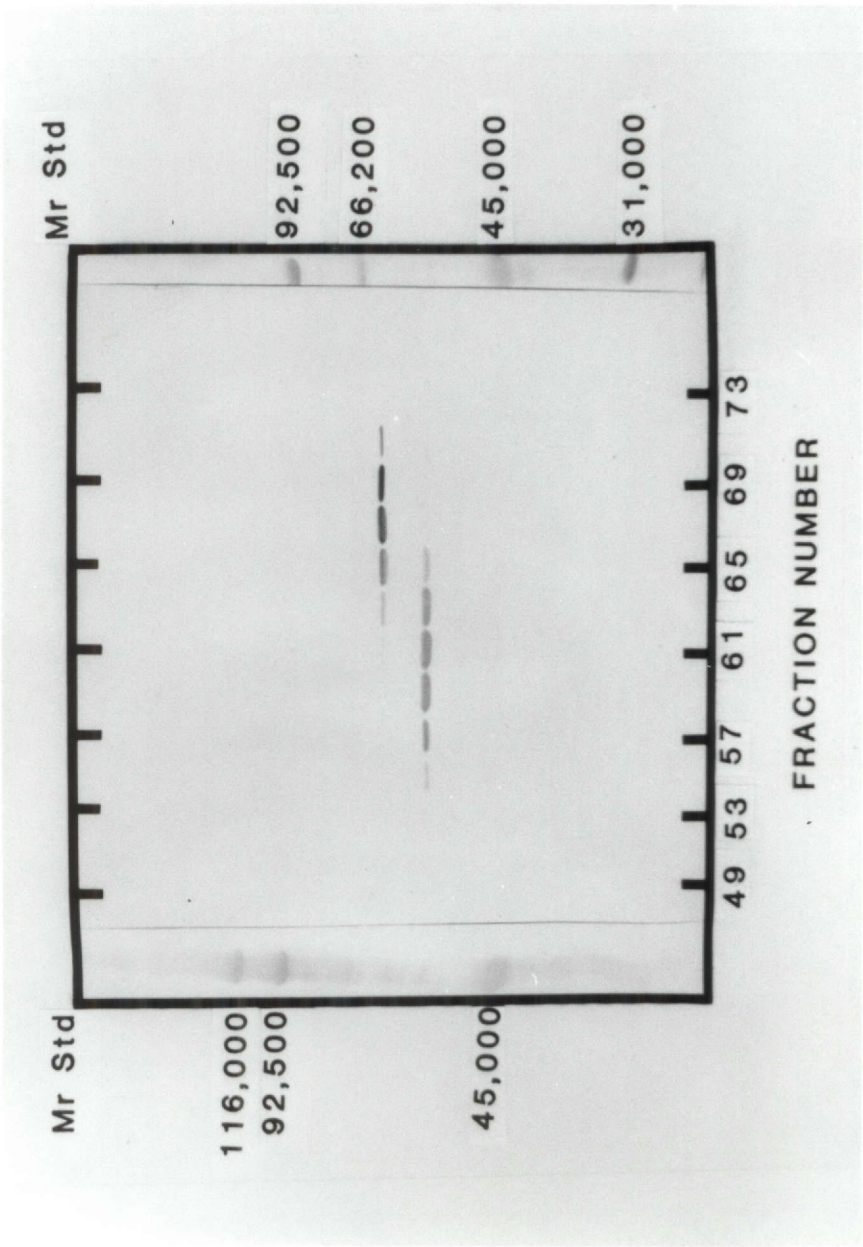

Mr Std
92,500
66,200
45,000
31,000
Mr Std
116,000
92,500
45,000
49 53 57 61 65 69 73
FRACTION NUMBER

Figure 21. DEAE Affigel Blue chromatography of ascites fluid. 23-H10D ascites fluid (2.5 ml, 55 mg) obtained 15 days after i.p. injection of the mouse with 23-H10D cells, was chromatographed on 10 ml DEAE Affigel Blue as described in Experimental Procedures. Protein was monitored at 280 nm (-O-) and the gradient monitored by conductivity (-●-). Fraction volume was 2 ml. Fractions #25-29 were pooled for further use.

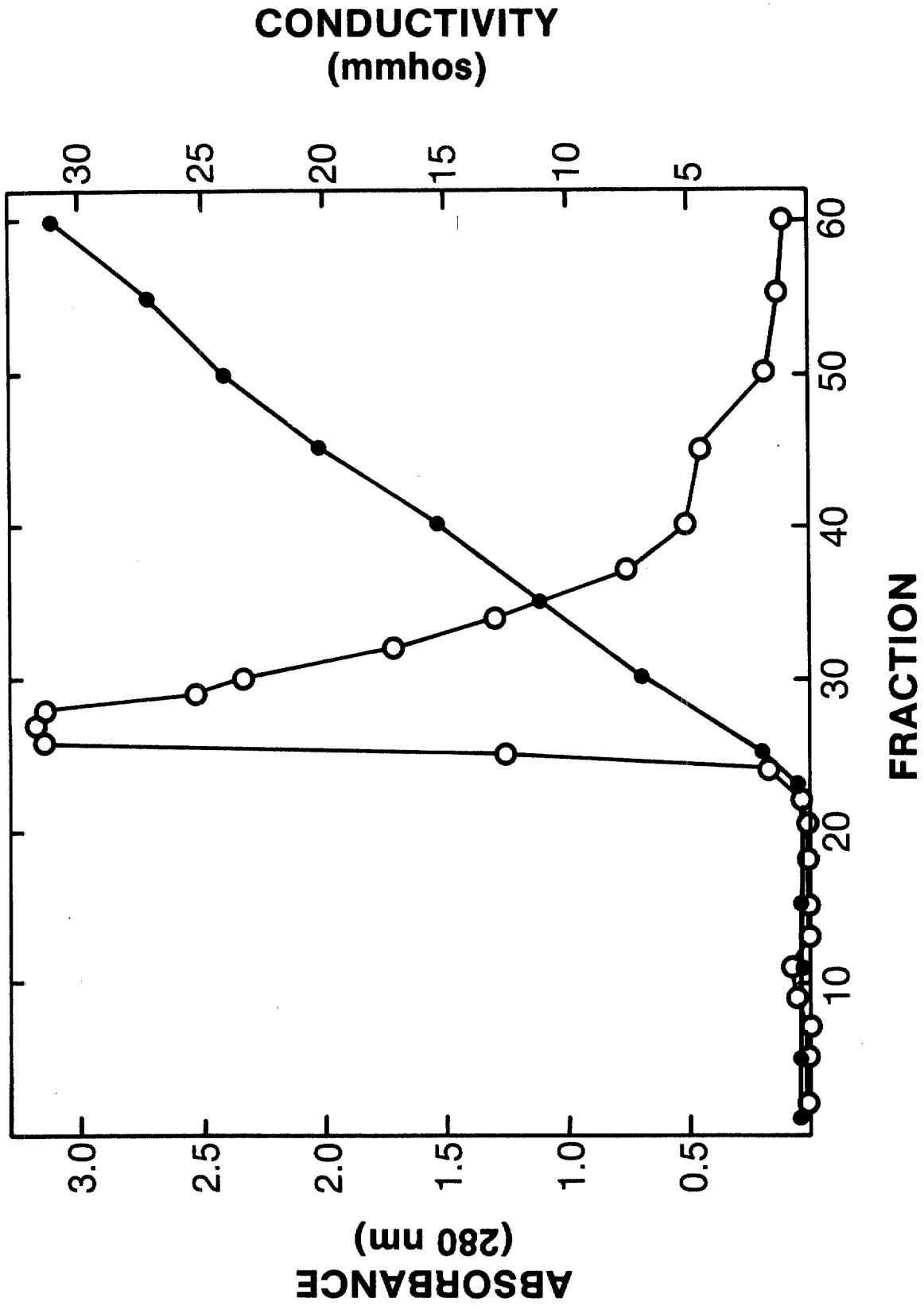

CONDUCTIVITY
(mmhos)
30
25
20
15
10
5
60
50
40
30
20
10
FRACTION
3.0
2.5
2.0
1.5
1.0
0.5
ABSORBANCE
(280 nm)

Figure 22. SDS-PAGE analysis of ascites fluid. Ascites fluid proteins were separated by 12.5% SDS-PAGE and stained with Coomassie Blue. Lanes B and C contain 46 µg 18-F9D ascites fluid and 44 µg 23-H10D ascites fluid, respectively. Lane D contains 20 µg of DEAE Affigel Blue purified 23-H10D IgG. Ascites fluid was purified as described in Figure 21. BioRad low molecular weight standard proteins are in Lane A.

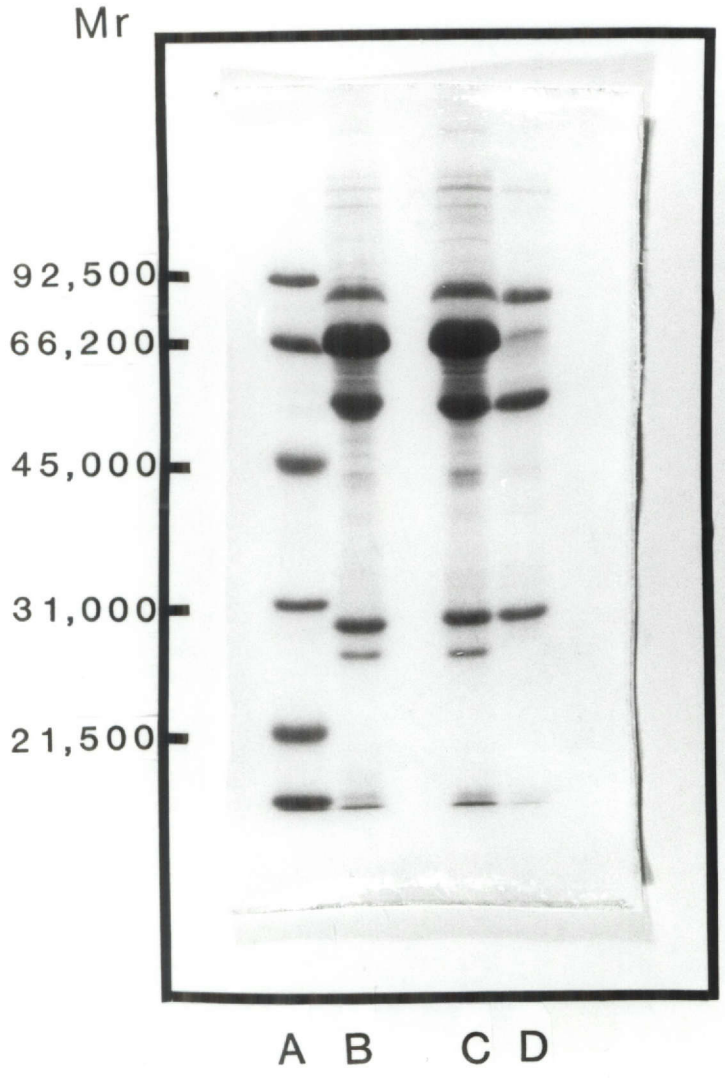

Mr
92,500
66,200
45,000
31,000
21,500
A B C D

Figure 23. Chromatography of the S6 kinase preparation on 23-H10D-Sepharose. S6 kinase (0.95 mg) prepared by the modified purification protocol was chromatographed on a 1 ml 23-H10D-Sepharose column as described in Experimental Procedures. Aliquots (10 µl) from fractions (0.5 ml total volume) were assayed for protein kinase with 2 mg/ml mixed histone and 92 µM [γ-^{32}P]ATP (69 dpm/pmol) as substrate. Phosphotransferase reactions were preceded by a 20 minute preincubation (-●-). Fractions were assayed for Activating Enzyme by addition of 1.9 µg S6 kinase preparation to the preincubation reaction (-O-). Total protein was monitored spectophotometrically at 280 nm (-X-).

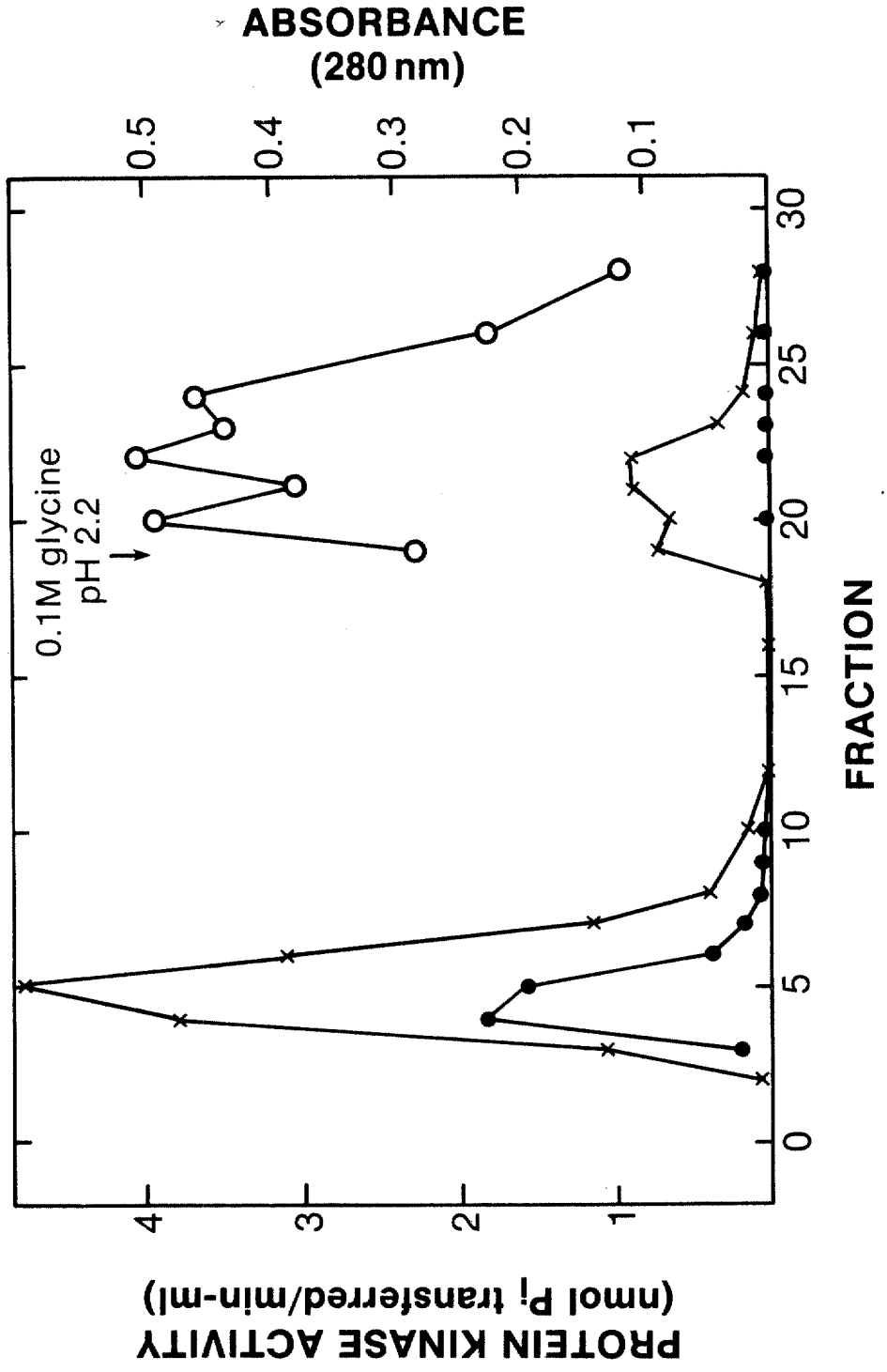

ABSORBANCE
(280 nm)
0.5
0.4
0.3
0.2
0.1
0.1M glycine
pH 2.2
0
5
10
15
20
25
30
FRACTION
PROTEIN KINASE ACTIVITY
(nmol Pi transferred/min-ml)
4
3
2
1

Figure 24. SDS-PAGE analysis of 23-H10D-Sepharose chromatography. Proteins from the 23-H10D-Sepharose chromatography of the S6 kinase preparation described in Figure 23 were analyzed by 12.5% SDS-PAGE. The bound protein remaining after assay (Table V) was lyophilized and dialyzed against distilled water prior to electrophoresis. Gels were stained with Coomassie Blue. Lane A: 25 µg S6 kinase; B: 66 µg unbound protein; C: 80 µg bound protein; D: BioRad low molecular weight standard proteins.

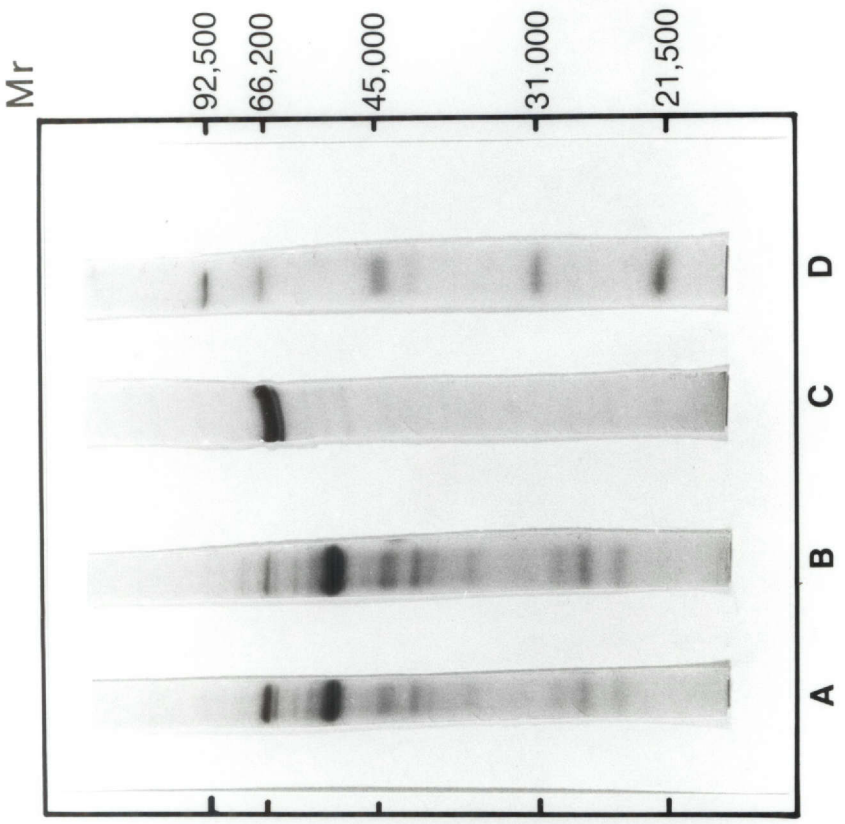

Mr
92,500
66,200
45,000
31,000
21,500
A
B
C
D

Figure 25. Chromatography of Activating Enzyme on 18-F9D-Sepharose. Activating Enzyme (0.2 mg) prepared by the modified purification protocol was chromatographed on a 0.5 ml 18-F9D-Sepharose column as described in Experimental Procedures. Aliquots (10 µl) from fractions (0.5 ml total volume) were assayed for Activating Enzyme with 2.8 µg S6 kinase preparation in a 20 minute preincubation reaction. The phosphotransferase assay was initiated by the addition of 2 mg/ml mixed histone as the protein substrate (-O-). Activating Enzyme histone phosphotransferase activity in the absence of S6 kinase was 25 pmol/min-ml. Total protein was monitored spectrophotometrically at 280 nm (-●-).

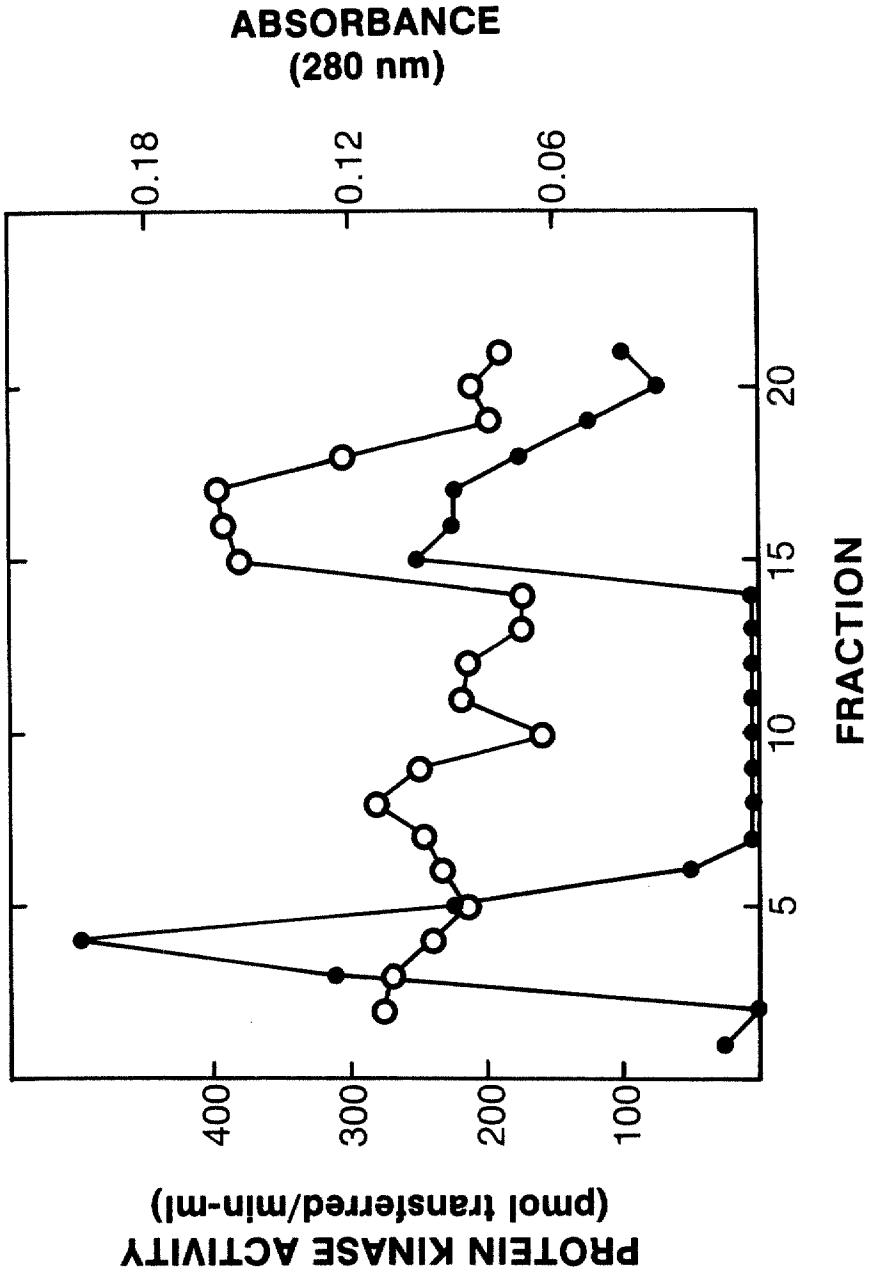

ABSORBANCE
(280 nm)
0.18
0.12
0.06
5
10
15
20
FRACTION
400
300
200
100
PROTEIN KINASE ACTIVITY
(pmol transferred/min-ml)

Figure 26. SDS-PAGE analysis of 18-F9D-Sepharose chromatography of Activating Enzyme. The bound protein fraction from chromatography of Activating Enzyme on 18-F9D-Sepharose was analyzed by 12.5% SDS-PAGE. The bound protein was concentrated on a Centricon centrifugal microconcentrator (Amicon) prior to electrophoresis. Lane A: 16 µg bound Activating Enzyme; B and C: BioRad molecular weight standard proteins.

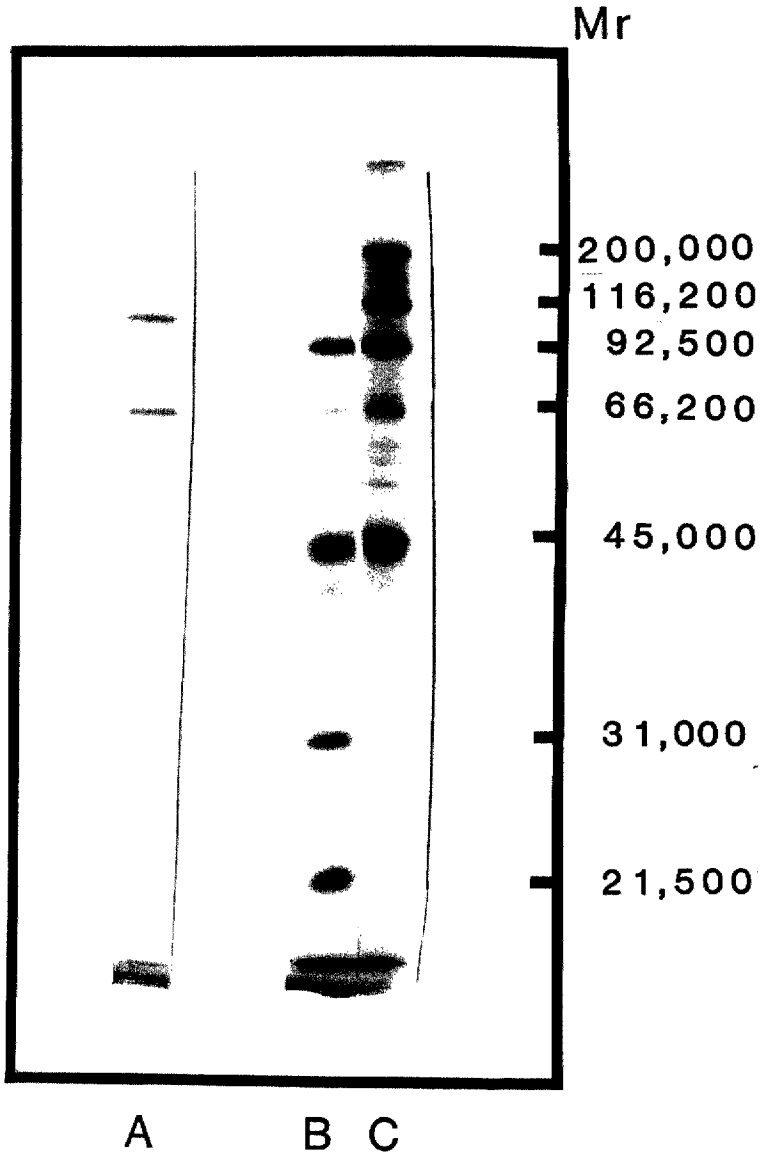

Mr
200,000
116,200
92,500
66,200
45,000
31,000
21,500
A
B C

Figure 27. SDS-PAGE analysis of 18-F9D-Sepharose chromatography of Activating Enzyme. Activating Enzyme (1.4 mg), prepared by the modified purification protocol, was chromatographed on a 1 ml 18-F9D-Sepharose column. Bound protein remaining after assay (Table VI) was lyophilized and dialyzed against water prior to 12.5% SDS-PAGE. Lane A: 20 µg S6 kinase; B: 28 µg Activating Enzyme; C: 100 µg bound Activating Enzyme; D: BioRad high molecular weight standard proteins.

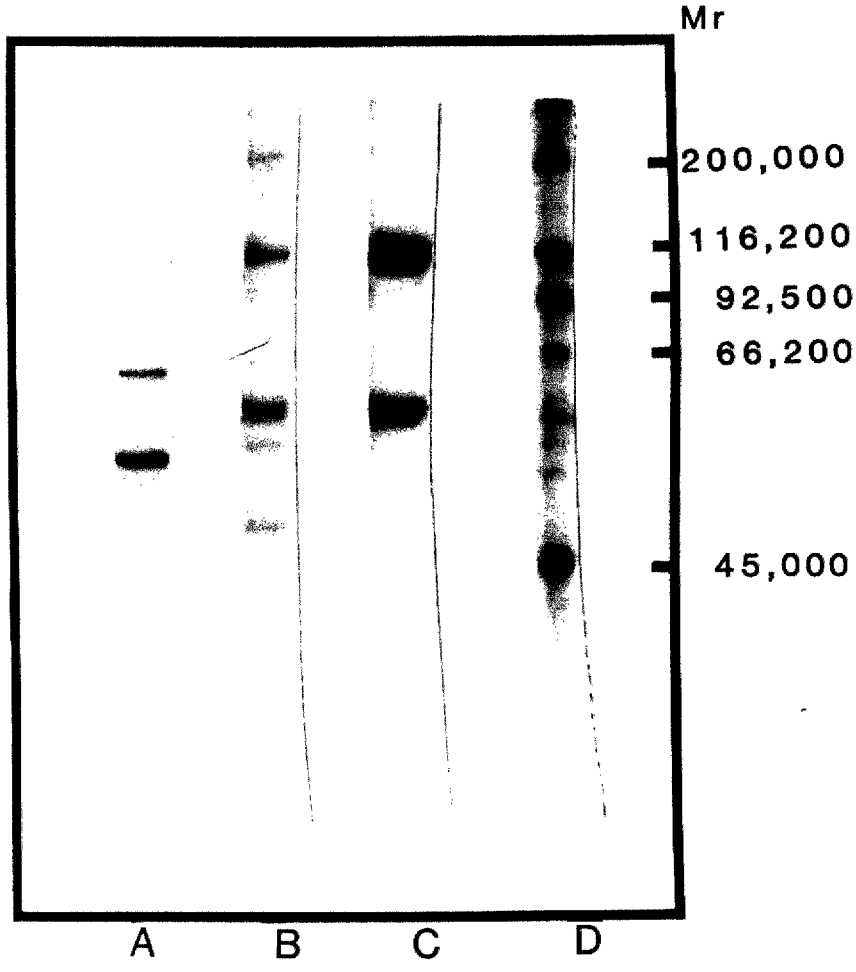

Mr
200,000
116,200
92,500
66,200
45,000
A
B
C
D

Figure 28. SDS-PAGE analysis of individual gel filtration fractions chromatographed on 18-F9D-Sepharose. Individual fractions from gel filtration chromatography as shown in Figure 7 were chromatographed on 18-F9D-Sepharose as described in Experimental Procedures. In lanes B, D and E the protein bound to 18-F9D-Sepharose was assayed for activity (Table VII). The remaining sample was lyophilized and dialyzed against water prior to 12.5% SDS-PAGE. Lane C represents 20% of the protein bound to 18-F9D-Sepharose. Gels were stained with Coomassie Blue. Lane B: Activating Enzyme (Fraction 49, 2 µg); C: Activating Enzyme (Fraction 57, 10 µg); D: Trypsin-activatable S6 kinase (Fractions 62-63, 20 µg); E: MgATP-activatable S6 kinase (Fractions 71-72, 2 µg). BioRad molecular weight standard proteins are shown in Lanes A and F. IgG heavy (Mr 55,000) and light (Mr 27,000) chains are present in each eluted sample.

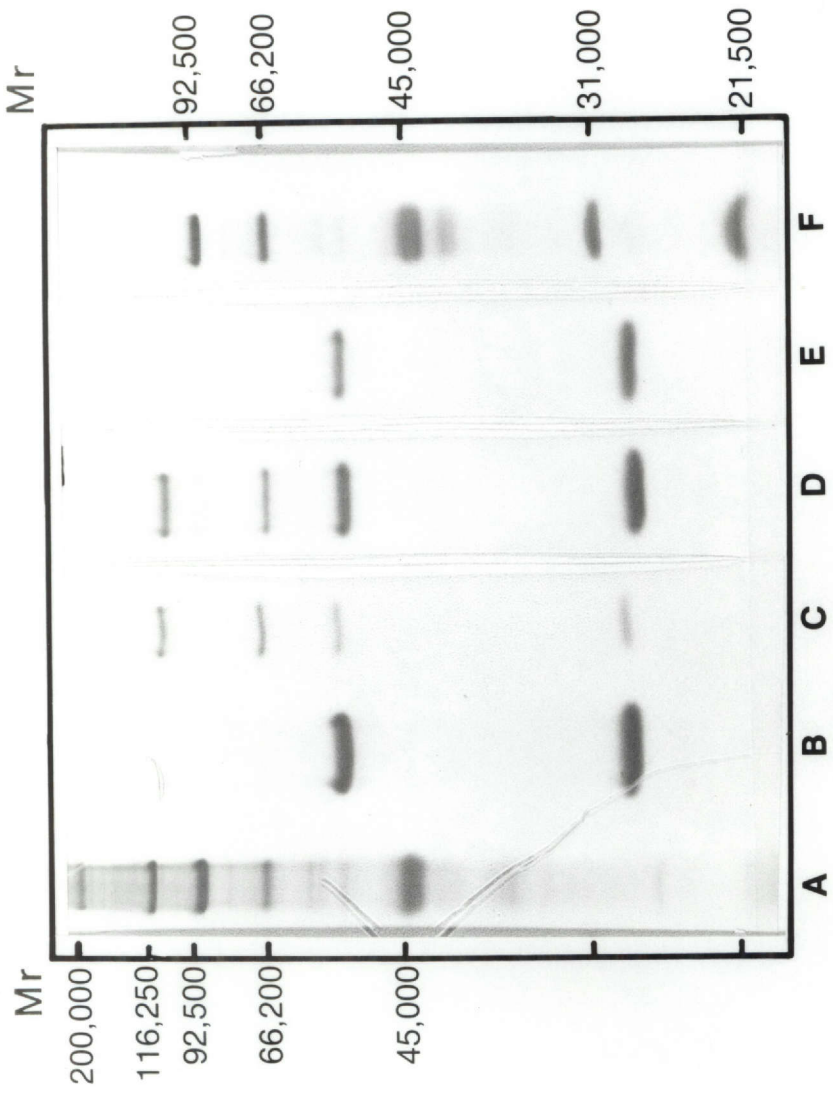

Mr
92,500
66,200
45,000
31,000
21,500
A
B
C
D
E
F
Mr
200,000
116,250
92,500
66,200
45,000

Figure 29. Phosphorylation of the Mr 116,000 protein by insulin receptor kinase. Activating Enzyme from each peak on gel filtration chromatography as shown in Figure 7 (A: fraction 49; B: fraction 57), was chromatographed on 18-F9D-Sepharose as described in Experimental Procedures. Insulin receptor kinase was incubated with 0.3 μM insulin in 20 mM Tris-Cl, pH 8.0, for one hour at room temperature. The activated insulin receptor kinase (0.8 μg/80 μl reaction) was incubated with 0.1 mM [γ-^{32}P]ATP (361 dpm/pmol) and 5 mM $MgCl_2$ for 5 minutes at 30° C prior to addition of the Activating Enzyme (A: 0.6 μg; B: 1.0 μg). Control reactions were performed without insulin receptor kinase (unshaded bars). Reactions were terminated after 20 minutes by the addition of SDS-PAGE sample buffer, proteins separated by 10% SDS-PAGE and phosphoproteins localized by autoradiography of the stained, dried gel. Radioactivity in the portion of the gel corresponding to the Mr 116,000 protein was quantitated by liquid scintillation counting. Shaded bars represent reactions in which the insulin receptor kinase was present.

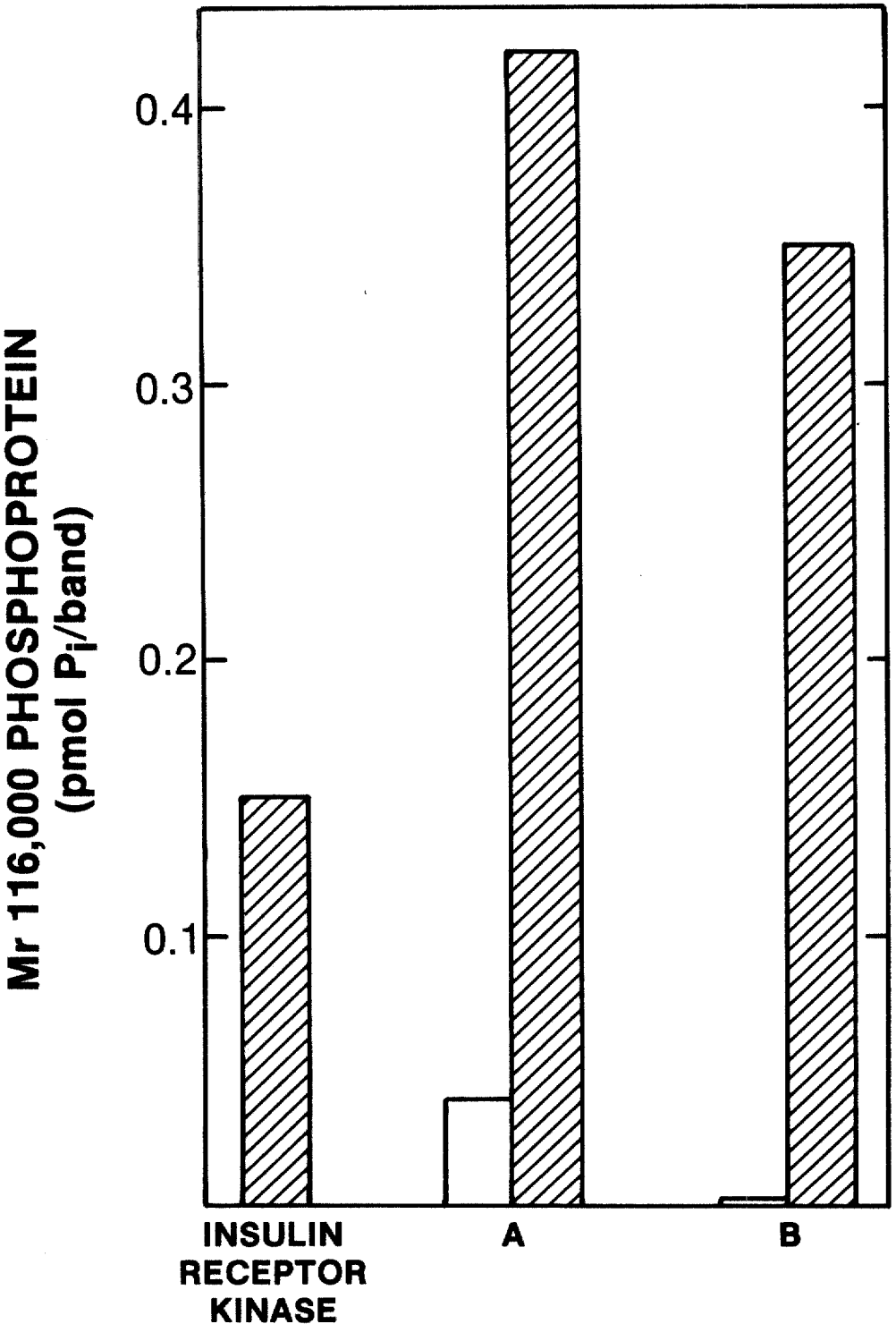

Mr 116,000 PHOSPHOPROTEIN
(pmol P_i/band)
0.4
0.3
0.2
0.1
INSULIN RECEPTOR KINASE
A
B

Figure 30. Western Blot analysis of S6 kinase with monoclonal antibody. S6 kinase was prepared by the modified purification protocol, proteins separated by 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose in Tris-glycine-methanol buffer. Each lane contained approximately 20 µg protein in the gel. The ELISA was performed with cell conditioned medium diluted 1:1 in TBS-Tween or a mix (1:1) of immune serum from mouse #1 and #2 diluted 1:200, peroxidase-linked goat anti-mouse IgG diluted 1:3500, and peroxidase-linked rabbit anti-goat IgG diluted 1:3500. The incubation times were 6 hours at 25° C, 10 hours at 4° C and 3 hours at 25° C respectively. Antigen Mr was estimated by comparison with BioRad molecular weight standard proteins from a comparable stained gel.

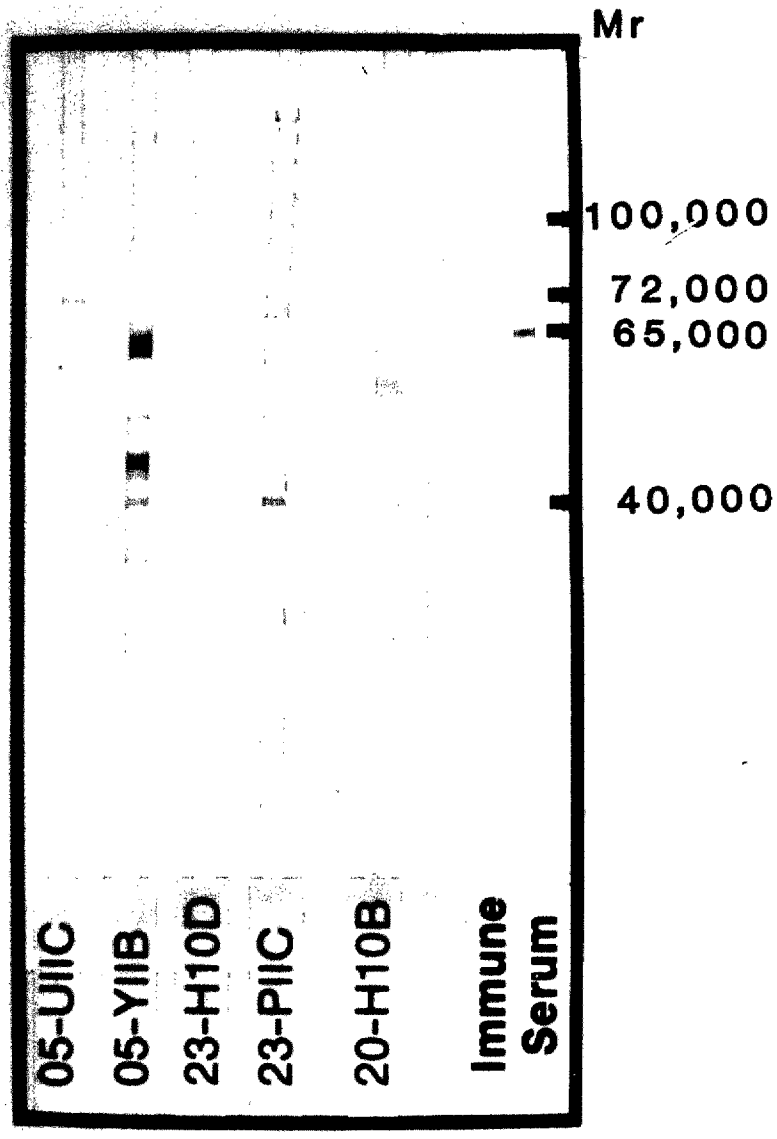

Mr
100,000
72,000
65,000
40,000
05-UIIC
05-YIIB
23-H10D
23-PIIC
20-H10B
Immune Serum

Figure 31. Immunodot analysis of phosphocellulose chromatography fractions with 18-F9D monoclonal antibody. Enzyme was prepared from human placenta by the modified purification protocol. Proteins from individual fractions of the phosphocellulose chromatography (30 µl aliquots) were applied directly to nitrocellulose in the BioDot apparatus (BioRad). The ELISA was performed with 18-F9D ascites fluid diluted 1:2000, peroxidase-linked goat anti-mouse IgG diluted 1:2000 and peroxidase-linked rabbit anti-goat IgG diluted 1:2000. Incubation times were 1, 0.5 and 0.5 hours, respectively. Reactions were quantitated by lasar scanning densitometry (-O-). Protein kinase assays were performed with 2 mg/ml mixed histone and without a preincubation step as described in Experimental Procedures (-●-).

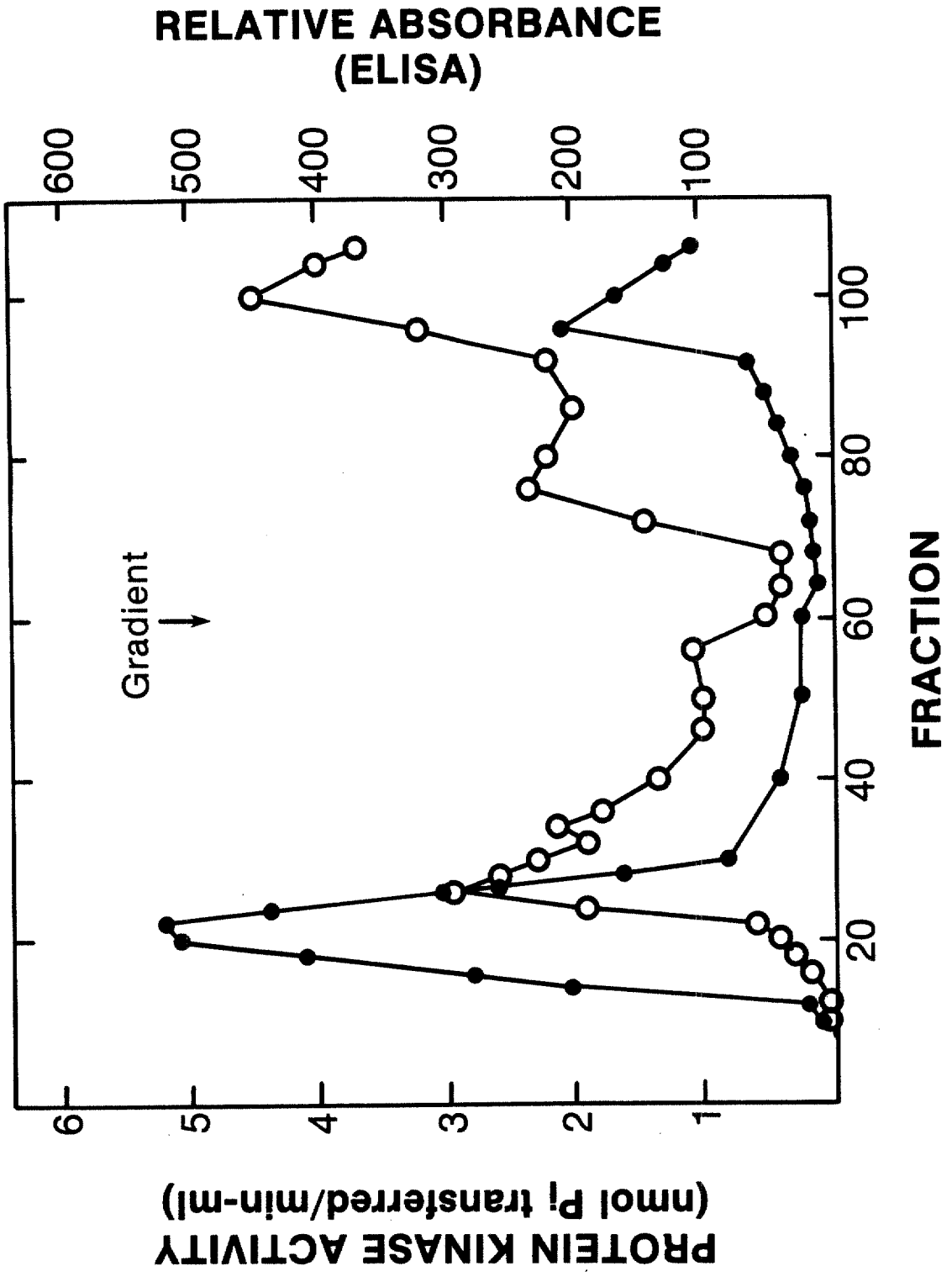
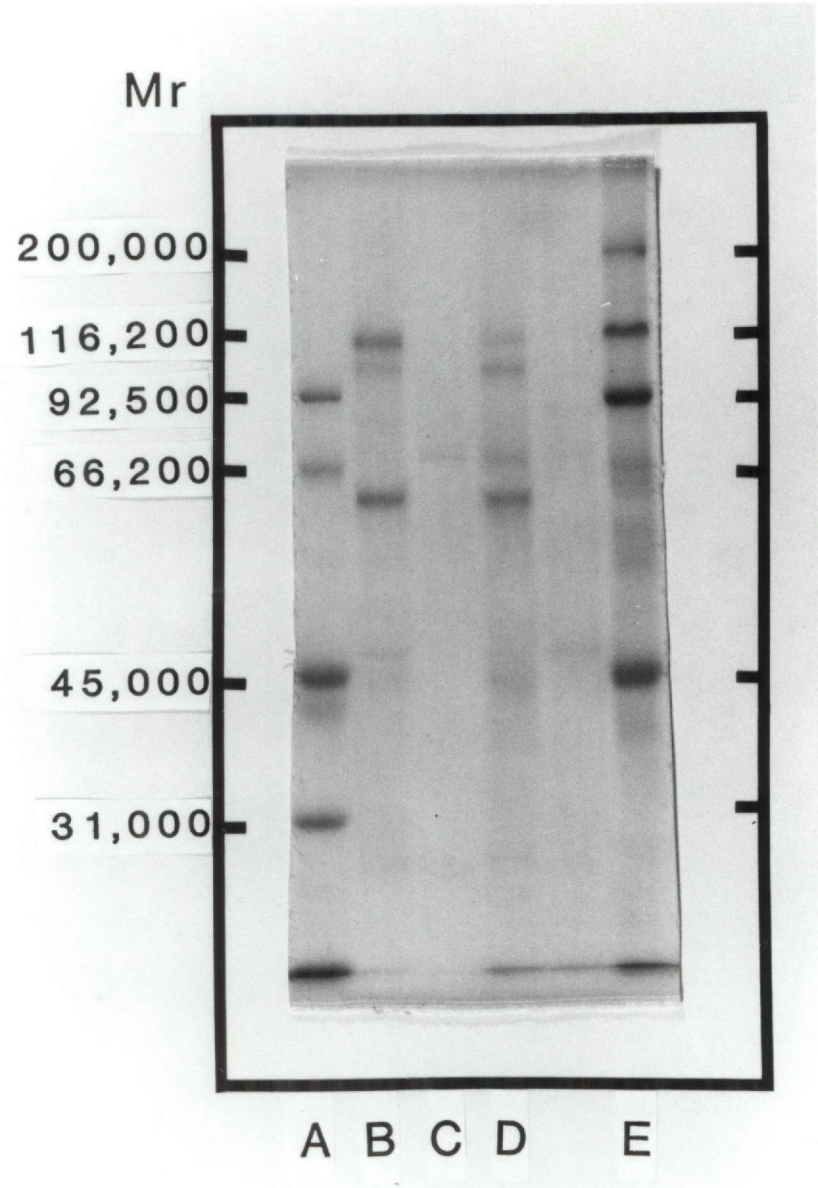

RELATIVE ABSORBANCE
(ELISA)
600
500
400
300
200
100
Gradient
20
40
60
80
100
FRACTION
PROTEIN KINASE ACTIVITY
(nmol Pi transferred/min-ml)
1
2
3
4
5
6

Figure 32. SDS-PAGE analysis of antibody affinity chromatography of proteins not bound to CM-Sephadex. Enzyme was prepared from human placenta by the modified purification protocol. Protein which failed to bind to CM-Sephadex was pooled and portions subjected to chromatography on monoclonal antibody-Sepharose as described in Experimental Procedures. Protein bound to the antibody-Sepharose was lyophilized, dialyzed against water and analyzed by 12.5% SDS-PAGE. Proteins were visualized with Coomassie Blue stain. Lane B: 20 µg protein bound to 18-F9D-Sepharose; C: 7 µg protein bound to 23-H10D-Sepharose; D: 28 µg protein bound to 05-E11C-Sepharose. BioRad molecular weight standard proteins are shown in Lanes A and E.


Mr
200,000
116,200
92,500
66,200
45,000
31,000
A B C D E

CHAPTER IV

DISCUSSION

A protein kinase which catalyzed the phosphorylation of histone H4 and 40S ribosomal protein S6 has been partially purified from human placenta (23, 24, 28, 64). This protein kinase has been designated S6 kinase. Histone H4 is a convenient protein substrate for *in vitro* assay. Saturating concentrations of histone can be achieved in solution and the phosphoprotein product separated from [γ-^{32}P]ATP substrate by trichloroacetic acid precipitation by the method of Reimann et al. (76). However, the S6 kinase is a cytoplasmic enzyme and no physiological significance is attached to its utilization of histone H4 *in vitro*.

Ribosomal protein S6 is located on the 40S ribosomal subunit and is the major site of ribosome phosphorylation observed in eukaryotic cells (1). Ribosomal protein S6 phosphorylation is a regulated event in response to extracellular signals in the cell (40, 60, 61, 88, 97). Insulin treatment of cells stimulates an increase in the degree of S6 phosphorylation on multiple sites (25, 56, 100). The S6 kinase preparation described in this study catalyzes the phosphorylation of ribosomal protein S6 on at least three sites (28); therefore, S6 is a likely *in vivo*

substrate for this enzyme.

The purification procedure employed yields a partially purified S6 kinase which retains regulatory properties. The S6 kinase preparation can be activated by incubation with MgATP in the absence of protein substrate. Additional S6 kinase activity is observed by limited trypsin treatment prior to the MgATP incubation. In either case, MgATP is required in the activation reaction and MnATP cannot be substituted. However, the phosphotransferase reaction can utilize either MgATP or MnATP as the metal-nucleotide substrate (22-24). The MgATP activation of S6 kinase displays a nonlinear dependence on the S6 kinase concentration (23, 24). A doubling in the S6 kinase concentration results in a greater than doubling of the activation rate, suggesting a bimolecular reaction. The different divalent cation requirement observed with the activation reaction as opposed to the phosphotransferase reaction suggests that a factor in the preparation other than S6 kinase is the MgATP-dependent activator of S6 kinase.

Several S6 kinases have been reported by other investigators. The protease-activated kinase II has been demonstrated to phosphorylate ribosomal protein S6 on sites comparable to those observed in insulin-treated cells (58, 72, 73). An S6 kinase which phosphorylates the ribosomal

protein S6 on multiple sites has been purified to homogeneity from frog eggs (34, 35). Cobb (17) has isolated an S6 kinase from the post-ribosomal supernatant of insulin-treated 3T3-L1 cells. The activity of this enzyme was stimulated nine fold in insulin-treated cells, but the sites of S6 phosphorylation were not determined.

The S6 kinase preparations display a number of similar characteristics. All the enzymes elute from DEAE cellulose chromatography between 100 to 150 mM salt. They also bind to cation exchange resins, with the exception of the activated S6 kinase preparation from insulin-treated 3T3-L1 cells. The enzymes also display an average Mr of 80,000 on gel filtration chromatography. In all cases examined, they catalyze phosphorylation of ribosomal protein S6 on multiple serine residues, which is comparable to the pattern observed in growth factor-stimulated cells. None of the enzymes can utilize GTP as the phosphate donor and they are inhibited by sodium flouride, β-glycerophosphate and phosphate salts. The enzyme from frog eggs, from 3T3-L1 cells and the protease-activated kinase II can all be activated in appropriately stimulated whole cells. The activated enzyme is lost during purification, if phosphatase inhibitors are not included in the purification buffers (17, 34, 35).

The greatest difference between the S6 kinase preparations is their relative substrate specificities. The

S6 kinase preparation employed in this study could catalyze the phosphorylation of histone H4, the synthetic peptides PK1 and Ser-47, or ribosomal protein S6 (24). The frog egg enzyme does not utilize histone H4 or any other common protein substrate, though it does utilize the synthetic peptide PK1. The protease-activated kinase II will catalyze the phosphorylation of eukaryotic initiation factor 2 as well as ribosomal protein S6. The 3T3-L1 cell S6 kinase preparation utilized ATP citrate lyase and histone H4 as protein substrates, but the activation of the enzyme in whole cells by insulin only generated a 1.5 to 2 fold increase in activity with these substrates. The increase in activity was 9 fold with S6 as the protein substrate. The specific activity of the S6 kinase preparations varies from 0.6 nmol/min-mg for the enzyme from insulin-treated 3T3-L1 cells to 300 nmol/min-mg for the enzyme from frog eggs. The S6 kinase preparation employed in this dissertation displayed a specific activity of 5-10 nmol/min-mg with histone H4 as the protein substrate and without trypsin activation.

A factor termed Activating Enzyme which apparently enhances the rate of MgATP activation of S6 kinase has been partially purified as previously reported (23, 24). This enzyme displays no phosphotransferase activity with histone, ribosomal protein S6, protamine, casein or phosvitin as the

protein substrate. Neither limited trypsin digestion nor inclusion of common protein kinase activators generated phosphotransferase activity of the Activating Enzyme toward these protein substrates. The only activity observed for this enzyme was the concentration-dependent activation of the S6 kinase preparation. The activation reaction requires MgATP and MnATP cannot be substituted. This is the same divalent cation requirement observed for the MgATP activation of S6 kinase in the absence of exogenous Activating Enzyme. An activator of S6 kinase which might be comparable to Activating Enzyme has not been reported by other investigators to date.

The heterogeneity of the final S6 kinase preparation precludes identification of the enzyme protein, possible subunit structure or regulatory components. The two major proteins are Mr 72,000 and Mr 55,000. However, the major phosphoproteins have Mr 56,000 and Mr 38,000, raising the possibility that the S6 kinase is a minor protein in the preparation. In addition, an Mr 95,000 protein present in the S6 kinase preparation is phosphorylated only in the presence of Activating Enzyme. Therefore, the Mr 95,000 protein is also a candidate for the S6 kinase, as Activating Enzyme may activate the S6 kinase via a phosphorylation reaction.

The experimental strategy employed in this study was to

produce monoclonal antibodies against proteins in the S6 kinase preparation. The specific aim was to obtain a panel of antibodies which would specifically detect single proteins in the S6 kinase preparation. This would allow regulatory as well as catalytic components of the enzyme system to be identified independently of enzyme activity. Monoclonal antibodies would also allow isolation of individual proteins and determination of their role in S6 kinase activity or regulation. This approach was chosen in preference to extensive purification of the S6 kinase activity, because the primary goal of this research is the delineation of regulatory mechanisms. The loss of regulatory properties of the S6 kinase during more extensive purification is a substantial risk. In fact, the fold MgATP activation of the S6 kinase preparation decreases at the last two steps of the purification protocol. In the absence of a method to monitor regulatory proteins, the extensive purification of S6 kinase activity may lead to the homogeneous preparation of a catalytic subunit. As an example, Maller and coworkers (34, 35) have purified an S6 kinase to homogeneity from frog oocytes. The enzyme displays a single protein with Mr 95,000 on SDS-PAGE, but no regulatory properties have been demonstrated.

A mouse immune serum response was obtained to an Mr 116,000 and Mr 72,000 protein in gel filtration fractions

prepared by the modified protocol. The Mr 116,000 protein elutes from the gel filtration chromatography with the two peaks of Activating Enzyme at the void volume and Mr 200,000. The Mr 72,000 protein elutes with the MgATP-activatable S6 kinase activity at Mr 85,000. An Mr 72,000 protein also elutes from gel filtration chromatography with the two peaks of Activating Enzyme, but the mouse immune serum did not detect this protein. On 10% or 12.5% SDS-PAGE analysis the two Mr 72,000 proteins are indistinguishable. The discrimination between these two proteins by the mouse immune serum suggests that they are different proteins. Alternatively, the antigenic site may be quite restricted and is not available in the form of the Mr 72,000 protein which coelutes with the Mr 116,000 protein.

The fusion experiment yielded monoclonal antibodies against the Activating Enzyme, which facilitated determination of this enzyme's structure and provided evidence for the mechanism of S6 kinase activation. A protein complex consisting of the Mr 116,000 and 72,000 proteins is observed when the S6 kinase preparation, Activating Enzyme or selected fractions from the gel filtration purification step are chromatographed on the 18-F9D-Sepharose antibody affinity resin. In all experiments the relative concentration of the two eluted

proteins, as estimated by Coomassie Blue staining, is apparently equal. The 18-F9D monoclonal antibody monospecifically recognizes the Mr 116,000 protein in Western Blot analyses. The data suggest that the Mr 72,000 protein is distinct from the Mr 116,000 protein and that it is the Mr 116,000 protein which specifically binds to the 18-F9D-Sepharose. The Mr 116,000 and 72,000 proteins form a complex with 1:1 stoichiometry which is not disrupted in the presence of 0.5 M NaCl and 0.05% Tween-20.

Immunodot, Western Blot and SDS-PAGE analysis of the individual gel filtration chromatography fractions were employed to determine the elution patterns for specific proteins and their correlation to enzyme activity. The Mr 116,000 and the nonimmunoreactive Mr 72,000 proteins elute in roughly equal quantities and are coincident with the Activating Enzyme activity. The highest concentration of the Mr 116,000 protein is eluted with the largest peak of Activating Enzyme activity with an elution Mr of 200,000. The largest portion of the Mr 116,000-72,000 protein complex is isolated from this peak by 18-F9D-Sepharose chromatography. The protein complex can also be isolated from the smaller peak of Activating Enzyme activity which elutes at the void volume. The Mr 116,000-72,000 protein complex isolated from either of these peaks contains Activating Enzyme activity. The protein complex is devoid

of S6 kinase activity and autophosphorylation is not observed. These data support the conclusion that the Mr 116,000-72,000 protein complex is the Activating Enzyme observed to elute from the gel filtration chromatography at the void volume and with Mr 200,000.

The molecular basis for elution of the Activating Enzyme as two forms from gel filtration chromatography is not clear at this time. However, this may be a significant feature of the enzyme as more than one form is also observed to elute from phosphocellulose chromatography and CM-Sephadex chromatography. In all cases, only the Mr 116,000-72,000 protein complex was present; however, the absolute number of subunits has not been determined. It is possible that the enzyme may exist as a dimer and a tetramer. In addition, a portion of the Activating Enzyme comigrates with the S6 kinase activity on phosphocellulose, CM-Sephadex and gel filtration chromatography. Interaction of a portion of the Activating Enzyme with the S6 kinase may influence its chromatographic behavior.

The S6 kinase preparation can be activated by preincubation with MgATP in the absence of exogenous Activating Enzyme. Although the S6 kinase preparation can utilize either MgATP or MnATP as the metal-nucleotide substrate, the autoactivation reaction requires MgATP. Enhancement of the rate of activation by Activating Enzyme

is also dependent on MgATP. These data suggest that the MgATP-dependent activation of S6 kinase preparation may be catalyzed by endogenous Activating Enzyme.

The 23-H10D monoclonal antibody recognizes an Mr 72,000 protein which elutes from gel filtration chromatography with an Mr 90,000 and corresponds to the peak of MgATP-activatable S6 kinase activity. The Mr 72,000 protein was obtained as a single protein from chromatography of the S6 kinase preparation on 23-H10D-Sepharose. Removal of this protein from the S6 kinase preparation results in a dramatic reduction of the MgATP-dependent activation of S6 kinase activity. Reconstitution of the system by addition of the Mr 72,000 protein restores the S6 kinase to full activity.

Other experiments performed in this laboratory (64) effected a more complete removal of the Mr 72,000 protein from the S6 kinase preparation. This was accompanied by the almost complete loss of MgATP-dependent activation of the S6 kinase activity. The S6 kinase preparation depleted of the Mr 72,000 protein could be activated by addition of Activating Enzyme to the MgATP preincubation reaction. These results suggested that the enzyme preparation had not been depleted in the protein kinase, but rather the activator had been removed. Similar results were obtained in experiments utilizing 40S ribosomal subunits as the protein substrate (64). The Mr 72,000 protein alone was

devoid of S6 kinase activity and autophosphorylation was not observed.

Experiments performed in this laboratory (64) also demonstrated that the Mr 72,000 protein obtained from 23-H10D-Sepharose chromatography could be labelled with fluorosulfonyl benzoyladenosine, 5'-p-[adenine-8-^{14}C] ([^{14}C]FSBA), indicating an ATP binding site. The Mr 72,000 protein from the Activating Enzyme Mr 116,000-72,000 protein complex was also labelled with [^{14}C]FSBA. These data support the conclusion that the Mr 72,000 protein is the endogenous activator in the S6 kinase preparation.

The simplest hypothesis is that the Mr 72,000 protein, which is the endogenous activator of the S6 kinase, is the catalytic subunit of the Mr 116,000-72,000 Activating Enzyme. However, the 23-H10D and 05-Y11B monoclonal antibodies fail to recognize the Mr 72,000 protein which elutes from gel filtration chromatography with the Activating Enzyme. Laser scanning densitometric analysis of SDS-PAGE proteins from individual gel filtration fractions reveals that the distribution of the Mr 116,000 protein closely parallels that of the nonimmunoreactive Mr 72,000 protein, suggesting that these proteins are present as the complex. The two monoclonal antibodies apparently recognize only the free Mr 72,000 protein.

The hypothesis that the free Mr 72,000 protein is the

catalytic subunit of the Mr 116,000-72,000 Activating Enzyme is supported by the observations that: 1) both preparations activate the S6 kinase, 2) the activation reaction in each case requires MgATP, 3) manganese cannot substitute for magnesium in either activation reaction, 4) the Mr 72,000 protein from both preparations was labelled with [^{14}C]FSBA, indicating an ATP binding site, 5) similar patterns of endogenous proteolysis are observed for the Mr 72,000 protein from both preparations.

S6 kinase activity in the cell is regulated in response to extracellular signals. The enzyme which directly activates the S6 kinase, must therefore be regulated as well. The identification of the Mr 72,000 protein as the catalytic subunit which can occur either free or complexed with the Mr 116,000 protein, leads to the hypothesis that the Mr 116,000 protein may represent a regulatory subunit. A model for regulation of the Activating Enzyme analogous to cyclic AMP-dependent protein kinase is considered. Allosteric or covalent modification of the Mr 116,000 regulatory subunit would lead to a release of the Mr 72,000 catalytic subunit from the complex.

Preliminary experiments have demonstrated phosphorylation of the Mr 116,000 protein by the insulin receptor kinase. The maximum stoichiometry of phosphorylation observed was 10%. This is a significant

degree of phosphorylation with a protein substrate concentration below 1 μM. S6 kinases are activated *in vivo* upon insulin or growth factor treatment of whole cells (8, 17, 69). However, the direct activation of the S6 kinase by the insulin receptor kinase *in vitro* has not been demonstrated. Based on data in this dissertation, the Activating Enzyme is a likely candidate for the link between the insulin-activated receptor kinase and the activated S6 kinase. Data from other laboratories have indicated the presence of a phosphoprotein with an approximate Mr of 116,000, which is a substrate for the insulin receptor kinase. An endogenous substrate for the insulin receptor kinase with an Mr 120,000 has been identified in whole cells by Rees-Jones and Taylor (75). This protein was not purified, thus neither its subunit structure nor its function was determined. Similar experiments by Van Obberghen and coworkers (79) have identified an endogenous substrate for the insulin receptor kinase with Mr 110,000.

The designation of the Mr 116,000 and 72,000 proteins as the regulatory and catalytic subunits, respectively, of Activating Enzyme, would suggest that the Mr 116,000-72,000 complex is a non-activated form of the enzyme. The data in this study are consistent with this hypothesis. The largest portion of the Mr 116,000-72,000 complex elutes from gel filtration chromatography with Mr 200,000. This peak of

Activating Enzyme overlaps the trypsin-dependent S6 kinase activity, which elutes at Mr 158,000. Incubation with MgATP fails to activate this peak of S6 kinase activity despite the presence of Activating Enzyme. Limited trypsin digestion is required for expression of the S6 kinase activity. These observations support the conclusion that the Mr 116,000-72,000 Activating Enzyme which elutes from gel filtration chromatography with Mr 200,000 is, in fact, an inactive form of the enzyme.

Activating Enzyme which elutes from gel filtration chromatography at the void volume and with Mr 200,000 does exhibit activity when assayed with the MgATP-activatable S6 kinase preparation. Analysis by 18-F9D-Sepharose chromatography and SDS-PAGE suggests that the predominant form of the Activating Enzyme in these two peaks is the Mr 116,000-72,000 complex. However, the MgATP-activatable S6 kinase preparation contains activated S6 kinase and the free Mr 72,000 protein, one of which apparently phosphorylates the Mr 116,000 subunit of the Activating Enzyme. This phosphorylation most likely occurs on a different site than observed with insulin receptor kinase. The S6 kinase specifically modifies serine residues, while the insulin receptor kinase modifies tyrosine residues. The functional consequence of the phosphorylation of Activating Enzyme catalyzed by either protein kinase has yet to be determined.

Activation of the Activating Enzyme may occur in response to either modification.

The antibody-purified Activating Enzyme also displays activity. Chromatography of the Activating Enzyme on 18-F9D-Sepharose yields an Mr 116,000 and Mr 72,000 protein. The evidence strongly supports the contention that these two proteins bind to the resin as a complex, as only the Mr 116,000 protein is recognized by the 18-F9D monoclonal antibody. However, the integrity of this association is unknown after elution from the resin at pH 2.2. The activity observed upon elution of the protein from the antibody resin may be due to subunit dissociation.

Identification of the proteins associated with Activating Enzyme has simplified identification of proteins associated with the S6 kinase activity. Three major proteins associated with S6 kinase activity with Mr 72,000, 95,000 and 55,000 were revealed by SDS-PAGE analysis of the individual gel filtration fractions. The Mr 72,000 protein is proposed to be the catalytic subunit of Activating Enzyme. Lasar scanning densitometry showed that the Mr 95,000 protein was entirely coincident with S6 kinase activity dependent on trypsin for expression. The Mr 55,000 protein was the only protein observed to be coincident with all S6 kinase activity. Experiments performed in this laboratory (64) demonstrated that both of these proteins

could be labeled with [^{14}C]FSBA. Based on these observations the catalytic subunit of S6 kinase is tentatively identified as the Mr 55,000 protein. The Mr 95,000 protein may be a regulatory subunit or a precursor form of the enzyme.

Additional evidence for the conclusion that the Mr 95,000 and 55,000 proteins are integral to the S6 kinase is their association as a complex in radioimmunoprecipitation experiments. The inclusion of the Mr 116,000 protein to the complex upon the addition of Activating Enzyme is evidence for the interaction of the two enzymes. The result of this interaction is the phosphorylation of the Mr 95,000 protein in the presence of Activating Enzyme. The phosphorylation of the Mr 55,000 protein also increases in the presence of Activating Enzyme. Activation of the S6 kinase may proceed via a phosphorylation on one of its subunits catalyzed by the Activating Enzyme.

The structure and Mr of the S6 kinase employed in this study can be compared to Mr determinations for other S6 kinases. The S6 kinase preparation employed in this study elutes from gel filtration chromatography as an inactive form with Mr 158,000 and as a MgATP-activatable form with Mr 85,000. MgATP activation of the enzyme prior to gel filtration chromatography resulted in a shift of the active S6 kinase elution to Mr 44,000 (23, 24). The inactive S6

kinase is associated with two proteins with Mr 95,000 and Mr 55,000 on SDS-PAGE, while the MgATP-activatable form is associated with the Mr 55,000 protein. The only S6 kinase which has been purified to homogeneity to date (from frog eggs) displays an Mr of 95,000 on SDS-PAGE and Mr 55,000 on glycerol gradients (32, 33). The enzyme elutes from gel filtration chromatography with Mr 70,000-80,000. The partially purified protease-activated kinase II displays an Mr of 80,000 on gel filtration chromatography (55). The Mr shifts to 35,000 if the enzyme is trypsin activated prior to the chromatography. The S6 kinase preparation isolated from insulin-treated 3T3-L1 cells displays an Mr of 70,000 on gel filtration chromatography, and an Mr of 55,000-65,000 in glycerol gradient analysis (17). The behavior of these S6 kinase preparations on gel filtration chromatography is substantially similar. However, more insightful comparisons await reports of the behavior of the purified enzymes on SDS-PAGE.

An immune response to the S6 kinase apparently was not achieved. The mouse immune serum did not inhibit the S6 kinase reaction. The immune serum also did not display crossreactivity with S6 kinase prepared from mouse lymphosarcoma in Western Blot analysis. An antigen which was coincident with either trypsin-dependent S6 kinase or total S6 kinase activity was not detected with immune serum

or monoclonal antibody in Western Blot or immunodot analyses. Identification of the Mr 55,000 protein as the catalytic subunit of S6 kinase presents a ready explanation for the failure to obtain an immune response to this enzyme. This failure may be the result of tolerization of the mouse at the time of immunization (2). An appropriate amount of a single antigen for mouse immunization is in the range of 1-50 µg. The quantity of Mr 55,000 protein introduced to the mouse was in the range of 150-400 µg per injection.

In contrast to the absence of an immune response to the S6 kinase, a strong immune response was obtained against the Activating Enzyme. The strongest immunoreactivity observed in Western Blot analysis of gel filtration fractions with mouse immune serum was against the Mr 116,000 protein, a protein which was not apparent in Coomassie Blue stained SDS-PAGE of the S6 kinase preparation used for immunization. The strongest immunoreactivity observed in Western Blot analysis of the S6 kinase preparation used for immunization with mouse immune serum was a doublet at Mr 80,000-83,000. This doublet was lost in favor of the Mr 116,000 antigen in Western Blot analysis of gel filtration chromatography fractions of enzyme prepared by the modified method. The enzyme used for immunization was prepared by the unmodified protocol and proteolysis was apparent. It is proposed that the Mr 80,000-83,000 protein served as the antigen in the

mouse immunization for antibody raised against the Mr 116,000 protein.

Three mechanisms for the activation of S6 kinase by the Activating Enzyme have been considered: proteolysis, allosteric binding and phosphorylation. The Mr shift of activated S6 kinase preparation on gel filtration chromatography coupled with the ability to activate the S6 kinase preparation by limited trypsin treatment supports an activation by proteolysis. Although activation of the S6 kinase preparation _in vitro_ by trypsin no doubt involves limited proteolytic cleavage which may release a regulatory subunit or loosen a regulatory domain, this is not an expected mechanism of _in vivo_ regulation due to its irreversible nature. In addition, work performed in this laboratory (23, 24) has demonstrated that a range of protease inhibitors failed to inhibit the MgATP-dependent activation of the S6 kinase preparation with exogenous Activating Enzyme. Furthermore, Mr shifts of major proteins are not observed by SDS-PAGE of the S6 kinase preparation activated by Activating Enzyme. The Mr shift observed on gel filtration chromatography upon activation of the S6 kinase preparation may be due to a conformational change or the loss of a regulatory subunit.

The available data are consistent with activation of the S6 kinase by the allosteric binding of the Mr 72,000

protein of Activating Enzyme or via a phosphorylation of the S6 kinase by Activating Enzyme. The preponderance of the data favor activation via a phosphorylation reaction as follows: 1) the activation reaction requires MgATP, 2) the Mr 72,000 protein of Activating Enzyme contains an ATP binding site, and 3) Activating Enzyme promotes the phosphorylation of S6 kinase preparation proteins. The increase in phosphorylation of the S6 kinase proteins by Activating Enzyme is much less than the concommitant increase in S6 kinase activity. This is the expected observation for a cascade type of activation.

The characteristics of the Activating Enzyme are analogous to cyclic AMP-dependent protein kinase. The structure of Activating Enzyme consists of two dissimilar subunits, one of which contains an ATP binding site. It has been demonstrated that the subunit with the ATP binding site can occur as a free protein. The free subunit displays S6 kinase activating activity and is the endogenous activator in the S6 kinase preparation. Other data which support this hypothesis are that the maintenance of active S6 kinase preparations from growth factor-treated cells requires the presence of phosphatase inhibitors (35, 69). This suggests that the phosphorylation of a site on the S6 kinase is required for the activated form of the enzyme. The evidence supports the hypothesis that the Activating Enzyme catalyzes

this phosphorylation of the S6 kinase.

The multiple effects of insulin on cells are not necessarily mediated by a single pathway. Recent work by Saltiel et al. (80) has identified a glycolipid which may act as a second messenger in the activation of the cyclic AMP-phosphodiesterase in response to insulin. However, the work presented in this dissertation combined with previous work in this and other laboratories suggests a direct phosphorylation cascade by which the insulin signal is translated to the substrate level phosphorylation of ribosomal protein S6. The mechanism of enzyme activation at each level of the cascade remains to be fully elucidated; however, the sequence of regulatory events can be postulated as follows: the binding of insulin to its receptor results in activation of the receptor associated tyrosine kinase. The Activating Enzyme is phosphorylated on the Mr 116,000 subunit by the receptor kinase and subsequently activates the S6 kinase by a phosphorylation. The activated S6 kinase accomplishes the multisite phosphorylation of ribosomal protein S6 which is observed in insulin-treated cells. The activation of S6 kinase proposed in this cascade is similar to the activation of phosphorylase kinase by cyclic AMP-dependent protein kinase. The major difference between the epinephrine and insulin systems is the replacement of the second messenger-regulated protein kinase step in the

epinephrine-initiated cascade with a receptor-associated protein kinase, which is directly activated by hormone binding in the insulin-initiated cascade.

The work presented in this dissertation makes a significant contribution to the knowledge of S6 kinase regulation and the mechanism by which substrate level phosphorylation is regulated by insulin in whole cells. Production of monoclonal antibodies against the Activating Enzyme has allowed determination of this protein's structure and function. The structure of the Activating Enzyme consists of a catalytic and regulatory subunit. The ability of the regulatory subunit to serve as a substrate for the insulin receptor kinase has been demonstrated. Identification of the Activating Enzyme catalytic subunit allowed this protein to be isolated from the S6 kinase preparation, and thus elucidate the mechanism of MgATP activation of the S6 kinase preparation. The subunits of the S6 kinase have also been tenatively identified, providing a firm basis for the further investigation of this enzyme's regulation. Knowledge of the subunit structure of the two enzymes has allowed a more accurate interpretation of autophosphorylation data. This has led to the hypothesis that the Activating Enzyme actually catalyzes the phosphorylation of a subunit of the S6 kinase. The definition of each component in this enzyme system has led

to a postulated insulin-intiated phosphorylation cascade, which can be tested at every level.

BIBLIOGRAPHY

Books

1. Bielka, Heinz, editor (1982), The Eukaryotic Ribosome, New York, Springer-Verlag.

2. Burnet, Macfarlane (1969), Self and Not-Self, Carlton, Australia, Melbourne University Press.

Articles

3. Adelstein, R. S. (1983), J. Clin. Invest., 72:1863-1866.

4. Ahmad, A., Camici, M., DePaoli-Roach, A. A. and Roach, P. J. (1984), J. Biol. Chem., 259:3420-3428.

5. Anderson, C. W., Baum, P. K. and Gesteland, R. F. (1973), J. Virol., 12:241-252.

6. Benjamin, W. B. and Singer, I. (1974), Biochim. Biophys. Acta, 252:28-42.

7. Blackshear, P. J., Witters, L. A., Girard, P. R., Kuo, J. F. and Quamo, S. N. (1985), J. Biol. Chem., 260:13304-13315.

8. Blenis, J. and Erikson, R. L. (1985), Proc. Natl. Acad. Sci. USA, 82:7621-7625.

9. Bradford, M. M. (1976), Anal. Biochem., 72:248-254.

10. Brownsey, R. W., Belsham, G. J. and Denton, R. M. (1981), FEBS Lett., 124:145-150.

11. Brownsey, R. W. and Denton, R. M. (1982), Biochem. J., 202:77-86.

12. Brownsey, R. W., Edgell, N. J., Hopkirk, T. J. and Denton, R. M. (1984), Biochem. J., 218:733-743.

13. Brownsey, R. W. and Hardie, D. G. (1980), FEBS Lett., 120:67-70.

14. Burkhard, S. J. and Traugh, J. A. (1983), J. Biol. Chem., 258:14003-14008.

15. Butcher, R. W., Robinson, G. A. and Sutherland, E. W. (1972), Biochemical Actions of Hormones, 2:21-50.

16. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982), J. Biol. Chem., 257:7847-7851.

17. Cobb, M. H. (1986), J. Biol. Chem., 261:12994-12999.

18. Cobb, M. H. and Rosen, O. M. (1984), Biochim. Biophys. Acta, 738:1-8.

19. Cochet, C., Gill, G. N., Meisenhelder, J., Cooper, J. A. and Hunter, T. (1984), J. Biol. Chem., 259:2553-2558.

20. Cohen, S., Carpenter, G. and King, L. (1980), J. Biol. Chem., 255:4834-4842.

21. Cohen, S., Ushiro, H., Stoscheck, C. and Chinkers, M. (1982), J. Biol. Chem., 257:1523-1531.

22. de la Houssaye, B. A., Echols, T. K. and Masaracchia, R. A. (1983), J. Biol. Chem., 258:4272-4278.

23. de la Houssaye, B. A., Murdoch, F. E. and Masaracchia, R. A. (1986), Fed. Proc., 45:1862.

24. de la Houssaye, B. A., Murdoch, F. E. and Masaracchia, R. A. (1986), Submitted.

25. Del Grande, R. W. and Traugh, J. A. (1982), Eur. J. Biochem., 123:421-428.

26. Denton, R. M., Brownsey, R. W. and Belsham, G. J. (1981), Diabetologia, 21:347-362.

27. de St. Groth, S. F. and Scheidegger, D. (1980), J. Immunol. Meth., 35:1-21.

28. Donahue, M. J. and Masaracchia, R. A. (1984), J. Biol. Chem., 259:435-440.

29. Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., Devare, S. G., Robbins, K. C., Aaronson, S. A. and Antoniades, H. N. (1983), Science, 221:275-277.

30. Downward, J., Yasden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M. W. (1984), Nature, 307:521-527.

31. Du Vernay, V. H., Jr. and Traugh, J. A. (1978), Biochemistry, 17:2045-2049.

32. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J-H., Masiarz, R., Kan, Y. W., Goldfine, I. D., Roth, R. A. and Rutter, W. J. (1985), Cell, 40:747-758.

33. Eckhart, W., Hutchinson, M. A. and Hunter, T. (1979), Cell, 18:925-933.

34. Erikson, E. and Maller, J. L. (1985), Proc. Natl. Acad. Sci. USA, 82:742-746.

35. Erikson, E. and Maller, J. L. (1986), J. Biol. Chem., 261:350-355.

36. Flockhart, D. A., Watterson, D. M. and Corbin, J. D. (1980), J. Biol. Chem., 255:4435-4440.

37. Fritz, I. B. (1972), Biochemical Actions of Hormones, 2:166-209.

38. Galfre, G. and Milstein, C. (1981), Meth. Enzymol., 73B:3-46.

39. Gilman, A. G. (1984), J. Clin. Invest., 73:1-4.

40. Gordon, J., Nielsen, P. J., Manchester, K. L., Towbin, H., Jimenez de Asua, L. and Thomas, G. (1982), Cur. Top. Cell. Reg., 21:89-98.

41. Hassell, T. C., Kemp, B. E. and Masaracchia, R. A. (1986), Biochem. Biophys. Res. Commun., 134:240-247.

42. Hathaway, G. M. and Traugh, J. A. (1979), J. Biol. Chem., 254:762-768.

43. Hayakawa, T., Perkins, J. P. and Krebs, E. G. (1973), Biochemistry, 12:574-580.

44. Hazen, R. J. and Huang, L. C. (1978), Adv. Cyclic Nucleotide Res., 9:425-439.

45. Heldin, C-H., Ek, B. and Ronnstrand, L. (1983), J. Biol. Chem., 258:10054-10061.

46. Herrera, R., Petruzzelli, L., Thomas, N., Bramson, H. N., Kaiser, E. T. and Rosen, O. M. (1985), Proc. Natl. Acad. Sci. USA, 82:7899-7903.

47. Hunter, T. and Sefton, B. M. (1980), Proc. Natl. Acad. Sci. USA, 77:1311-1315.

48. Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1976), J. Biol. Chem., 251:4476-4478.

49. Kasuga, M. Karlsson, F. A. and Kahn, C. R. (1982), Science, 215:185-187.

50. Kasuga, M., Zick, Y., Blith, D. L., Karlsson, F. A., Haring, H. U. and Kahn, C. R. (1982), J. Biol. Chem., 257:9891-9894.

51. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980), J. Biol. Chem., 255:2273-2276.

52. Krebs, E. G. and Beavo, J. A. (1979), Annu. Rev. Biochem., 48:923-959.

53. Laemmli, U. K. (1970), Nature, 227:680-685.

54. Lake, R. S. (1973), J. Cell. Biol., 58:317-331.

55. Larner, J., Galasko, G., Cheng, K., DePaoli-Roach, A. A., Huzng, L., Daggy, P. and Kellogg, J. (1979), Science, 206:1408-1410.

56. Lastick, S. M. and McConkey, E. H. (1981), J. Biol. Chem., 256:583-585.

57. Le Peuch, C. J., Ballester, R. and Rosen, O. M. (1983), Proc. Natl. Acad. Sci. USA, 80:6858-6862.

58. Lubben, T. H. and Traugh, J. A. (1983), J. Biol. Chem., 258:13992-13997.

59. Magnino, P. E., de la Houssaye, B. A. and Masaracchia, R. A. (1983), Biochem. Biophys. Res. Commun., 116:675-681.

60. Maller, J. L., Pike, L. J., Freidenberg, G. R., Cordera, R., Stith, B. J., Olefsky, J. M. and Krebs, E. G. (1986), Nature, 320:459-461.

61. Martin-Perez, J., Siegmann, M. and Thomas, G. (1984), Cell, 36:287-294.

62. Masaracchia, R. A., Kemp, B. E. and Walsh, D. A. (1977), J. Biol. Chem., 252:7109-7117.

63. Merril, C. R., Goldman, D., Sedman, S. A. and Ebert, M. H. (1981), Science, 211:1437-1438.

64. Murdoch, F. E., Magnino, P. E., de la Houssaye, B. A. and Masaracchia, R. A. (1986), Submitted.

65. Nishikawa, M., Hidaka, H. and Adelstein, R. S. (1983), J. Biol. Chem., 258:14069-14072.

66. Nishimura J., Huang, J. S. and Deuel, T. F. (1982), Proc. Natl. Acad. Sci. USA, 79:4303-4307.

67. Nishizuka, Y. (1984), Nature, 308:693-697.

68. Nishizuka, Y. (1984), Science, 225:1365-1370.

69. Novak-Hofer, I. and Thomas, G. (1985), J. Biol. Chem., 260:10314-10319.

70. Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D. and Ullrich, A. (1986), Science, 233:853-858.

71. Pelech, S. L., Olwin, B. B. and Krebs, E. G. (1986), Proc. Natl. Acad. Sci. USA, 83:5968-5972.

72. Perisic, O. and Traugh, J. A. (1983), J. Biol. Chem., 258:9589-9592.

73. Perisic, O. and Traugh, J. A. (1983), J. Biol. Chem., 258:13998-14002.

74. Pires, E. M. V. and Perry, S. V. (1977), Biochem. J., 167:137-146.

75. Rees-Jones, R. W. and Taylor, S. I. (1985), J. Biol. Chem., 260:4461-4467.

76. Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1971), J. Biol. Chem., 246:1986-1995.

77. Roach, P. J. (1981), Curr. Top. Cell. Regul., 20:45-105.

78. Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M. and Cobb, M. H. (1983), Proc. Natl. Acad. Sci. USA, 80:3237-3240.

79. Sadoul, J-L., Peyron, J-F., Ballotti, R., Debant, A., Fehlmann, M. and Van Obberghen, E. (1985), Biochem. J., 227:887-892.

80. Saltiel, A. R., Fox, J. A., Sherline, P. and Cuatrecasas, P. (1986), Science, 233:967-972.

81. Scholey, J. M. (1986), Nature, 320:215-216.

82. Seals, J. R. and Czech, M. P. (1980), J. Biol. Chem., 255:6529-6531.

83. Sheorain, V. S., Ramakrishna, S., Benjamin, W. B. and Soderling, T. R. (1985), J. Biol. Chem., 260:12287-12292.

84. Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T. and Stanley, E. R. (1985), Cell, 41:665-676.

85. Shia, M. A. and Pilch, P. F. (1983), Biochemistry, 22:717-721.

86. Shoemaker, C. B. and Chalkley, R. (1978), J. Biol. Chem., 253:5802-5807.

87. Shulman, M., Wilde, C. D. and Kohler, G. (1978), Nature, 276:269-270.

88. Smith, C. J., Wejksnora, P. J., Warmer, J. B., Rubin, C. S. and Rosen, O. M. (1979), Proc. Natl. Acad. Sci. USA, 76:2725-2729.

89. Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkeler, F. L., Walsh, D. A. and Krebs, E. G. (1970), J. Biol. Chem., 245:6317-6328.

90. Stadtmauer, L. A. and Rosen, O. M. (1983), J. Biol. Chem., 258:6682-6685.

91. Szewczyk, B. and Kozloff, L. M. (1985), Anal. Biochem., 150:403-407.

92. Tabarini, D., Heinrich, J. and Rosen, O. M. (1985), Proc. Natl. Acad. Sci. USA, 82:4369-4373.

93. Tahara, S. M. and Traugh, J. A. (1981), J. Biol. Chem., 256:11558-11564.

94. Tahara, S. M. and Traugh, J. A. (1982), Eur. J. Biochem., 126:395-399.

95. Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977), J. Biol. Chem., 252:7603-7609.

96. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1976), J. Biol. Chem., 251:3692-3695.

97. Thomas, G., Martin-Perez, J., Seigmann, M. and Otto, A. M. (1982), Cell, 30:235-242.

98. Traugh, J. A. and Porter, G. G. (1976), Biochemistry, 15:610-616.

99. Traugh, J. A. and Traut, R. R. (1974), J. Biol. Chem., 249:1207-1212.

100. Trevillyan, J. M., Perisic, O., Traugh, J. A. and Byus, C. V. (1985), J. Biol. Chem., 260:3041-3044.

101. Tuazon, P. T., Merrick, W. C. and Traugh, J. A. (1980), J. Biol. Chem., 255:10954-10958.

102. Ushiro, H. and Cohen, S. (1980), J. Biol. Chem., 255:8363-8365.

103. Villar-Palasi, C. and Larner, J. (1971), Curr. Top. Cell. Regul., 3:195-236.

104. Walseth, T. and Johnson, R. (1979), Biochim. Biophys. Acta, 526:11-31.

105. Walsh, M. P., Dabrowski, R., Hinkins, S. and Hartshorne, D. J. (1982), Biochemistry, 21:1919-1925.

106. Wettenhall, R. E. H. and Cohen, P. (1982), FEBS Lett., 140:263-269.

107. Witte, O. N., Dasgupta, A. and Baltimore, D. (1980), Nature, 283:826-831.