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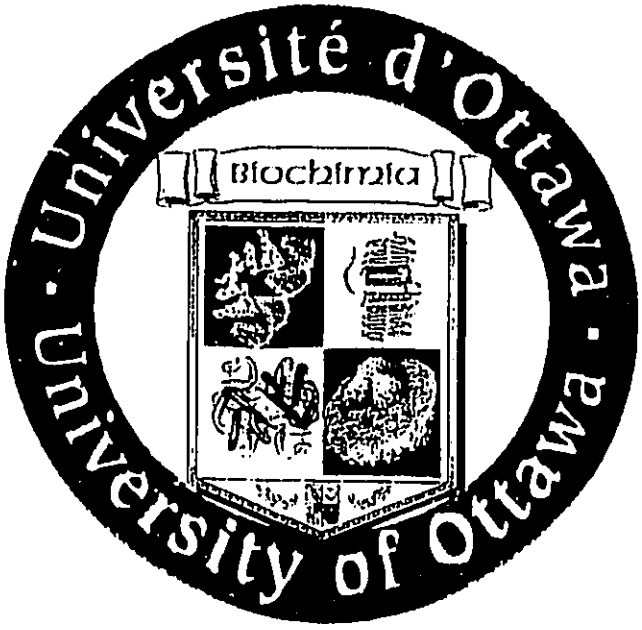
A Protein Tyrosine Kinase Associated with the ATP-dependent Inactivation of Adipose Diacylglycerol Acyltransferase[1]

by

Timothy E. Lau*

A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfilment of the requirements for the degree of Master of Science



University of Ottawa, Department of Biochemistry, Faculty of Medicine
Ottawa, Ontario, Canada, K1N 6N5.

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When you consider physical and chemical reactions,
molecules and energy, their complex interaction,
remember that our bodies are just collections
of atoms in nerves and tissues, in cross section.

But what about free will which we exert each day,
can you spend your life without having to say,
"I could do this or I could do that.
Can I deny my conscience by turning my back"?

Many believe our choices are made,
when possible options are visibly laid,
based on heridity, environment and something unknown,
all acting together, or separately on their own

If that unknown component were proven not to exist
our immoral decisions would be reduced to a societal cyst.
All of man's free choices would, in essence, become
actions of molecules their interaction the sum

ABSTRACT

An enzyme activity that reversibly inactivates adipose glycerolphosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT), *in vitro*, in the presence of ATP, has been partially purified from adipose tissue with an apparent molecular weight of 68 kDa. The activity responsible for inactivating DGAT is associated with a kinase activity as determined by phosphate incorporation into microsomes and a tyrosine containing peptide. Major substrates of this kinase are two microsomal polypeptides of 53 and 69 kDa. Both DGAT inactivation and kinase activities assayed from the purified sample and the cytosol, have been found to be insensitive to the Ser/Thr kinase inhibitor H-7 while being sensitive to the inhibitors genistein and tyrphostin 25. A crude protein phosphatase preparation from the liver was capable of reversing the effects of both activities. The purified sample was also shown to inactivate GPAT in the presence of ATP. These results suggest that a protein tyrosine kinase, in concert with a protein tyrosine phosphatase, may regulate the activities of DGAT and GPAT by a phosphorylation-dephosphorylation mechanism.

DEDICATION

This thesis is dedicated to the ones I love, my parents who instilled in me the importance of striving for excellence and to Amy who was there to support me by her understanding, encouragment and patience but whose name says it all. I would also like to dedicate this thesis to my supervisor for giving me a project that worked out so well. I can appeciate how the best supervisors and teachers often never get the recognition they deserve.

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1
INTRODUCTION

Physiological relevance of adipose TAG synthesis. Triacylglycerols are actively synthesized in the cells of vertebrates predominately in the liver and adipose tissue. In higher animals the highly specialized adipocyte is the primary repository of energy, stored as triacylglycerol in the intracellular lipid droplet. White adipose tissue has evolved, particularly in mammals for the storage of long chain fatty acids in times of energy surplus and the mobilization of fatty acids out of the triacylglycerol stores in times of anticipated or actual energy demand. Whereas in a normal day up to 80% of the energy requirements of a man can be met by fatty acids (1), in times of energy surplus and the requirement for net fat deposition, rat adipocytes can increase their triacylglycerol content by an incredible 5% per hour (2). There is a continuous turnover of the stores, but net mobilization or deposition will depend on the balance of the relative activities of the hydrolytic and esterification processes. Although the enzymes involved in both the synthesis and lipolysis are known, the molecular process by which co-ordinated regulation is achieved remains a mystery. The elucidation of these processes may be important for understanding not only how organisms regulate their daily energy requirements and hence general metabolism, but also disorders of excessive lipid storage such as obesity or the severe depletion of energy reserves, such as occurs with the cachexia of chronic illness. Moreover abnormal lipid depositions

have been identified in a number of pathophysiological conditions in which cells may assume adipocyte characteristics, including foam cells of athersclerotic plaques (3,4) and certain rare conditions such as ichthyosis in which a variety of cells become lipid laden (5).

As mentioned above, the amount of FA released by adipose tissue into circulation is determined by the balance between TAG synthesis and hydrolysis. Recent reports suggests that plasma levels of free fatty acids are involved in the regulation of lipid and glucose oxidation and of glycogen synthesis in humans [6,7]. Impaired FA delivery to the adipose tissue, low levels of adipose TAG synthesis due, for instance to the abnormal regulation of the enzymes in the TAG synthetic pathway, may cause an imbalance of lipolysis and esterification preventing the uptake of fatty acids into adipose tissue. As a consequence, more fatty acids might either be released from adipose tissue or fail to be stored there, in either case reaching the liver and causing hepatic TAG synthesis to increase and VLDL secretion to rise. *In vitro* stimulation of VLDL synthesis and secretion in hepatocytes incubated with FA has been shown (8-10). Thus it has been suggested that hypertriglyceridemia (hyper TG) and hyperapobetalipoproteinemia (hyperapoB) might often be due to a reduced rate of adipose tissue TAG synthesis (11-17) . This may thereby result in an array of pathological conditions from

obesity to athersclerosis. For instance it has been shown that *in vitro* incorporation of exogenous fatty acid into diacylglycerol and triacylglycerol proceeds at a slower rate in adipose tissue as well as in fibroblasts from hyperapoB patients in whom hyperTG is also frequent (17,18). Moreover as will be discussed later high levels of plasma fatty acid could also explain some cases of diabetes (NIDDM) associated with obesity.

Liver vs. adipose TAG synthesis. Considering their different respective roles in metabolism, TAG synthesis in liver and white adipose tissue must be regulated in a different manner since conditions of adipose lipolysis correspond to hepatic esterification, as exemplified in the fasting state when TAG synthesis and subsequent packaging into VLDL is elevated while in adipose tissue net TAG synthesis is reduced (19). In liver it has been proposed that control of glycerolipid synthesis is significantly influenced in a manner secondary to events regulating the alternative fate of fatty acids in that tissue, namely β-oxidation (20). This is most likely not the case in white adipose tissue since rates of fatty acid oxidation are extremely low relative to esterification (21) and therefore changes in oxidation would have neglible effects on esterification. Whereas most of the previous work on the regulation of TAG synthesis has been performed on the liver, we focused on adipose tissue where TAG synthesis is much higher and plays a central role in cell function. Since the regulation in both tissues is expected to be different,

precaution is required when comparing results from both tissues. However, it would be interesting to perform comparative studies between both tissues.

Regulation of TAG synthesis and lipolysis. It is generally accepted that the coordinated hormonal regulation of both lipolysis and synthesis allows the adipocyte to respond rapidly and efficiently to the energy demands of the organism while reducing to a minimum the energy waste associated with the futile cycle of fatty acid esterification and hydrolysis. Although the molecular mechanisms of the hormonal regulation of lipolysis have been extensively studied and generally agreed upon, the molecular events involved in the hormonal regulation of triacylglycerol remains undetermined.

The rate of triacylglycerol mobilization of fatty acids from adipose tissue is dependent upon the interplay between a number of factors both in the short and long term which have been elucidated in the early eighties (22-24). The central feature of lipolysis is the hormone sensitive lipase (HSL) which catalyzes the hydrolysis of triacylglycerol to diacylglycerol and diacylglycerol to monoacylglycerol. The hydrolysis of the resulting monoacylglycerol is mainly catalyzed by a separate monoacylglycerol lipase. Hormone sensitive lipase is activated by protein kinase A (PKA) (25-29) and it is now established that this is due to the phosphorylation of the enzyme at a single serine residue (30). Dephosphorylation and deactivation

of the lipase can be achieved by cellular protein phosphatases 1A, 2A, and 2C(24).

An assortment of lipolytic and antilipolytic agents acting through plasma membrane receptors (32) are able to influence the activity state of adenylate cyclase, through G-proteins, and thereby the level of cAMP and the activity of protein kinase A (PKA). The most frequently considered lipolytic hormones are adrenaline and noradrenaline (acting at β-adrenoreceptors), corticotropin, and glucagon all of whose receptors are coupled to the catalytic subunit of adenylate cyclase by its stimulatory G-protein (33). On the other hand, receptors for antilipolytic agents such as adenosine, PGE's, and nicotinate are coupled to adenylate cyclase by an inhibitory G-protein (33). The antilipolytic action of insulin, on the other hand, may in part be attributable to the decrease in cAMP levels [34] and PKA activity, although part of the hormone's effect is cAMP independent and is suggested to be due to the activation of phosphoprotein phosphatases (35).

A majority of the research focusing on the hormonal regulation of TAG synthesis was also performed from 1975-1985, but progress was hampered by the fact that the corresponding enzymes are integral membrane proteins and remain to be purified. As will be discussed below the research was focused on the

involvement of PKA and little attention was given to other possible phosphorylation systems. The existence of long term (e.g., starvation) and short term (i.e., hormonal) regulation of adipose triacylglycerol synthesis is well documented although the particulars are unknown. Starvation and streptozotocin diabetes result in decreased synthetic enzyme activities (2). Traditionally, changes in circulating levels of insulin in response to variations in blood glucose concentration, have been considered the physiological signal determining the conversion between glucose and fatty acid metabolism. In addition to increases in LPL activity/secretion and substrate transport in response to glucose and insulin, adipose tissue responds to that switch by modifying the rate of fatty acid release(2). There appears to be evidence that adipose tissue can overide the glucose-insulin signal system and impose fatty acid utilization even in the face of high levels of circulating glucose and insulin (7), as it seems to be the case of obesity-related insulin resistance which, in some cases, may be secondary to high levels of circulating fatty acids.

The rate of esterification of long chain fatty acids into neutral glycerides is dependent on the availibility of the respective substrates and the *in situ* activity of the participating enzymes(36). Regulation of esterification could either occur via changes in substrate availability (or delivery) and/or enzyme modification. However some have suggested that TAG synthesis is regulated **exclusively** by

substrate availability but this is unlikely for the following reasons.

1) Regulation by fatty acid availability alone should result in changes in levels of intermediates in response to changes in the delivery of substrates. Some of these intermediates are toxic to the cell and there appears to be no significant changes in their levels(36,37).

2) Moreover, increased levels of lipolytic hormones and/or diminished levels of insulin cause triacylglycerol mobilization, and *in vitro* a certain proportion of mobilized fatty acid is recycled into triacylglycerol when adipocytes are incubated in closed systems (38-40). Although *in vivo* blood flow may diminish this to an extent, there is nevertheless evidence for triacylglycerol/fatty acid substrate cycling (41). It has been suggested previously that rapid down regulation of the TAG synthetic enzyme activities under lipolytic conditions is important to restrain wasteful recycling of mobilized fatty acids back into triacylglycerol stores (2).

3) Experiments have shown the modification of enzyme activities and the corresponding changes in net TAG synthesis during both fed/fasting states and hormonal treatment (2). This implies a direct regulation of the enzymes as opposed to regulation of TAG synthesis by the availability of respective substrates.

It is therefore believed that regulation occurs by substrate availability in addition to changes in the activities of the corresponding enzymes. There is no clear rate limiting step (except possibly at the DGAT step (36)) in the synthesis pathway and the enzymes appear to be functioning well below saturating

conditions (37). Since hormonal regulation at a single step would also result in an accumulation of intermediates which is not observed (36,37), it is logical to assume that regulation occurs at more than one step by the same hormonal signal. This may be accomplished through a phosphorylation/dephosphorylation of multiple enzymes in the synthetic pathway by a kinase/phosphatase system.

Other mechanisms of TAG synthesis regulation may involve control of the delivery of fatty acid and product inhibition. The mechanism of regulation of both transport of extracellular fatty acid to TAG synthesis loci and the TAG synthetic enzymes are not yet clear. Whatever the transport mechanism, there appears to be in the cell a free fatty acid pool exchangeable with the extracellular fatty acid pool. The intracellular pool itself may bind reversibly to cellular components, both membranes and proteins (42). This pool must somehow be compartmentalized, since exogenous radiolabelled fatty acid can be readily esterified before the complete mixture with the intracellular free fatty acids is attained (43). The distribution of free fatty acids between the different compartments may play an important role in the regulation of fatty acid release and uptake. For instance under lipolytic conditions and in the absence of albumin in the medium, the accumulation of free fatty acid in the adipocytes inhibits lipolysis (42,45). It has been shown that incubation of adipocyte microsomes with micromolar concentrations of oleic acid results in the inhibition of fatty acid esterification to

either glycerol phosphate or diglycerides (46). Another possible mechanism which has been suggested by Abumrad et al. is the catecholamine-mediated stimulation of fatty acid transport out of the adipocyte (47). Thus the results suggest that free fatty acid and its delivery may play a role in the modulation of TAG synthesis enzymes.

Additionally fatty acid binding proteins (FABP's) have been proposed to be involved in the transport of free fatty acid across the plasma membrane and the cytosol to the ER for esterification, and to the mitochondria and peroxisomes for oxidation (48,49). Fatty acids bound to these FABPs may account for a good part of the active intracellular pool of free fatty acids. FABPs as well as other uncharacterized cytosolic proteins, have been shown to stimulate the TAG synthetic enzymes (50-55), although it is not clear whether they cause their effect by improving substrate delivery or by interacting with the synthetic enzymes and modifying their activities. FABP may also modulate lipid synthesis by preventing excessive accumulation of fatty acid in the ER membrane.

Adipose TAG synthesis pathway. Adipose tissue glycerolipid synthesis is primarily devoted to the synthesis of TAGs with only a small fraction of the pathway flux arising in phospholipid products (2)(refer to the pathways of glycerolipid synthesis shown in Figure 1). The intracellular lipid droplet where

TAGs are stored has at its periphery a complex network of filaments (56,57) as well as endoplasmic reticulum cisternae and tubules (58,59) which may extend into the core of the lipid droplet forming aqueous channels (59). In metabolically active adipocytes the luminal leaflet of the channels contain fatty acid products of triacylglycerol hydrolysis which are visualized under appropriate preparatory procedures as lamellar whorls (59), or lipid domains within the membrane leaflet (60). TAG formation in adipose tissue takes place on the ER membranes primarily through the glycerol phosphate pathway supplemented by the dihydroxyacetone pathway (2,61). The sequential enzyme-facilitated esterification of long chain fatty acids in the biosynthesis of triacylglycerols in adipose tissue is depicted in Figure 2. The effect of a number of hormones on various TAG synthetic enzymes have been studied including glucocorticoids, catecholamines, glucagon, insulin, and growth hormone. A recent study with obese premenopausal women suggests that triglyceride synthesis is shut down in adipose tissue following growth hormone administration, an effect that is not secondary to its affects on lipolysis [62]. Assuming there is hormone mediated multi-step regulation of the pathway by enzyme modifications, then at which points in the synthetic pathway will regulation occur?

Figure 1. Pathways of Glycerolipid Synthesis

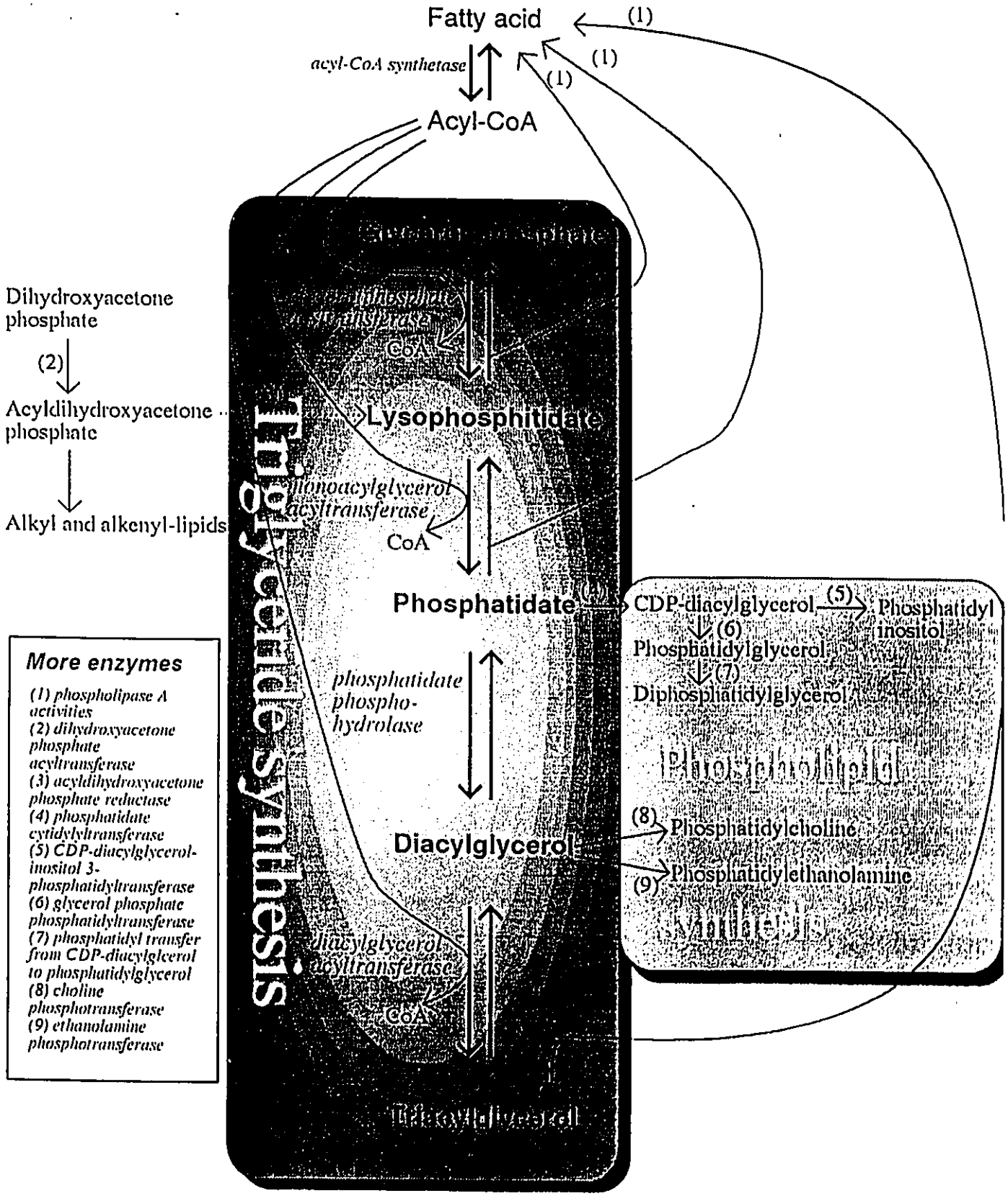
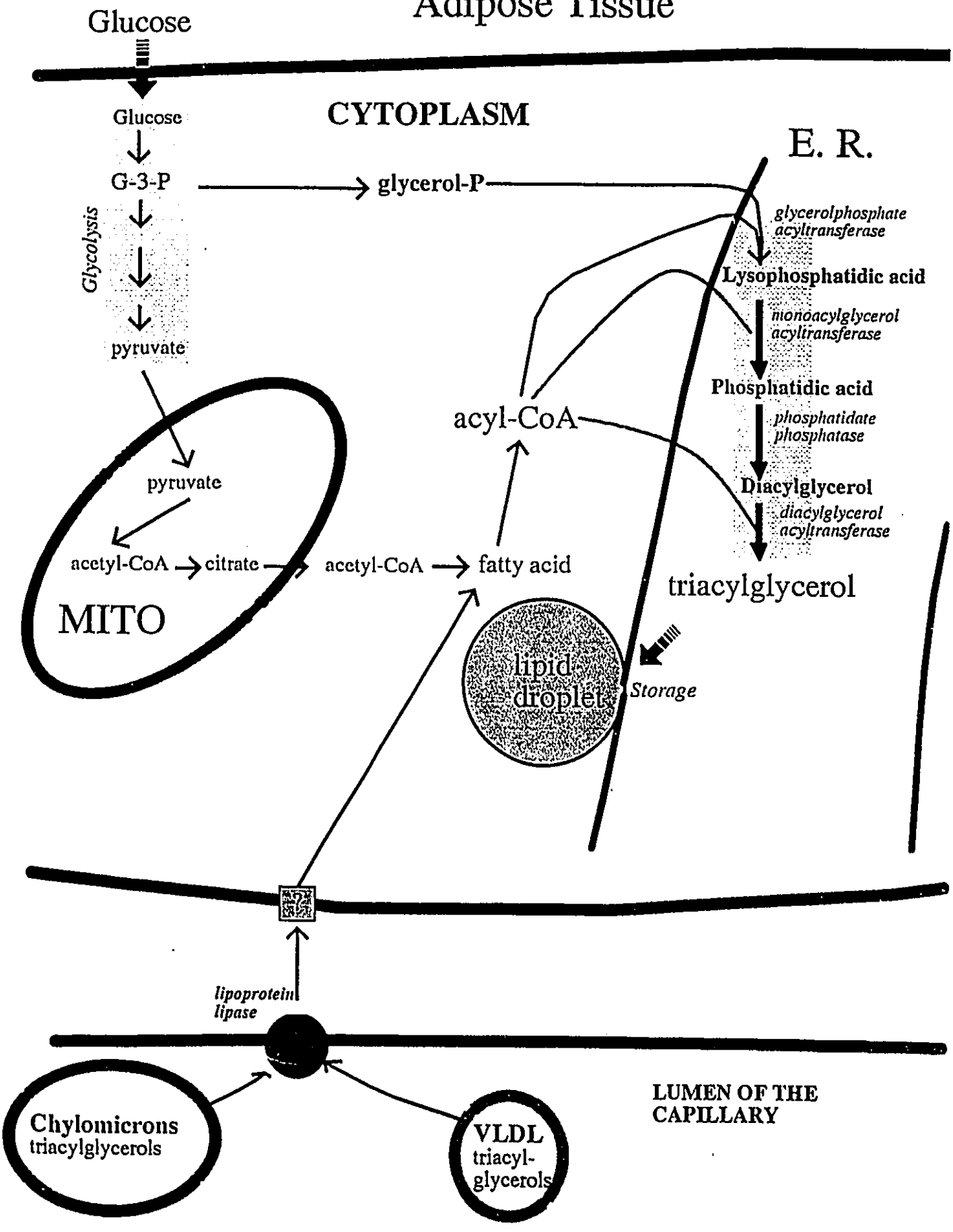


Figure 2.

Triglyceride Synthesis in Adipose Tissue



Specific multi-step regulation. Logically when considering the pathway and the intermediates, regulation at the level of glycerophosphate acyltransferase (GPAT) is certainly logical since this step results in committed TAG or PL synthesis. Catecholamines have been previously reported to decrease the activity of glycerolphospate acyltransferase (GPAT) (63,64). Stimulation of GPAT activity measured *in vitro* was reported in adipocytes preincubated with insulin (65) and insulin both blocks the effects of catecholamines (64) and reverse the induced decreases in the activity of GPAT (66). The regulation of MGAT to make it rate limiting would cause the accumulation of LPA which is toxic to the cell since it is a strong detergent. Furthermore, *in vitro* studies suggest that it has the highest specific activity; therefore not an effective point of regulation (67). Regulation at the level of phosphatidate phosphatase as suggested for the liver by Brindley (68) could be relevant since PA is the branch point for the formation of non-polar PLs and triacylglycerols. Moreover catecholamines have also been previously reported to decrease the activity of phosphatidic acid phosphatase (PAP) (69). Insulin also blocks the effects of the catecholamines on PAP and rapidly reverse the effects of catecholamines (70). It has been suggested that hormonal regulation is achieved at the level of translocation of the enzyme between the cytosol and ER membranes (67) but this has been the subject of debate because there appears to be little correlation between net TAG synthesis and translocation (36,37). Moreover since adipose cells do not significantly synthesize these PLs,

translocation of the enzyme to the cytosol during hormonal down regulation would result in the accumulation of phosphatidic acid which would be unfavourable. Additionally it has been difficult to detect consistent alterations in the steady state of PA levels (36,37).

Regulation at the level of DGAT may be important because it is the branch point for the formation of polar PLs and triacylglycerols (see Figure 1). In view of the different function of PLs and triacylglycerols, differential regulation at this branchpoint in all cell types is plausible, as it appears to be the case in hepatocytes (71,72). There is evidence that alterations in DGAT activity affects both PL and TAG synthesis (73,74). For instance a recent study demonstrates that inactivation of DGAT results in an increase in DAG incorporation into PL's (75) while other studies demonstrate that the availability of cellular diacylglycerol modulates TAG and PL synthesis (73,76-77). Of all the steps in the TAG synthesis pathway, recent studies with permeabilized rat hepatocytes suggest that only DGAT has been found to be rate limiting (36). In adipose tissue, accumulation of diacylglycerol (DAG), during the attenuation of DGAT activity, would not pose a significant threat to membrane stability and function, and could therefore be an important rate determining step in the regulation of synthesis. In the liver, adipocytes, soybean, and developing seeds, there is evidence that DGAT plays an important role in the regulation of TAG synthesis (36,37,67,76,78,79).

Catecholamines have been previously reported to decrease the activity of DGAT (64) however a more recent report suggests that norepinephrine does not directly regulate triacylglycerol synthesis in adipocytes (47) but rather it is the liberation of fatty acids through lipolysis mediated by HSL which inactivates the enzymes. Glucagon has also been reported to decrease the activity of DGAT (71,81) without affecting PL synthesis (71) although this is less important in human adipose tissue since there does not appear to be expression of glucagon receptors.

It is conceivable that in white adipose tissue DGAT, in addition to other TAG synthetic enzymes, demonstrates complex regulatory properties both in the short and long term. It is probably too simplistic to assign a dominant role to any regulatory enzyme in the overall control of glycerolipid synthesis. Rather it is better to think in terms of the multistep close-knit interplay between several key enzymes possibly mediated by kinases and phosphatases.

Involvement of protein kinases in regulation. Several *in vitro* experiments suggest that triacylglycerol synthetic enzymes, including GPAT, PAP and DGAT from various tissues, may be regulated by a phosphorylation-dephosphorylation mechanism. There are however, different kinases which have been implicated and conflicting reports on their effects on the enzymes has been reported (63,66,72,82-86). It has been suggested previously that GPAT from cardiocytes (63) and

adipocytes (82) are regulated by protein kinase A through the actions of the β-adrenergic receptor. However other laboratories have been unable to observe any GPAT inactivation upon incubation of rat adipocyte microsomes with PKA and its substrates (66,87). PAP from rat liver was also suggested to be regulated by a phosphorylation-dephosphorylation mechanism (83,85) although this has yet to be confirmed.

Regulation of DGAT activity by reversible phosphorylation-dephosphorylation mechanism associated with signal transduction in exocrine cells was suggested by Soling et al. (84) although phosphorylation was reported to increase the activity of DGAT and the link of TAG synthesis to signal transduction at the plasma membrane is unclear. Although DGAT phosphorylation may be important in signal transduction, considering the differences in function of adipocytes and purely exocrine cells Soling's hyporthesis is probably irrelevant to studies of adipocyte TAG synthesis. Another recent report with 3T3 adipocytes suggests that there appears to be the ATP-dependent formation of lipid protein complexes which stimulates DGAT activity (88) but the exact mechanism and physiological relavence with respect to regulation is obscure. Furthermore we have never detected an increase in DGAT activity following incubation of adipose tissue cytosol with microsomes and ATP. Modulation of DGAT activity in the liver by a phosphorylation-dephosphorylation mechanism was suggested previously by

Haagsman and coworkers (72). Rat liver microsomes were shown to be inactivated *in vitro* by incubation with ATP/Mg^{2+} and the liver cytosolic fraction but not with PKA and its substrates. The authors reported reactivation of DGAT activity from ATP treated microsomes upon incubation with liver cytosol. They also observed a decrease in DGAT activity of tissue homogenized in the presence of 40 mM NaF. By contrast, we were unable to detect either of these latter effects in adipose tissue (89). We did find a consistent stimulation of both DGAT and GPAT activities by the cytosolic fraction. Similar stimulation has been reported previously in different laboratories although the mechanism of stimulation has not yet been demonstrated (87,89).

Previous studies from our laboratory indicate the presence in adipose tissue of an ATP-dependent activity that *in vitro* reduces both GPAT and DGAT activities by 30-40% (87,89). As was reported, the activity responsible for GPAT and DGAT is associated with the cytosolic fraction, is heat sensitive, requires Mg^{2+}, and an intact cleavable β-γ-phosphodiester bond on the ATP. DGAT and GPAT activites from ATP treated microsomes can be restored to control levels by incubation with a crude preparation of liver protein phosphatase. New addition of ATP and cytosol results in reinactivation close to the level for ATP-treated microsomes (89). These results support the hypothesis of a soluble protein kinase involved in the regulation of adipose DGAT and GPAT. We also determined that DGAT inactivation was not

affected by the inhibitors of PKC and or PKA such as H7 analogue, staurosporine, amiloride, or PKA inhibitor. Furthermore since the stimulation of DGAT was not observed by the addition of Ca^{2+}, cAMP, or the cAMP analogue chlorophenylthio-cAMP, and because neither PKC from rat brain nor the catalytic subunit of PKA from bovine heart could replace the cytosolic activity, the postulated DGAT kinase is neither PKC nor PKA.

STATEMENT OF THE PROBLEM

As was discussed in the introduction, our lab has recently described the presence of an enzyme activity in adipose tissue which in the presence of ATP reversibly inactivates GPAT (87) and DGAT (89). Our hypothesis was that the ATP-dependent inactivation of both acyltransferases is associated with a protein tyrosine kinase activity. To prove this hypothesis, experiments were designed in order to 1) purify from adipose tissue cytosol, the enzyme activity associated with inactivating DGAT, 2) determine if there is a kinase activity associated with the DGAT inactivation throughout the purification steps, 3) if a kinase is co-purified determine its substrate specificity (ie. ser/thre, dual, tyr), 4) determine the sensitivity pattern of both activities to inhibitors, 5) examine if the purified activity can also act on GPAT, 6) examine if a crude phosphatase preparation could reverse effects of DGAT inactivation and microsomal phosphorylation, and 7) identify polypeptide substrates of the purified kinase which may be components of adipose DGAT and GPAT.

MATERIALS AND METHODS

Materials. Oleic acid, oleoyl-CoA, diolein (1,2-dioleoyl-sn-glycerol), L-glycerol 3-phosphate, ATP, coenzyme A (CoA), DL-dithiothreitol (DTT), ethylenediaminetetraacetate (EDTA), bovine serum albumin (BSA) (essentially fatty acid free), 4',5,7-trihydroxyisoflavone (genistein), 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H7), 2-aminopurine, staurosporine, [3,4,5-trihydroxybenzylidene]-malononitrile (tyrphostin 25), tyrosine kinase substrate peptide (Arg-Arg-Leu-Ile-Glu-Tyr-Ala-Ala-Arg-Gly), Reactive Blue 2-Sepharose, S-Sepharose, Sephacryl S-200, DEAE sepharose CL-6B, polyethylene glycol compound (MW 15,000-20,000), sodium dodecyl sulfate (SDS), acrylamide (TEMED, bisacrylamide), Brilliant Blue G colloidal, Fuji NIF-RX film, and molecular weight standards were obtained from the Sigma Chemical Co. (St. Louis, MO). Filters (0.22 μm pore size) were obtained from Millipore (Bedford, MA). Whatman P-81 paper was obtained from Canadawide Scientific (Ottawa, Ontario, Canada). [9,10-^{3}H]Oleic acid (7.4 Ci/mmol) and L-[2-^{3}H]Glycerol-3-phosphate (10.6 Ci/nmole, 1 Ci = 37 GBq) were obtained from Du Pont Canada Inc. (Mississauga, Ontario, Canada). [9,10-^{3}H]Oleoyl-CoA (100 Ci/mol) was prepared from the radioactive fatty acid following the method of Bishop and Hajra [90] as previously described [91]. ^{32}P-γ-ATP (3 Ci/mmol) was obtained from Amersham (Oakland, Ontario, Canada).

Tissue preparation. Epididymal adipose tissue microsomes from male Sprague Dawley rats (Charles River Canada Inc., St. Constant, Quebec, Canada) fed at libitum (except Table I) were prepared by diffential centrifugation of the homogenized tissue as previously described [92,93]. After excision of blood vessels and debris, epididymal adipose tissue was homogenized in three volumes of a 10 mM Tris-HCl (pH 7.5) buffer containing 0.25 M sucrose, and 1 mM DTT (buffer A). The homogenate was centrifuged at 600*xg* and the fat cake and pellet were discarded while the supernatant was filtered through glass wool. The filtrate was centrifuged at 16 000*xg*. The supernatant (post-mitochondrial supernatant) was resolved into cytosol and microsomes by centrifugation at 100 000*xg* for 1h. The microsomes were washed once, resuspended in buffer A containing 1 mM EDTA, to a final concentration of 1-2 mg protein/mL and kept in aliquots at -60°C. When indicated, to remove any endogenous kinase, the microsomes were further washed with a buffer containing 200 mM Tris-HCl (pH 9.0), 1 M NaCl, 1 mM EDTA, 0.25 M sucrose and 1 mM DTT. Amounts of microsomes used in the experiments are indicated throughout this thesis as the mass of microsomal protein. Concentration of samples when desired, was performed by dialysis against solid polyethylene glycol (PEG).

Purification of the activity responsible for DGAT inactivation. The activity responsible for DGAT inactivation was purified by fractionation of 50 mL (150 mg

protein) of the 100,000*xg* supernatant from homogenized adipose tissue, with fractions of the purification sequence tested for DGAT inactivation on washed microsomes (8 µg). The cytosol was first mixed with solid ammonium sulfate to a concentration of 55% ammonium sulfate saturation and kept stirrred at 4°C for 1 hour. After centrifugation the pellet (38 mg) was resuspended in TED buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 2 mM DTT) dialyzed against TED and applied to a 20 mL Blue-Sepharose column followed by elutions with TED buffer containing 0, 0.3 and 1 M NaCl. The protein peak which elutes at 0.3 M NaCl (9.4 mg) was dialyzed against TED buffer, applied to a 20 mL S-Sepharose column and eluted with 0.5 M NaCl in TED buffer. The pool containing the activity (0.57 mg) was dialyzed, applied to a 7 mL DEAE Sepharose column and eluted with 1 M NaCl in TED buffer. Fractions containing the activity (0.29 mg) were pooled, concentrated with PEG and applied to a 45 cm Sephacryl S-200 column equilibrated with TED buffer. The final pool (0.14 mg) of the fractions containing the activity was made 20% (v:v) in glycerol and stored at -20°C.

High performance liquid chromatography (HPLC) analysis was performed with a Bio-Gel TSK Phenyl 5-PW hydrophobic Bio RAD column. The HPLC pump was a Beckman Model 110A attached to a Beckman 340 flow regulator. Visualization of the protein peaks was achieved with an Hitachi Model 110-40 spectrophotometer. A cytosolic fraction (50 µg) from a purification sequence

including an ammonium sulfate precipitation, blue sepharose, phosphocellulose, DEAE-sepharose, and sephacryl chromatographies, followed by dialysis of the final pool with polyetheylene glycol to a concentration of 100 µg/mL was applied to the HPLC column followed by a 1 M to 10 mM ammonium phosphate gradient to achieve maximal resolution of the peaks. Flow rate was set at 1 mL/min and analysis and trials were performed by Celine Clement.

Protein phosphatase was partially purified from rat liver up to the ethanol precipitation step of the method of Brant et al. (94) as previously described (89). The liver tissue was homogenized in three volumes of a 10 mM Tris buffer (pH7.5) containing 0.25 M sucrose, 1 mM DTT and 1 mM EDTA (buffer B). 1 gm Norit A charcoal/L homogenate and acetic acid was added to achieve a pH of 5.8 for the acid precipitation. The mixture was centrifuged at 10 000*xg* for 20 min. and the supernatant was adjusted to a pH of 7.2 with Tris base. Ammonium sulfate was added to a concentration of 70%, for the precipitation of protein. The pH was again readjusted to 7.2 and the mixture was centrifuged at 10 000*xg* for 20 min. The pellet was redisolved in a minimum volume of a 20 mM Tris buffer containing 1 mM $MgCl_2$. The solution was then poured into five volumes of 95% ethanol and centrifuged at 5000*xg* for 5 min. The pellet was extracted by homogenization with 0.5 mL buffer B per gram of liver. The suspension was centrifuged at 16 000*xg* for 15 min and the pellet was extracted again. The supernatants were combined

and dialyzed against buffer B. A final concentration of 5.0 mg protein/mL was achieved by concentration with solid polyethylene glycol and an equal volume of glycerol was added to the extract and kept at -90°C.

Assays. GPAT was assayed as previously described (87). The assay was performed at 37°C, normally for 4 min, in a final volume of 0.5 mL containing 0.1 M Tris-HCl (pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, 25 µM BSA, 100 µM oleoyl-CoA, 1 mM [^{3}H]glycerol-3-phosphate (1.2 Ci/mol), and microsomes (5-10 µg). Incubation for 4 minutes following the addition of [^{3}H]glycerol-3-phosphate and oleoyl-CoA was terminated by the addition of 1 mL of butanol saturated with water. The aqueous and organic phases were separated by centrifugation in a clinical centrifuge. The organic phase was washed three times with 1.5 mL of water saturated with butanol. A 0.5 mL aliquot of the organic phase was counted in 7 mL of Universol.

DGAT was assayed as we previously described [89]. DGAT was assayed by incubating the microsomes (10-20 µg protein) for 6-8 min at 37°C with exogenous([95](Rodriguez, Dias, Charoui, and Lau) 20 µM 3H-oleoyl-CoA (7.5 Ci/mol) and 150 µM 1,2-dioleoyl-sn-glycerol (delivered in 5 mL acetone) in 0.5 mL of 0.1 M Tris-HCl (pH 7.5), containing 1mM DTT, 10 mM $MgCl_2$ and 5 uM BSA (TMB buffer). The reaction was stopped by the addition of 1.5 mL of

isopropanol/hexane (1:1, vol/vol). The organic phase was washed three times with 0.75 mL of isopropanol/hexane (4:1, vol/vol) and 0.75 mL of 0.05% KOH. A 0.75 mL aliquot from the organic (hexane) phase was counted in 7.5 mL of Universol scintillation cocktail in an LKB Wallac 1214 beta counter. ATP-dependent GPAT and DGAT inactivation were determined by performing the corresponding assays with the cytosolic fraction, in the presence and absence of 0.5 mM ATP.

DGAT activity was assayed in the liver without resolution of heptane soluble products by TLC because when a saturating concentration of diolein is achieved in the incubation mixture TAG formation greatly exceeds cholesterol ester synthesis (Rodriguez, M.A., and Lau, T.E. unpublished results).

Kinase activity was assayed by measuring the incorporation of ^{32}P from ^{32}P-γ-ATP into either microsomes or the tyrosine kinase synthetic-peptide substrate (96). Phosphorylation of microsomes was carried out in a total volume of 0.1 mL containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ^{32}P-γ-ATP (40 Ci/mole), and microsomes (30 µg). Reaction was initiated by the addition of the kinase sample or, in the case of microsomes containing the endogenous kinase, by the addition of the ^{32}P-γ-ATP. After an incubation of 5 minutes at 37°C, 0.22 µm pore size filters (2 cm diameter) which were prewashed with 0.5 mL of 50 mM phosphate, were blotted with 75 µL of the incubation mixture. For microsomes treated with phosphatase, the microsomes retained on the filters were incubated

with a crude preparation of liver phosphatase (90 µg) in a 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM $MgCl_2$ in a total volume of 100 µL for 5 minutes at room temperature. For all incubations, the reaction was terminated by passing 2.5 mL of 50 mM phosphate through the filter disc under suction. Discs were dryed and counted in 7 mL of Universol (ICN).

Phosphorylation of the synthetic peptide was carried out as described by Pike et al. [96] with some minor modifications. The incubation was carried out at 37°C in a total volume 20 µL containing 100 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$, 1 mM synthetic peptide, 0.5 mM ^{32}P-γ-ATP. Reaction was initiated by the addition of the kinase sample and terminated after 5 minutes by the addition of 30 µL of 5% trichloroacetic acid. After centrifugation, the supernatants were blotted on P-81 paper which was prewashed with 5 mM ATP in 50 mM phosphate buffer. The blotted paper was then washed three times with 75 mM phosphoric acid. After drying, the paper was counted in 7 mL of Universol in an LKB Rack beta scintillation counter.

Protein concentrations were measured by the dye-binding assay of Bradford (97) using bovine serum albumin as the standard.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis was carried out in a mini-protean gel electrophoresis apparatus (Bio-Rad), under reducing conditions using the discontinous system of Laemmli (98), with 7.5% and 4.5% acrylamide resolving and stacking gels. Gels were stained with Brilliant Blue G colloidal (Sigma), dried and, when indicated, exposed to Fuji NIF-RX X-ray film.

RESULTS

Localization of the activity responsible for DGAT inactivation. As we have reported previously most of the activity responsible for DGAT inactivation resides in the cytosol[89], however, depending on the microsome preparation and the types of washes performed, a fraction of the activity remains associated with the microsomes. Incubation of microsomes from rat adipose tissue with 0.5 mM ATP results in the partial inactivation of diacylglycerol acyltransferase (DGAT) (33 $\pm$ 1 % ATP-dependent DGAT inactivation) as shown in Figure 3. Interestingly these microsomes contain also an endogenous kinase activity that catalyzes the incorporation of ^{32}P from ^{32}P-γ-ATP into microsomes. Washing the microsomes with a high salt, high pH buffer causes the removal of both the ATP-dependent DGAT inactivating activity and the kinase activity. Adipose tissue cytosolic fraction is able to restore both the DGAT inactivating activity and the kinase activity when added to the washed microsomes.

Effect of inhibitors on cytosolic activity. To further characterize the kinase and DGAT inactivating activities, their sensitivity to some protein kinase inhibitors was examined (Figure 4). As we have previously shown, EDTA decreases the level of DGAT inactivation by the cytosol and ATP (89). Concurrently we have found that there is a decrease in the level of phosphorylation of the microsomes (Figure 4). Inhibitors of serine/threonine kinases such as H7 (99) and the protein inhibitor

Figure 3. **Localization of the activity responsible for DGAT inactivation.** Microsomes (8 µg) with and without cytosol (120 µg) were incubated in the presence and absence of 0.5 mM ATP for DGAT inactivation, and assayed for DGAT activity, as described in Materials and Methods. The corresponding decrease in DGAT activity caused by ATP incubation is given as mean ± SEM. For microsomal phosphorylation, microsomes (30 µg) with and without cytosol (60 µg) were assayed for the incorporation of ^{32}P-phosphate from ^{32}P-γ-ATP (0.5 mM, 40 Ci/mole) into microsomes as described in Materials and Methods. Results are given in nmole phosphate incorporated/mg microsomal protein. Washed microsomes refers to microsomes washed with a high pH, high salt buffer as described in Materials and Methods.

Figure 3. Localization of activity

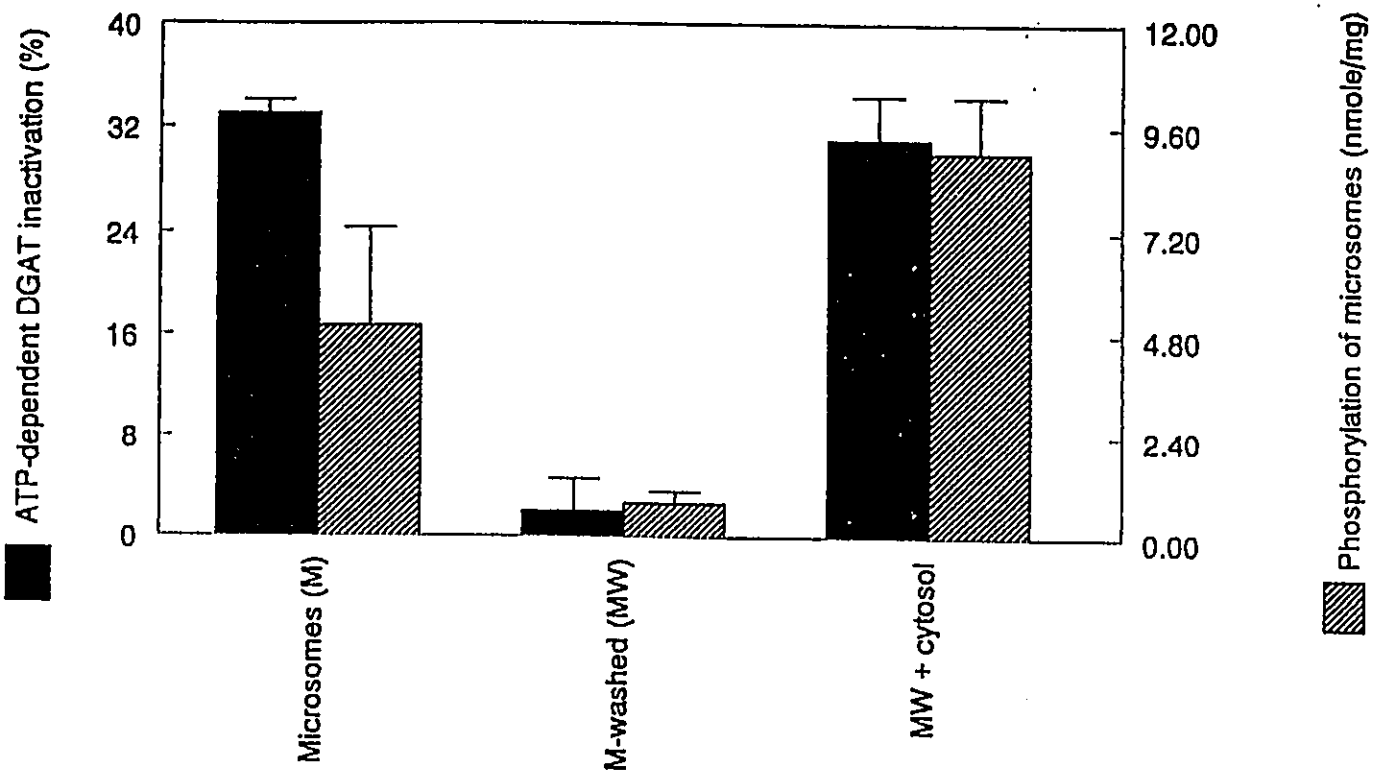
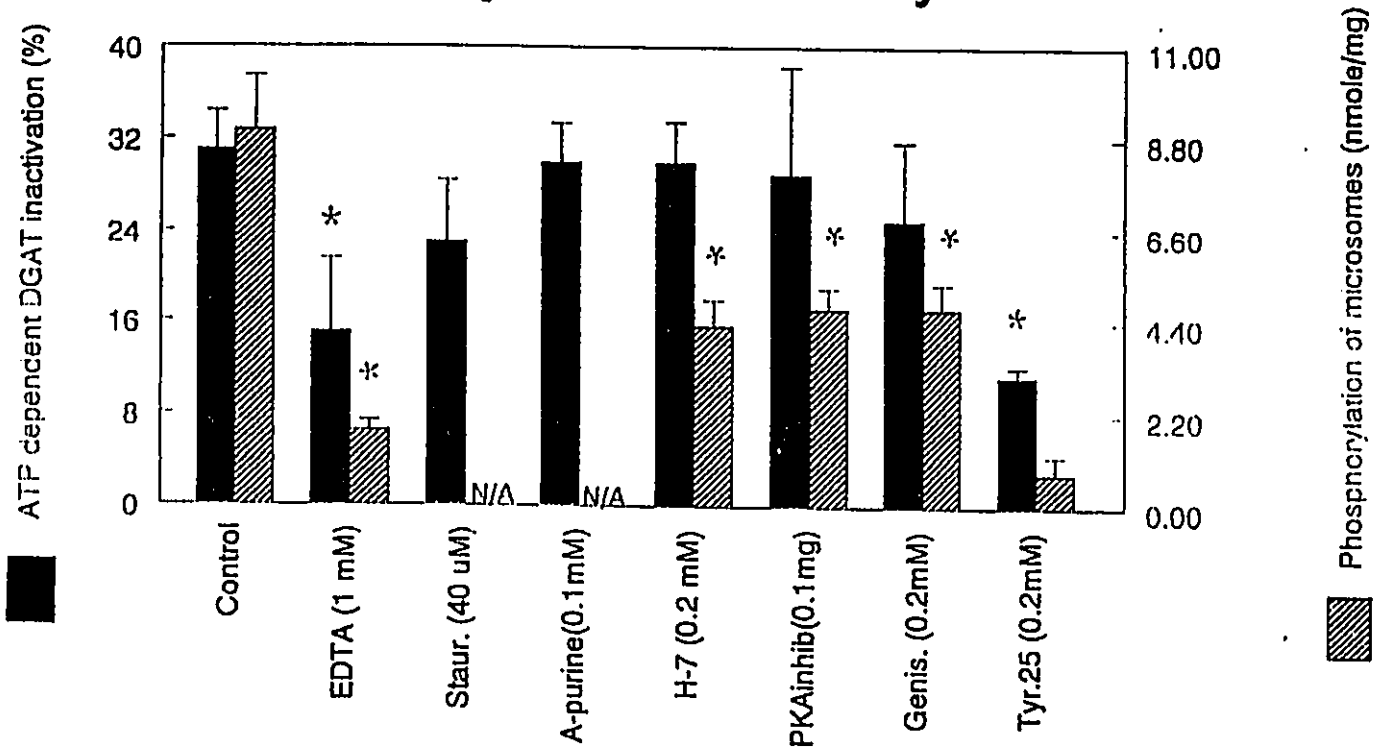


Figure 4. **Effect of kinase inhibitors on cytosolic activity.** Microsomes with added cytosol and the indicated inhibitor were assayed for DGAT inactivation and for phosphorylation of microsomes as described for Figure 1. * Indicates significant difference $p < 0.05$. The p values were calculated using a t-test with the control (no inhibitor) as reference.

Figure 4. Effect of kinase inhibitors on cytosolic activity



of PKA, did not significantly affect the ATP-dependent inactivation of DGAT, but they did affect the level of phosphorylation in the microsomes, suggesting the presence of cytosolic serine/threonine kinases acting on microsomal proteins other than DGAT. The ATP-dependent DGAT inactivation by the cytosol was also not inhibited by 100 μM 2-aminopurine which is an established protein kinase inhibitor with a wide spectrum of inhibition (100). Although staurosoporine was originally thought of as a serine/threonine kinase inhibitor it has also been reported to be an inhibitor of certain tyrosine kinases, including one isolated from rat adipose soluble fraction (101,102). However, we have not observed inhibition of DGAT inactivation with staurosporine. By contrast when tyrphostin 25, a tyrosine kinase inhibitor(99), is present there is a substantial decrease in both the level of ATP-dependent inactivation of DGAT and the level of phosphorylation in microsomes.

Effect of nutritional state on cytosolic activity. DGAT activity and its inactivation by cytosol/ATP was determined in adipose microsomes from rats kept on different diets: starved, fed at libitum, and fed a fat enriched diet. From Table I, the level of microsomal DGAT activity was greatest in the rats kept on fatty diets, least in the starved rats, and intermediate with rats fed at libitum which is consistent with what has been reported previously. Levels of ATP dependent DGAT inactivation were not different (all between 35-40%) between the three groups which could be explained if short term regulation (eg. with hormones) and long term regulation were

Table I

Different nutritional states and the corresponding DGAT activity and DGAT inactivation

Nutritional State[a]	DGAT activity[b] (nmole/min/mg)	ATP-dependent DGAT inactivation (%)
Starved	4.9 ± 0.9	33 ± 6
Fed at libitum	6.9 ± 0.8	31 ± 3
Fed fatty diet	9.2 ± 0.6	35 ± 3

[a]Male sprague dawley rats (4 per group) were kept on three different diet regimes; starved rats were deprived of food for 48 hrs; rats fed *at libitum* had free access to their regular diets; and rats fed on a high fatty diet had free access to chow supplemented with corn oil for one week (18% protein, 47% carbohydrate, 35% fat). Microsomes from epididymal tissue from rats were prepared as described in Materials and Methods.

[b]Microsomes (8 µg protein) with and without cytosol (120 µg protein) from adipose tissue were incubated in the presence and absence of 0.5 mM ATP for DGAT inactivation, and assayed for DGAT activity, as described in Materials and Methods.

achieved independently and through a different mechanism, or during the process of microsomal and cytosolic isolation the regulated enzymes lose their level of regulation (ie. if regulation is achieved by phophorylation-dephosphorylation then the enzymes may become dephosphorylated). The former is partially true in view of cell function and the importance of TAG synthetic enzymes in adipose tissue, since the long term regulation would likely involve increase in protein synthesis relative to the constititively expressed genes which would explain the observed increase in specific activity (Table 1). If the latter is true then *in vitro* studies of the effect of the hormone may be difficult to access because its action may involve phosphorylation of the regulated enzymes which may be reversed in the process of isolation of the enzyme activities, unless specific phosphatase inhibitors were found.

Cytosolic activity from liver vs adipose tissue. Cross inactivation of microsomes from adipose and liver tissues with cytosolic fractions from adipose and liver tissues (Table II) demonstrates that optimal inactivation is achieved by fractions from the same tissue. This suggests that either the enzymes from different tissues may represent differing isoforms with alternate specificities or the enyzmes are regulated in different manner as would be expected since adipose and liver tissue are regulated inversely. In fact, we have found evidence that some of the TAG synthetic enzymes of liver and adipose tissue may be different isoforms

Table II

Cross inactivation of DGAT from adipose and liver tissue fractions

	DGAT Inactivation[a] (%)	
Cytosol	Microsomes	
	Liver	Adipose
Liver	35 ± 4 (3)	11 ± 1 (3)
Adipose	12 ± 1 (3)	38 ± 2 (3)

[a]Microsomes (8 µg) with and without cytosol (120 µg) from either liver or adipose tissue were incubated in the presence and absence of 0.5 mM ATP for DGAT inactivation, and assayed for DGAT activity, as described in Materials and Methods. The corresponding decrease in DGAT activity caused by ATP is given as mean ± SEM for the number of independent experiments indicated in parenthesis.

because of differences in sensitivity to various inhibitors we have studied (results not shown) (103).

Purification of DGAT inactivating activity. The activity responsible for the ATP-dependent inactivation of DGAT was partially purified from the cytosol by a sequence of steps involving a 55% ammonium sulfate precipitation and Blue Sepharose, S-sepharose, DEAE-sepharose, and Sephacryl S-200 chromatographies as described in "Materials and Methods" and summarized in Figure 5. Other columns that were attempted but were inefficient in resolving activity from protein were hydroxyl-appatite, AMP-sepharose, phosphocellulose, histone-agarose, and sephadex G-75 (results not shown). The protein elution profiles for the purification sequence, in addition to the level of DGAT inactivation are given in Figures 6-9. From figure 6, the majority of the activity responsible for inactivating DGAT is associated with the protein peak which elutes at 0.3 M NaCl although some activity is associated with the 1 M peak. At each step, with the exception of the Sephacryl column, using the capacity to inactivate DGAT to follow the activity the chromatographic columns were able to resolve the activitiy from other protein peaks without activity, with the majority of the activity binding to Blue Sepharose (Figure 6), S-Sepharose (Figure 7) and DEAE sepharose (Figure 8) and coelutes with the protein peaks at NaCl concentrations of 0.3, 0.5 and 1.0 M respectively. In the case of the gel filtration column (Figure 9), the protein elutes

Figure 5. **Flowchart of the purification sequence.** Rat epididymal tissue was homogenized and applied to a purification sequence as described in Materials and Methods. Purification factors calculated based on protein recovery are indicated in parenthesis.

Figure 5. Flowchart of the Purification Sequence.

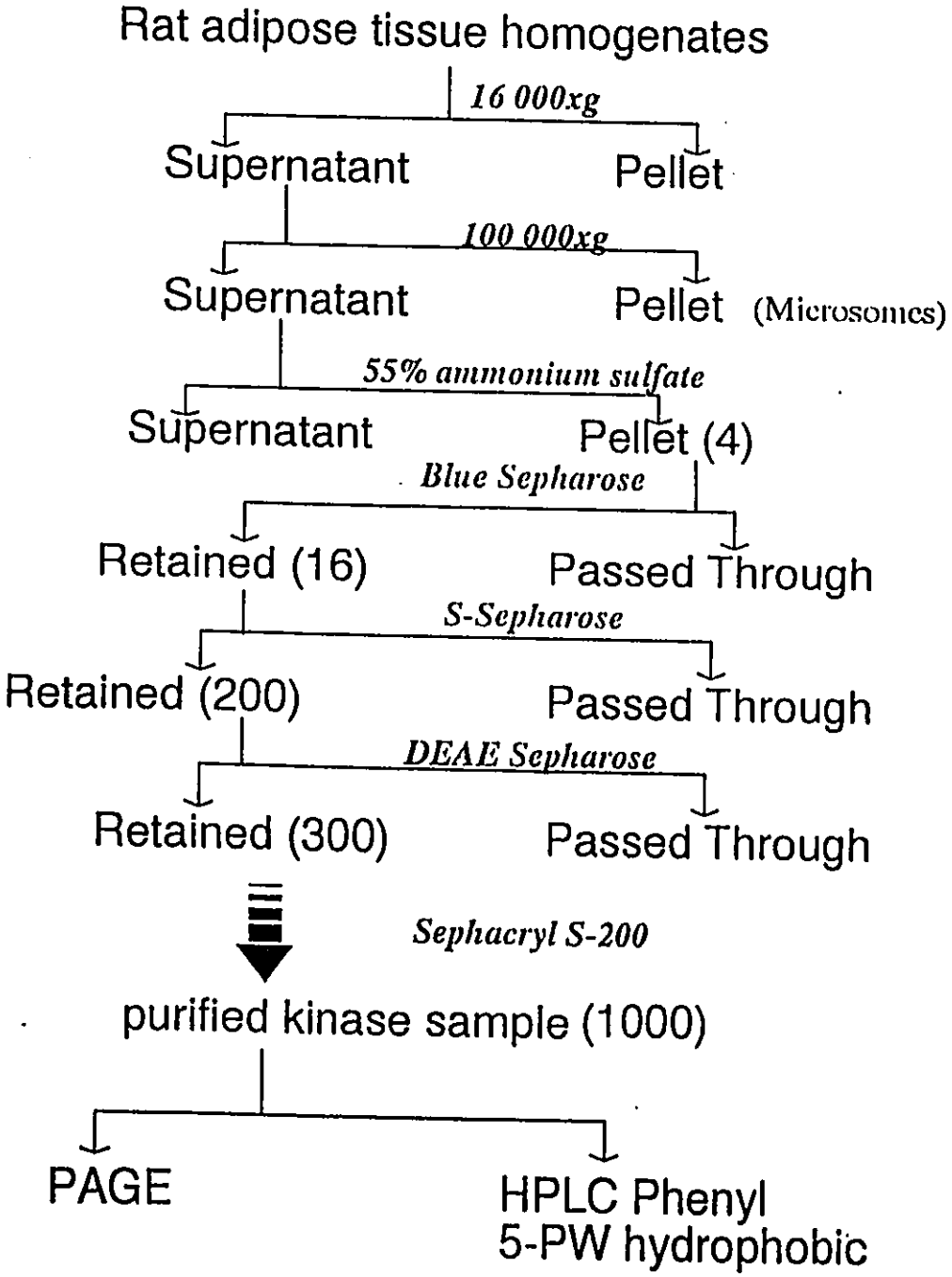


Figure 6. **Elution profile for the blue Sepharose column.** The pellet (38 mg) from the 55% ammonium sulfate precipitation was resuspended in TED buffer and applied to a 30 mL of blue Sepharose column followed by a step elution of TED buffer containing 0, 0.3 amd 1 M NaCl. Fractions were assayed for protein content. Pools were made of the protein peaks as indicated by the bar diagrams and assayed for DGAT inactivation as described in Materials and Methods by incubating the pooled fractions with washed microsomes and with the DGAT substrates in the presence and absence of ATP. The percentage decrease in activity caused by the addition of ATP is given.

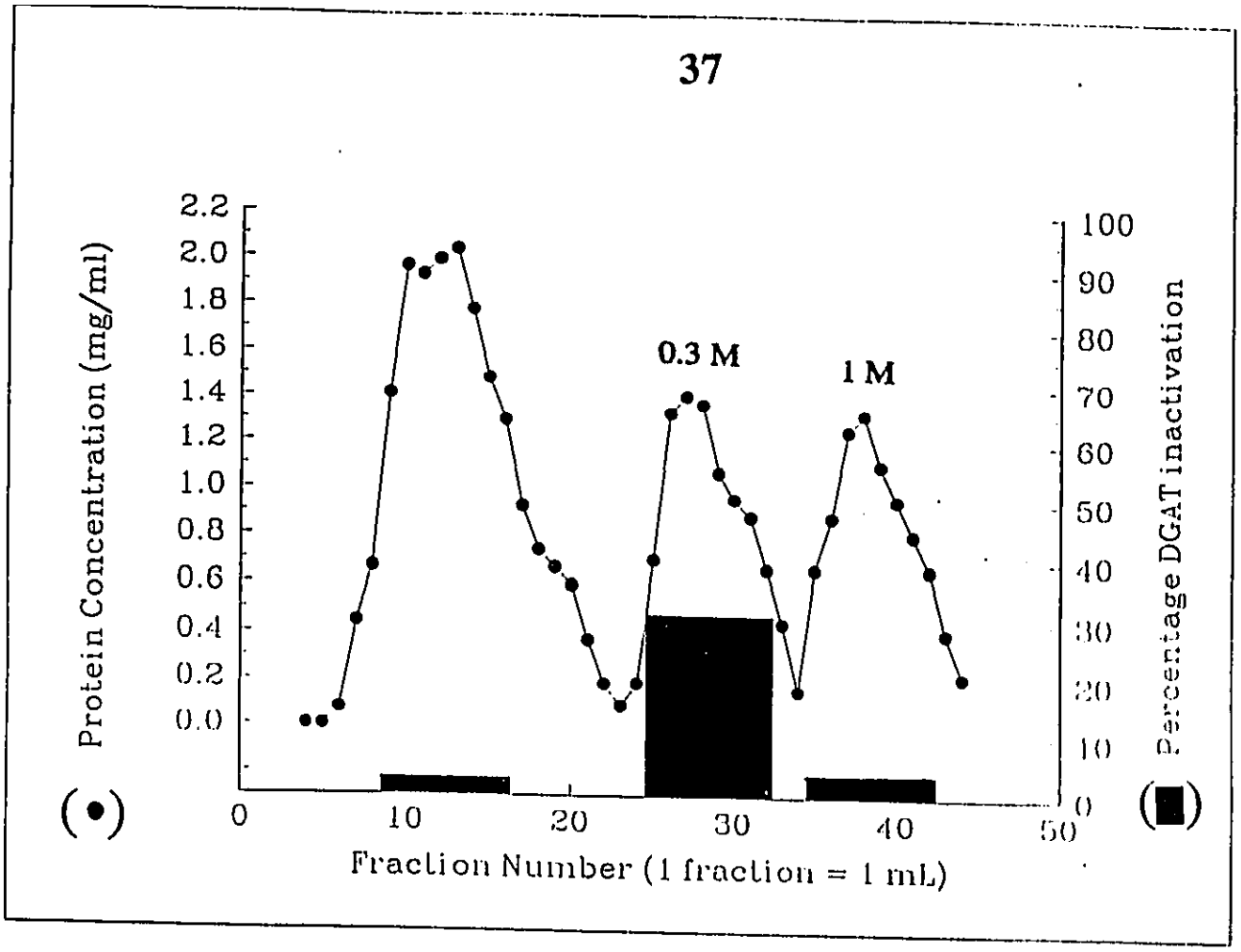


Figure 6. Elution profile for the blue sepharose column.

Figure 7. **Elution profile for the S-Sepharose column.** The protein peak (9.4 mg) which eluted at 0.3M NaCl from the previous column was pooled and dialyzed against TED buffer and applied to a 20 mL S-Sepharose column and eluted in step wise fashion with 0, and 0.5 M NaCl in TED buffer. DGAT inactivation was assayed with the pooled fractions 8-11, 19-21 as described in Figure 6.

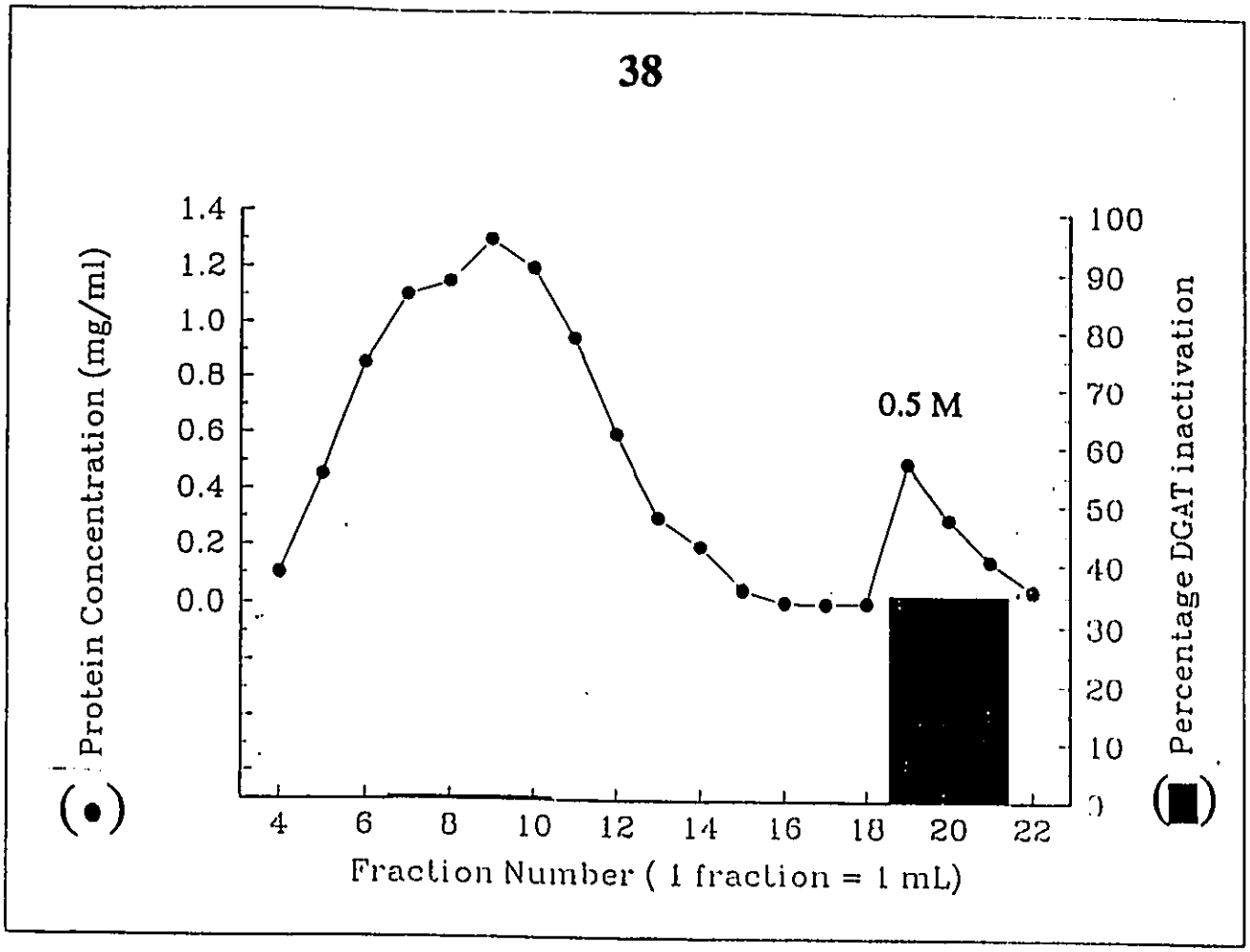


Figure 7. Elution profile for the s-sepharose column.

Figure 8. **Elution profile for DEAE sepharose.** The protein peak/pooled fraction (0.570 mg) which eluted at 0.5 M NaCl in TED buffer from the S-sepharose column was dialyzed against TED and applied to a 7 mL DEAE sepharose column and eluted with 1 M NaCl in TED. DGAT inactivation was assayed with the pooled fractions 6-10, 14-16, and 20-21 as described in Figure 6.

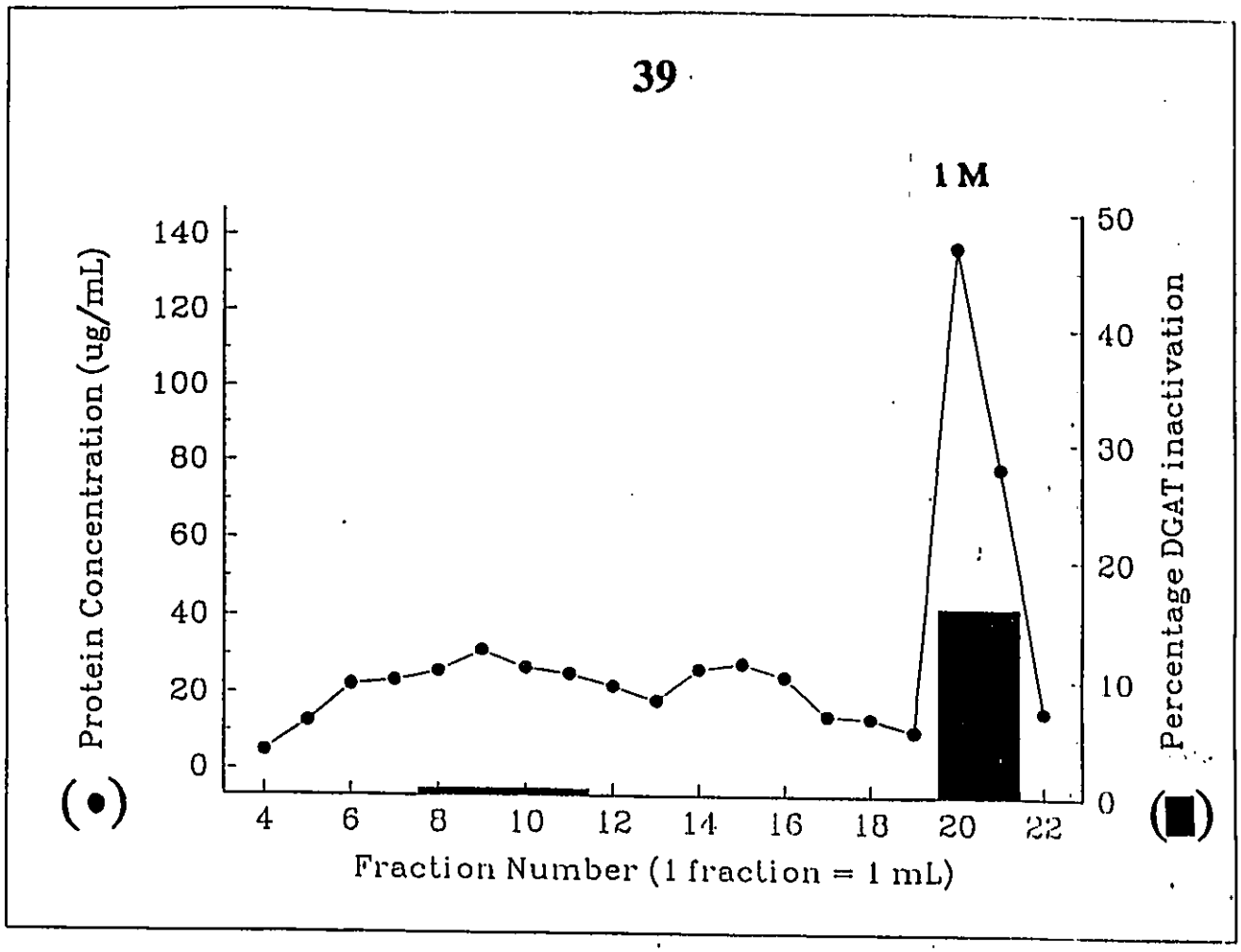


Figure 8. Elution profile for the DEAE-sepharose column.

Figure 9. **Elution profile for the Sephacryl column.** The pooled fraction/protein peak (0.29 mg) which eluted from the DEAE Sepharose column was applied to a 45 cm Sepharcryl column equilibrated with TED buffer. DGAT inactivation was assayed with fractions 11, pooled fractions 12-15, and fraction 16 as described in Figure 6.

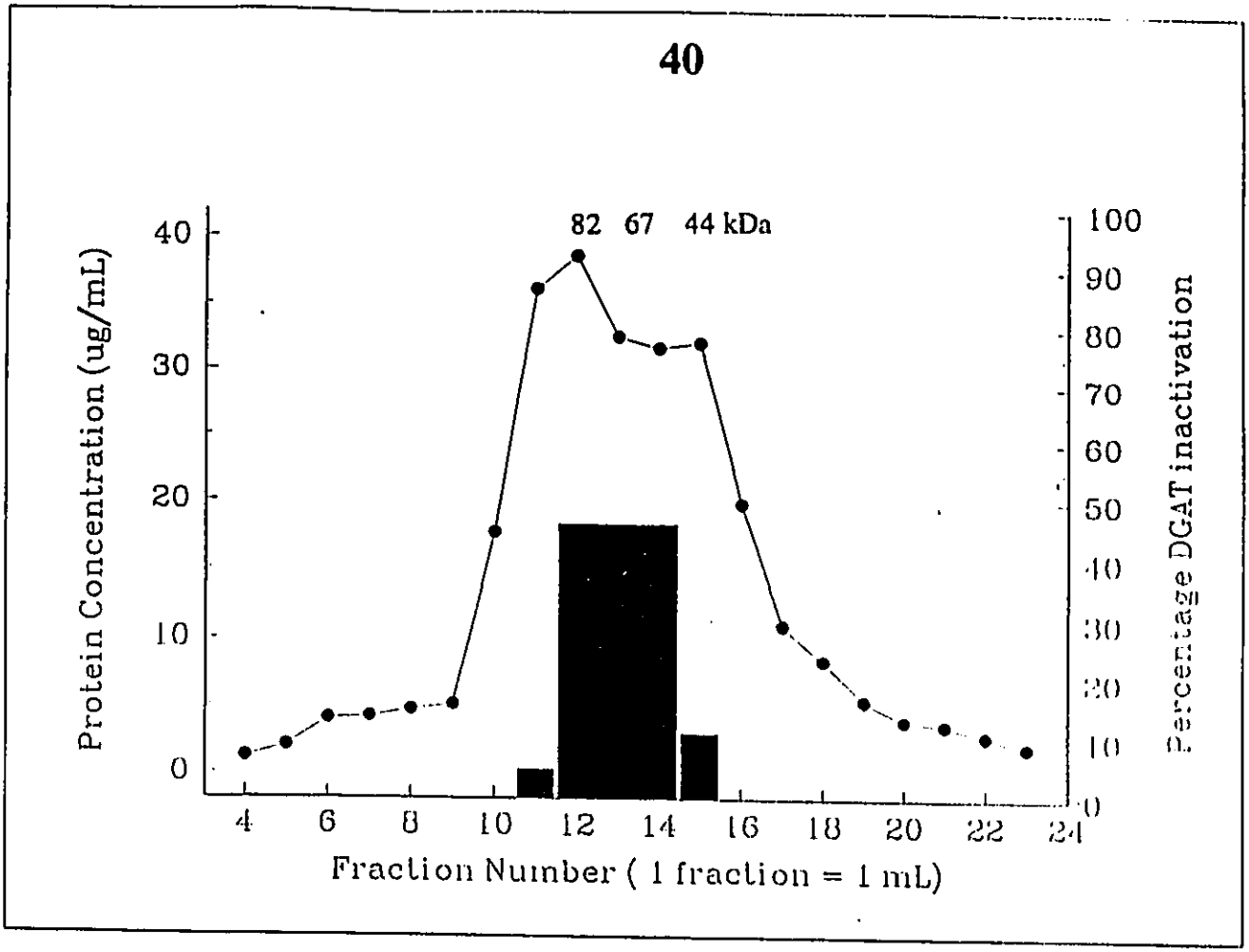


Figure 9. Elution profile for the Sephacryl column.

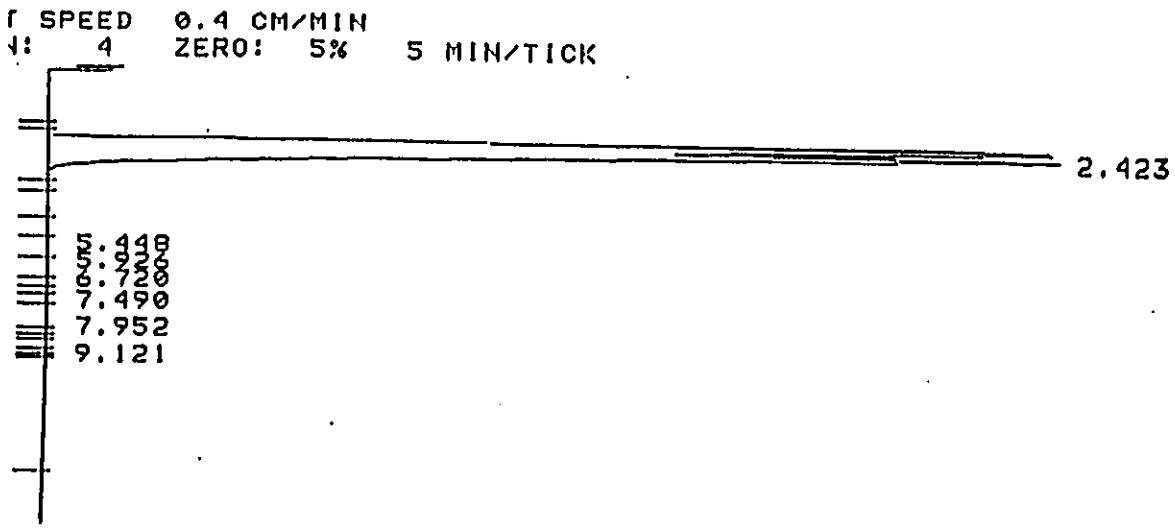
in two overlapping peaks corresponding to 82 and 44 kDa, with the pool of activity centered at 67 kDa. The final fraction containing the activity represented less than 0.1% of the starting protein.

During one of several previous attempts at the purification of the DGAT inactivating activity a sequence of chromatographic steps was run under conditions similar to those described above but a phosphocellulose column was substituted for the S-sepharose column. The level of purification achieved as determined by protein recovery was less than 300x. The final pool which contained the activity responsible for DGAT inactivation was analyzed for purity by a HPLC Bio-Gel TSK Phenyl 5-PW hydrophobic Bio RAD column. After several runs we were able to partially resolve two peaks as shown in Figure 10. As a diagnostic tool suggesting we had two or more polypeptides in our final pool, we attempted to separate the two polypeptides in a more efficient purification sequence. As a consequence, the S-sepharose column was substituted for the phosphocellulose anion exchanger to achieve a more efficient purification.

Previously, in an attempt to follow protein kinase activity throughout our purification, an assay was developed for kinases employing the phosphorylation of a mixture of histones (Calbiochem, San Diego, C.A.) by ^{32}P-γ-ATP followed by binding of the products to phosphocellulose paper (Whattman p-81) (results not

Figure 10. **HPLC Analysis of sephacryl pool of DGAT inactivating activity.** High performance liquid chromatography column was a Bio-Gel TSK Phenyl 5-PW hydrophobic Bio RAD column. The kinase sample (50 µg) was prepared by concentration of the final pool of the sephacryl column by dialysis with polyetheylene glycol to a concentration of 100 µg/mL and applied to the column followed by a 1 M to 10 mM ammonium phosphate gradient to achieve maximal resolution of the peaks.

Figure 10. HPLC analysis.



shown). During the process of the purification of the activity responsible for inactivating DGAT the histone phosphorylation did not coincide with the protein pools corresponding to DGAT inactivating activity at each chromatographic step of the purification. This indicated that histones, which are good substrates for several ser/thr kinases, were not good substrates of our kinase activity.

Our later results with a tyrosine kinase substrate suggested an explanation; our cytosolic fraction preparations may contain a tyrosine kinase activity and hence did not phosphorylate either the mixture of histones (or caseine) appreciably. Indeed, the activity which was responsible for inactivating DGAT was shown to co-purify at each step with a kinase activity as measured with a non-specific assay for tyrosine kinases employing a synthetic peptide. From Table III the specific activity of tyrosine phosphorylation went from 0.48 to 74 pmoles phosphate incorporated into the peptide per µg microsomal protein, an increase of approximately 150x, from the pellet of the ammonium sulfate precipitation to the final pool of the sephacryl column. Reducing SDS-PAGE of the purified fraction shows a single major band at about 68 kDa (see Figure 11).

Characterization of the purified kinase. By measuring the level of phosphorylation of microsomes it became evident that the final pool of the purification has kinase activity (6 $\pm$ 1.7 nmole phosphate incorporated/mg microsomal protein) (Table IV).

Table III

Purification sequence of the kinase associated with DGAT inactivation.

Purification Step	Total Protein (mg)	DGAT inactivation[a] (%)	Protein Tyrosine Kinase Activity[b]	
			Total Activity[c] (pmoles)	Specific Activity[d] (pmoles/µg)
Cytosol	150	31	149	0.991
55% Ammonium Sulfate	37.5	49	18	0.475
Blue Sepharose 0.3 M NaCl	9.38	31	26	2.81
S-Sepharose 0.5 M NaCl	0.57	35	10	17.6
DEAE 1 M NaCl	0.29	13	9	31.8
Sephacryl	0.14	46	10	74.0

[a]Microsomes in the presence of the indicated fractions were assayed for DGAT inactivation in the presence and absence of ATP.

[b]Fractions from the purification steps were assayed for tyrosine kinase activity with the synthetic peptide as described in Methods.

[c]Results are expressed as pmoles phosphate incorporated into the synthetic peptide for the total amount of the cytosolic fraction.

[d]Results are expressed as pmoles phosphate incorporated into the synthetic peptide per µg of protein (cytosolic fractions).

Figure 11. **Partial purification of the activity responsible for DGAT inactivation.** SDS-PAGE was run with 80 µg of the unfractionated cytosol (lane 1) and 10 µg of the Sephacryl S-200 pool (lane 2) and the gel stained for protein with Brilliant Blue G colloidal. The number on the right indicates the apparent MW (kDa) of the purified protein.

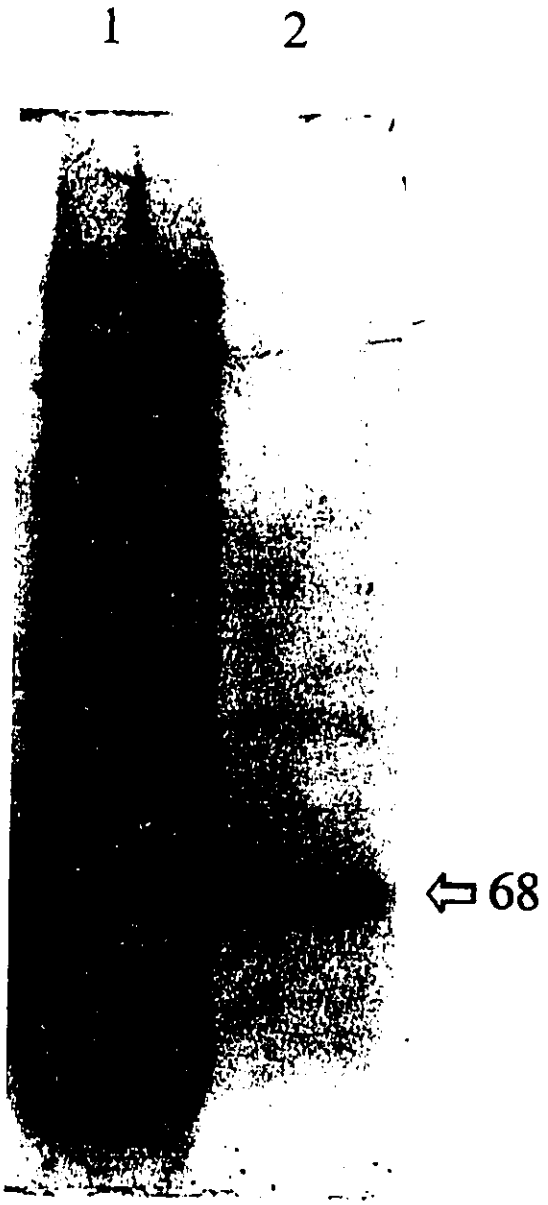


Figure 11. SDS-PAGE of the purified kinase

Table IV

Effect of cytosol fractions on different substrates.

Addition	DGAT inactivation[a] (%)	GPAT inactivation[a] (%)	Phosphorylation	
			Microsomal proteins[b] (pmole/mg protein)	Synthetic peptide[c] (pmole/µg protein)
Control	6 ± 4.7 (3)	7.3±4.5 (3)	1 ± 0.3 (3)	$(25\pm14)\times10^{-5}$ (3)
Cytosol	31 ± 3.4 (6)	38 ± 5 (9)	9 ± 1.3 (4)	0.99±0.15(8)
Purified kinase	46 ± 3.8 (8)	34.3±3.5 (3)	6 ± 1.7 (4)	74.0 ± 0.1 (8)

[a] Microsomes were assayed for DGAT and GPAT inactivation as described in Methods in the presence of either 1.2 µg of the purified kinase or 20 µg of the S-sepharose flow through fraction (control).

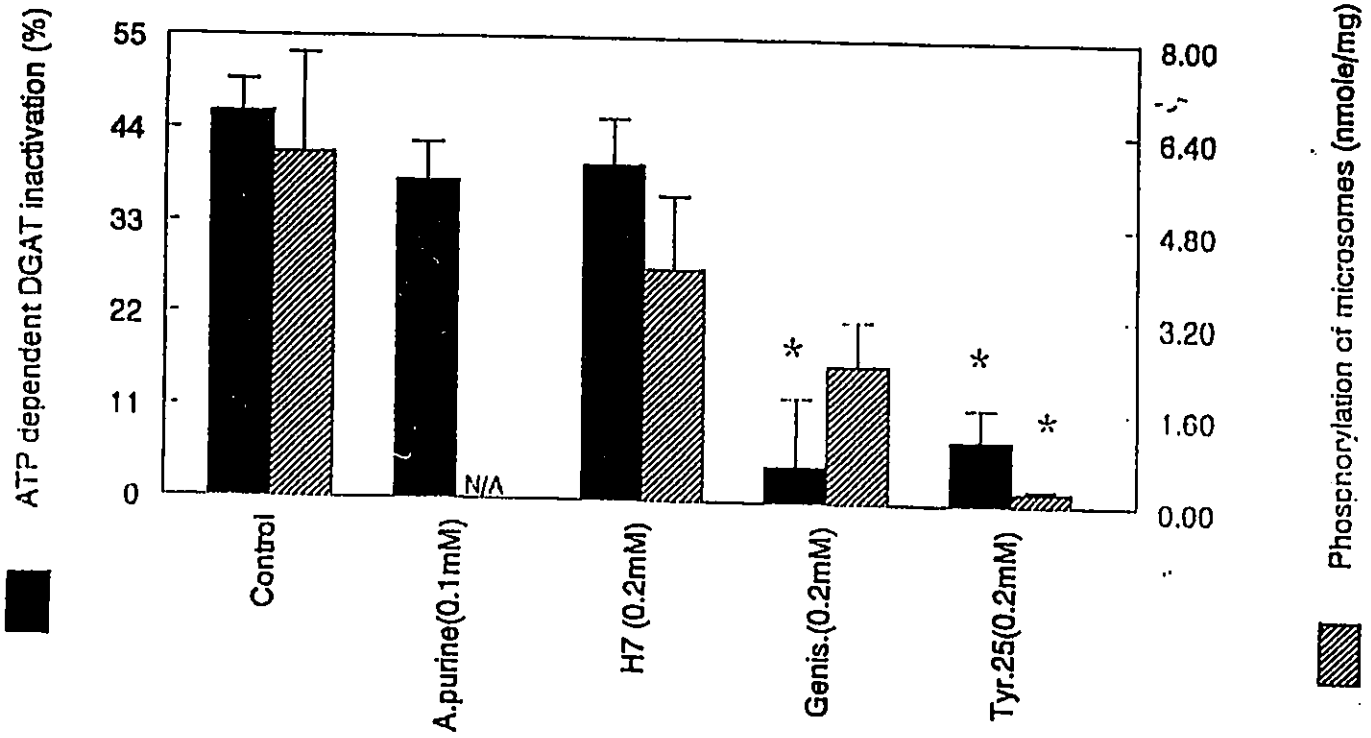
[b] Microsomes were assayed for phosphorylation as described in Methods in the presence of either 60 µg cytosol, 10 µg control, or 100 ng purified kinase.

[c] The purified kinase (165 ng average) and 5 µg of the S-sepharose flow through fraction (control) were assayed for tyrosine kinase activity as described in Methods.

Again, as for the cytosol, this kinase activity is almost completely blocked by tyrphostin 25, and to a lesser degree by genistein (Figure 12), which are both tyrosine kinase inhibitors (99). By contrast, the kinase preparation is less sensitive to inhibition by H7, suggesting that most of the serine/threonine kinases present in the cytosol have been removed. Additionally, the ATP dependent DGAT inactivation by the sephacryl pool is prevented by genistein and tyrphostin 25 but not by H7, although the effect of genistein is now more apparent than with the cytosol (Figure 12). Furthermore, our preparation is also able to phosphorylate the synthetic peptide Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly which is a specific substrate of protein tyrosine kinases (96) (Table III and IV). Since we have previously shown that microsomal GPAT is also reversibly inactivated by an ATP-dependent activity in rat adipose cytosol (87), we determined whether or not the activity purified from the cytosol, as followed by DGAT inactivation, could inactivate GPAT *in vitro*. From Table IV it is apparent that the partially purified kinase preparation is capable of inactivating GPAT in the presence of ATP. As shown for the phosphorylation of the synthetic peptide, the flow-through fractions of S-sepharose column, which were unable to inactivate DGAT were also unable to inactivate GPAT significantly.

Figure 12. **Effect of kinase inhibitors on the purified kinase.** Microsomes were assayed for DGAT inactivation and phosphorylation of microsomes in the presence of different inhibitors as described in Figure 4 but purified kinase (final concentration, 2.4 µg/mL) substituted for cytosol.

Figure 12. Effect of kinase inhibitors on the purified kinase



Phosphorylation of microsomal proteins and SDS PAGE. Figure 13 shows the autoradiograph of ^{32}P-phosphorylated microsomal proteins run on SDS-PAGE. Two major bands at 53 ± 1.3 and 69 ± 1.1 kDa (average of four gels) are visible as well as a third one at the top of the resolving gel which may represent an aggregate of proteins. Other minor bands are also visible in the regions of 40 and 100 kDa. Addition of our purified kinase to the incubation medium results in increased phosphorylation of all bands, and this effect is largely prevented if the inhibitor tyrphostin 25 is present in the incubation. The fact that the addition of the kinase and the inhibitor does not change the pattern of phosphorylated bands in the microsomes suggests that the endogenous kinase activity remaining in our microsomal preparation is the same tyrosine kinase that is present in our purified fraction. Surprisingly, when the purified kinase (Figure 14-lane 2) or a previous fraction of the sequential purification sequence, the S-sepharose pool (Figure 14-lane 3), was incubated with ^{32}P-γ-ATP and run on SDS-PAGE followed by autoradiography, no phosphorylated bands were apparent, suggesting that the enzyme does not autophosphorylate. However a similar incubation with the cytosol exhibited multiple phosphorylated proteins including a protein of the same molecular weight (68 kDa) as the protein from the purified fraction suggesting that in the presence of other proteins (perhaps other kinases in the phoshorylation cascade), our purified kinase may be phosphorylated (Figure 14). From lane 4 and 5 of Figure 14, which represent the phosphorylation of microsomes by two

Figure 13. **Phosphorylation of microsomal proteins.** Phosphorylation of microsomes was carried out by incubating microsomes (20 µg) in a 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$ buffer with ^{32}P-γ-ATP (0.375 mM, 1 Ci/mmole) with and without purified kinase (0.5 µg) for 5 min at 37°C and tyrphostin 25 (0.2 mM) in a total volume of 40 µL. The reaction was terminated by the addition of 20 µL sample buffer (3 fold concentration) then incubated at 95°C for 4 min. A 50 µL aliquot was applied to each lane; Lane 1) control (microsomes alone); lane 2) kinase added; and lane 3) kinase and tyrphostin 25 (0.2 mM) added. Gels were stained, dried and exposed to Fuji NIF-RX film in an X-ray cassette for 3 days. The numbers on the right indicate the MW (kDa) values of the marker proteins and the numbers on the left the MW (kDa) of the major phosphoproteins labelled.

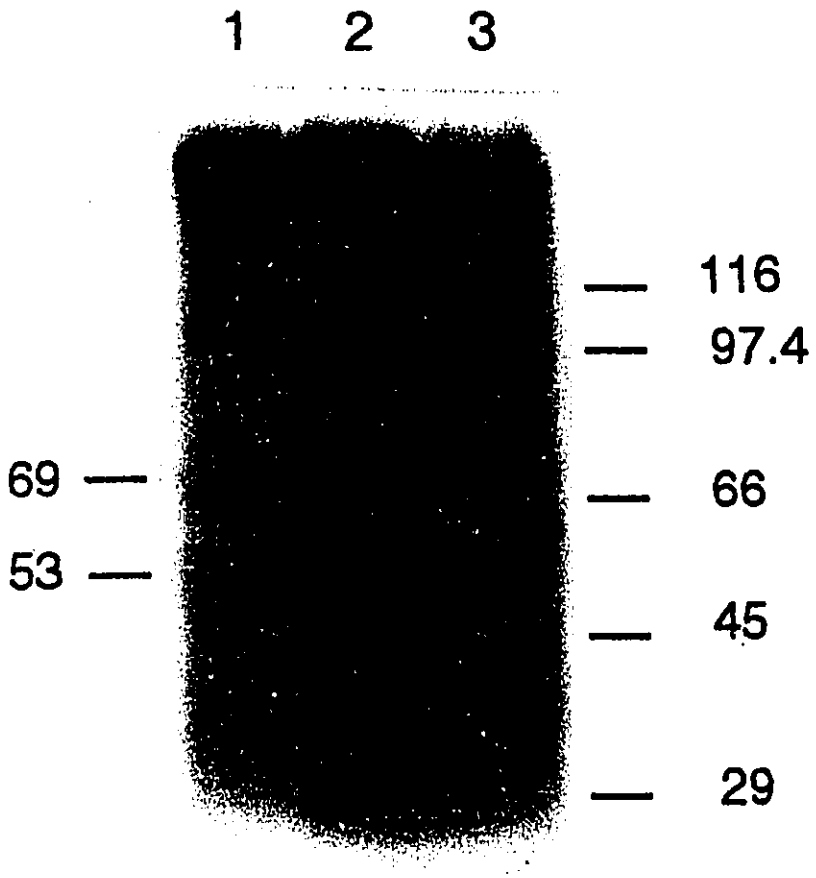


Figure 13. Phosphorylation of microsomal proteins.

Figure 14. **Protein phosphorylation by different cytosolic fractions.** Phosphorylation of proteins was carried out by incubating either microsomes (20 µg) or the equivalent amount of buffer A with a kinase sample (cytosolic fractions) in a 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$ buffer with ^{32}P-γ-ATP (0.250 mM, 1 Ci/mmole) for 5 min at 37°C in a total volume of 40 µL. The reaction was terminated by the addition of 20 µL sample buffer (3 fold concentration) then incubated at 95°C for 4 min. A 50 µL aliquot was applied to each lane; Lane 1) cytosol alone (50 µg); lane 2) S-sepharose pool alone (10 µg); lane 3) Sephacryl pool alone (10 µg); lane 4) microsomes with cytosol (20 µg); lane 5) microsomes with the S-sepharose pool (2 µg); lane 6) microsomes with Sephacryl pool (2 µg); lane 7) microsomes alone. Gels were stained, dried and exposed to Fuji NIF-RX film in an X-ray cassette for 3 days.

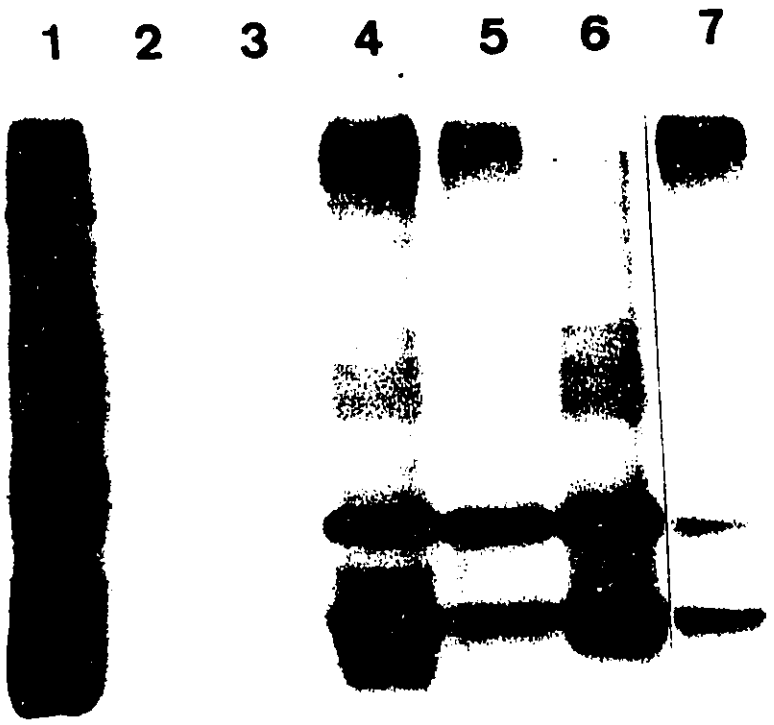


Figure 14. Protein phosphorylation by cytosolic fractions.

different fractions of the purification sequence, it is apparent that the kinase activities present in the two fractions may exhibit different microsomal substrate specificity.

Phosphatase experiments. We have previously reported that cytosol/ATP dependent inactivation of microsomal GPAT (87) and DGAT (89) activities could be reversed by incubating the inactivated microsomes with a partially purified protein phosphatase from rat liver in the absence of ATP. Our previous attempts at reactivation of adipose DGAT by various cytosolic fractions as described by Haagsman in rat hepatocytes (72) were unsuccessful. The failure may be direct results of the reported phosphatase inhibitors present in adipose tissue (104,105). However it appears that one preparation of cytosol did have a factor which stimulated the activation of DGAT by the protein phosphatase from liver. The factor was determined to have a molecular weight of less than 30 000 as determined by membrane filtration but further attempts to purify the factor were unsuccessful since the activity is easily lost. Therefore we decided to reverse the kinase mediated phosphorylation of microsomal proteins by using a phosphatase preparation from liver that was able to reactivate DGAT. From Table V it is shown that the reactivation of DGAT from kinase treated microsomes by the phosphatase preparation is Mg^{2+} dependent and is inhibited by 1 mM EDTA, 10 mM phosphate, 40 mM NaF, and vanadate but not by the presence of 0.1 μM okadaic acid. Since

Table V

Inhibition of DGAT reactivation with protein phosphatase

Addition	DGAT reactivation[a] (% ± SDM)	Reactivation inhibition[b] (%)	p[c]
None	92 ± 9 (4)	0.0	-
0.1 µM okadaic acid	88 ± 9 (4)	4 ± 10	n.s.
40 mM NaF	26 ± 4 (4)	72 ± 5	<0.001
10 mM phosphate	12 ± 15 (4)	87 ± 17	<0.01
1 mM EDTA	6 ± 11 (3)	93 ± 12	<0.01
2 mM vanadate	45 ± 9 (3)	52 ± 10	<0.05

Microsomes (1 mg/mL) were pretreated for 10 min at room temperature with cytosol (2.4 mg/mL) and ATP (0.5 mM) in a 60 mM Tris (pH 7.4), 1 mM DTT, 10 uM BSA, 5 mM $MgCl_2$. Aliquots of treated microsomes containing from 10-15 ug were incubated for 10 min at 37°C in a similar buffer with and without a crude preparation of liver protein phosphatase as described in Table V in the presence of the indicated inhibitors and 20 uM of carried over ATP, and then assayed for DGAT activity.

[a]Over the controls with inhibitors and no phosphatase

[b]Over the control with no inhibitor (92%)

[c]From a t-test using the control with no inhibitor as reference.

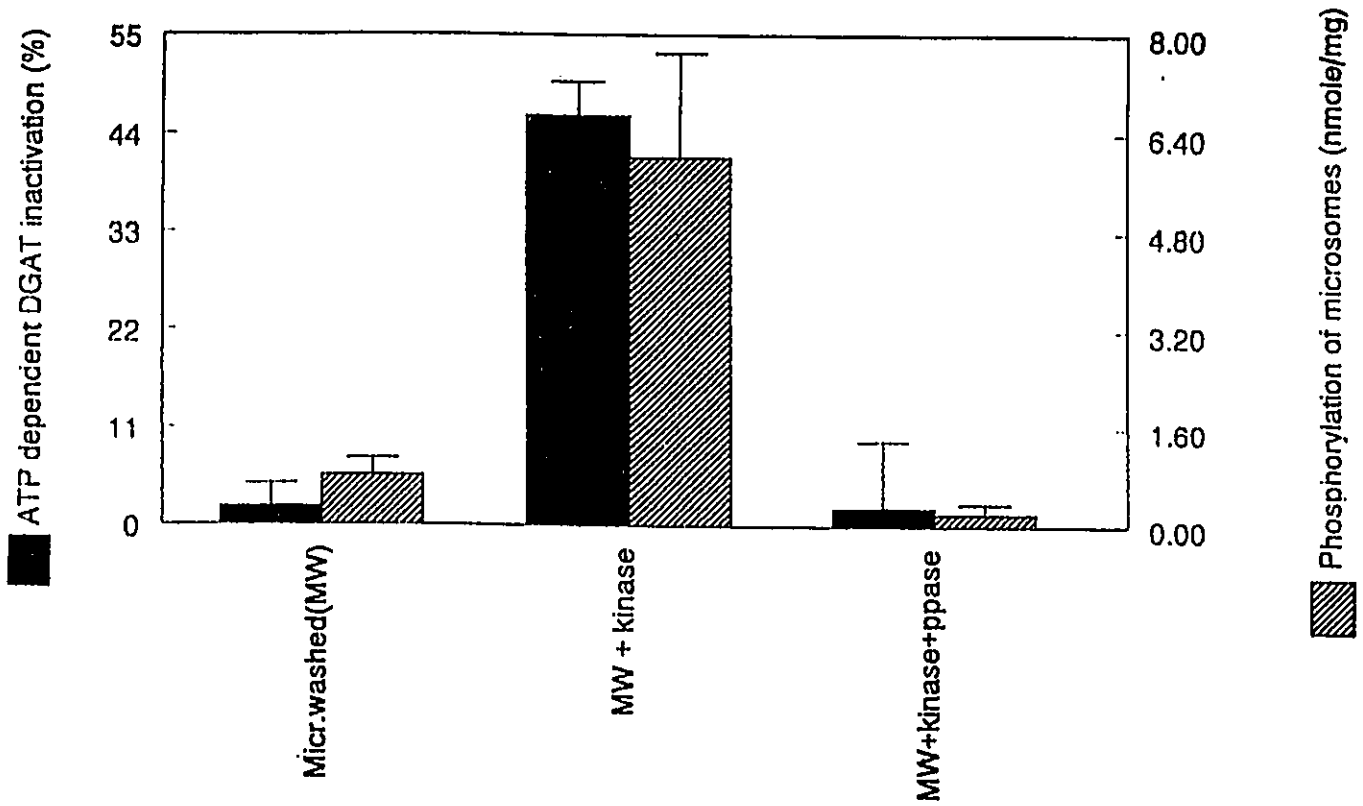
vanadate is an inhibitor of tyrosine phosphatases this suggests that the phosphatase which functions antagonistically against our kinase may be of the tyrosine variety (106). The resistance to 0.1 μM okadaic acid (Table V) and to EGTA, implies that it is neither protein phosphatases 1, 2a, or 2b (107). Furthermore a liver cytosolic fraction enriched with a Mg^{2+} dependent, okadaic acid insensitive, phosphatase did not correspond to the activity responsible for DGAT stimulation which led us to believe that the phosphatase was not of the type 2C (results not shown).

Reversibility of microsome phosphorylation by the liver phosphatase preparation was investigated by pretreatment of washed microsomes with the purified kinase, which stimulated microsomal phosphorylation from 0.8 ± 0.28 to 6 ± 1.7 nmol/mg microsomes (Figure 15). Incubation of the microsomes retained on the filter with the crude phosphatase preparation, as described under Materials and Methods, reduces the phosphorylation to 0.2 ± 0.16 nmol/mg microsomes (Figure 15).

We have observed that if both the purified kinase and liver phosphatase are present in excess with microsomes, in the presence of 1 mM ATP, the kinase is able to overcome the phosphatase resulting in net inactivation of DGAT and the corresponding increase in microsomal phosphorylation over the control (4.5 vs 0.8

Figure 15. **Reversibility of DGAT inactivation and microsomal phosphorylation.** Washed microsomes were assayed for DGAT activity in the presence of the purified kinase in the presence and absence of ATP as described in Figure 10. Microsomal phosphorylation was assayed as described in Figure 4. For the DGAT inactivation of the kinase/phosphatase incubation, after the preincubation of microsomes with the kinase (without the oleoyl-CoA and diolein) the ATP was removed by passing the sample through an Amicon P30 membrane. The microsomes were resuspended in the incubation buffer with 100 µg of the crude liver phosphatase preparation and after correction for protein loss on the membrane, DGAT was then assayed. For the microsomal phosphorylation, microsomes incubated with the purified kinase were retained on the filters and incubated with a crude preparation of liver phosphatase (90 µg) in a 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM $MgCl_2$ in a total volume of 100 µL for 5 minutes at room temperature. For all incubations, the reaction was terminated by passing 2.5 mL of 50 mM phosphate through the filter disc under suction. Discs were dryed and counted in 7 mL of Universol (ICN).

Figure 15. Reversibility of effects



nmole/min/mg), although not to the levels achieved by the purified kinase acting on microsomes alone (6.0 nmole/min/mg (Figure 15)).

Although a DGAT reactivating phosphatase from adipose tissue has yet to be identified, during the fractionation of the cytosol to purify the kinase, we detected the presence of an activity which stimulated DGAT. This activity was insensitive to okadaic acid but was prevented by 2 mM ATP, and was present in both the 55% ammonium sulfate pellet and the flow through fraction of the Blue sepharose column (Figure 6) (Lau and Rodriguez unpublished results).

Figure 16 shows the desphoshorylation of phospho proteins from the kinase treated microsomes. Treatment with the kinase stimulated the level of ^{32}P-phosphate incorporation into microsomal polypeptides as in Figure 13. Treatment with the liver phosphatase reversed the level of phoshorylation close to control levels (lane 3).

Figure 16. **Phosphatase treatment of kinase phosphorylated microsomal proteins.** Phosphorylation of microsomes was carried out by incubating microsomes (20 µg) in a 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$ buffer with ^{32}P-γ-ATP (0.25 mM, 1 Ci/mmole) with (lane 2 and 3) and without purified kinase (lane 1) (0.5 µg) for 5 min at 37°C in a total volume of 40 µL. For lane 3, the kinase treated microsomes were then retained on a 30 000 molecular weight cut-off membrane and resuspended in a 35 µL of a 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$ buffer. 5 µL of the phosphatase preparation was added to the incubation mixture. The reaction was terminated by the addition of 20 µL sample buffer (3 fold concentration) then incubated at 95°C for 4 min. A 20 µg protein aliquot from each incubation mixture was applied to each lane. Gels were stained, dried and exposed to Fuji NIF-RX film in an X-ray cassette for 5 days. The numbers on the right indicate the MW (kDa) values of the major phosphoproteins labelled.

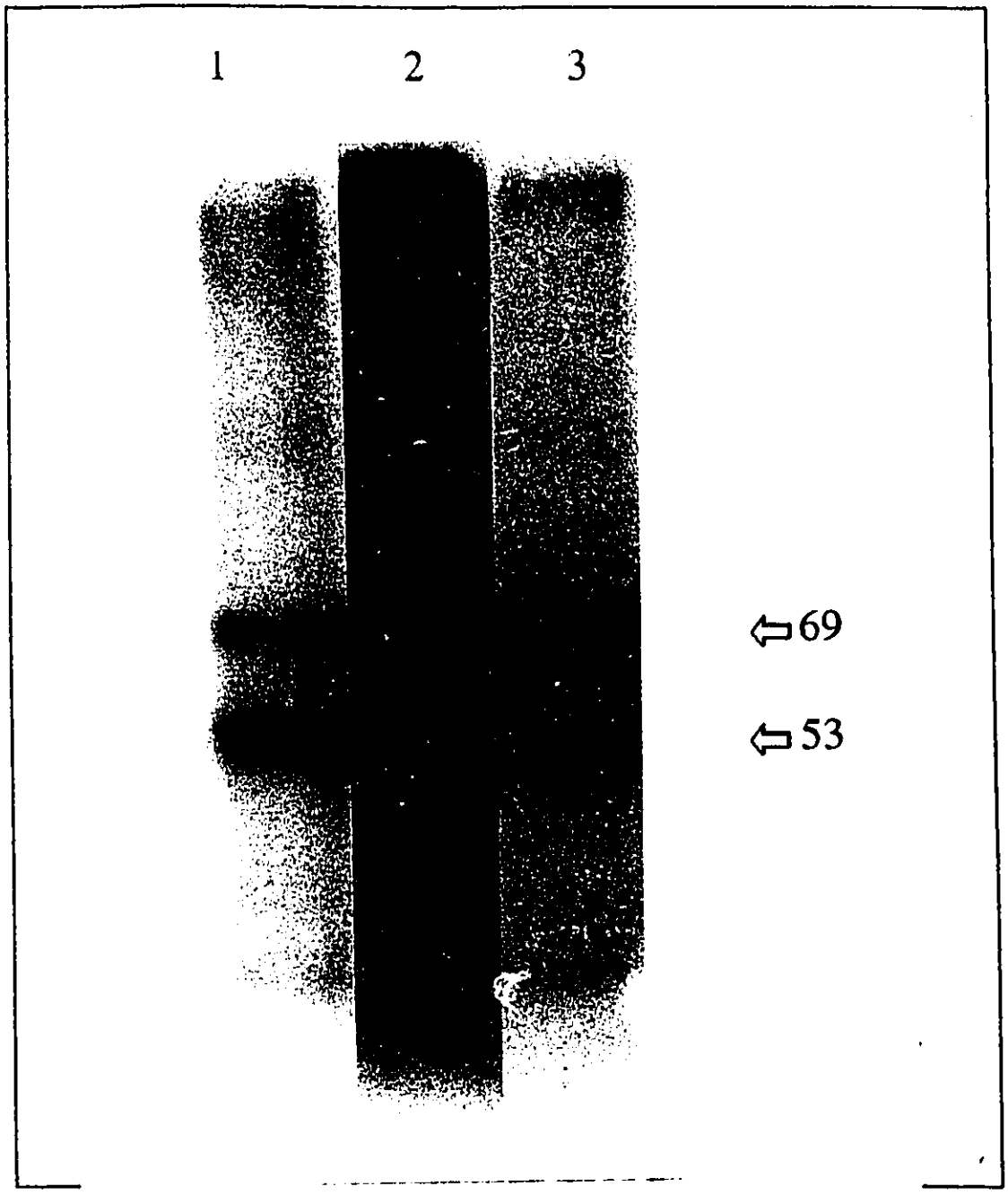


Figure 16. Phosphatase treatment and microsomal phosphorylation.

DISCUSSION

Our results confirm our previous findings of an ATP-dependent activity present in the cytosol of adipose tissue, which *in-vitro* reduces both microsomal glycerolphosphate acyltransferase and diacylglycerol acyltransferase activities by 30-40% (87,89). We have now partially purified that activity from cytosol and found it to run at about 67 kDa on gel filtration (Figure 9) and at 68 kDa on SDS-PAGE (Figure 11). It is probably important to note that the calculation of molecular weight from the calibrated gel filtration column may be inaccurate if the molecule is asymmetric, interacts with the gel, or interacts with other proteins. Although electrofocusing or another dimension of PAGE or N-terminal amino acid analysis would better confirm the homogeneity of our preparation, the fact that both SDS-PAGE and gel filtration estimation of molecular weight (MW) yield the same result suggests a monomeric enzyme of an approximate MW 67-68 kDa.

Since we measure only levels and not rates of DGAT inactivation, which probably correspond to maximal changes, we were unable to determine the degree of purification of the DGAT inactivating activity. In an attempt to quantify the activity throughout the purification steps the fractions were assayed for tyrosine kinase activity under conditions of linearity in response to enzyme concentration. Although the specific kinase activity increased by a factor of 150 from the ammonium sulfate precipitation to the final pool the total activity decreased

significantly from the cytosol to the final pool indicating that other tyrosine kinases unrelated to DGAT inactivation were removed during the process of the purification, especially at the salt precipitation and S-sepharose steps (Table III). However, according to protein recovery in the final pool, the degree of purification attained is in the order of one thousand fold (Table III). Thus the lack of a quantitative yet specific assay for our enzyme prevents us from an accurate estimation of the degree of purification achieved.

Our hypothesis that the activity responsible for DGAT inactivation in the presence of ATP is a protein kinase is supported by the following observations: 1) the kinase activity, as expressed as the phosphorylation of microsomes, is associated with the DGAT inactivating activity through all of the purification steps, 2) when endogenous microsomal activity responsible for DGAT inactivation is removed by appropriate washing of microsomes, the microsomal phosphorylating activity is concomitantly lost, 3) both activities require Mg^{2+} and are inhibited by EDTA, 4) DGAT inactivation does not occur when ATP is substituted by β,γ-methylene-ATP (89), 5) both activities are sensitive to the tyrosine kinase inhibitors genistein and tyrphostin 25, and 6) both activities can be reversed by treatment with a crude preparation of protein phosphatase from rat liver. Moreover, our putative DGAT kinase appears to be tyrosine kinase, both because of its sensitivity to established tyrosine kinase inhibitors (genistein and tyrphostin 25) (99) and

because of its ability to phosphorylate a synthetic peptide which is used as a general artificial substrate of protein tyrosine kinases(96).

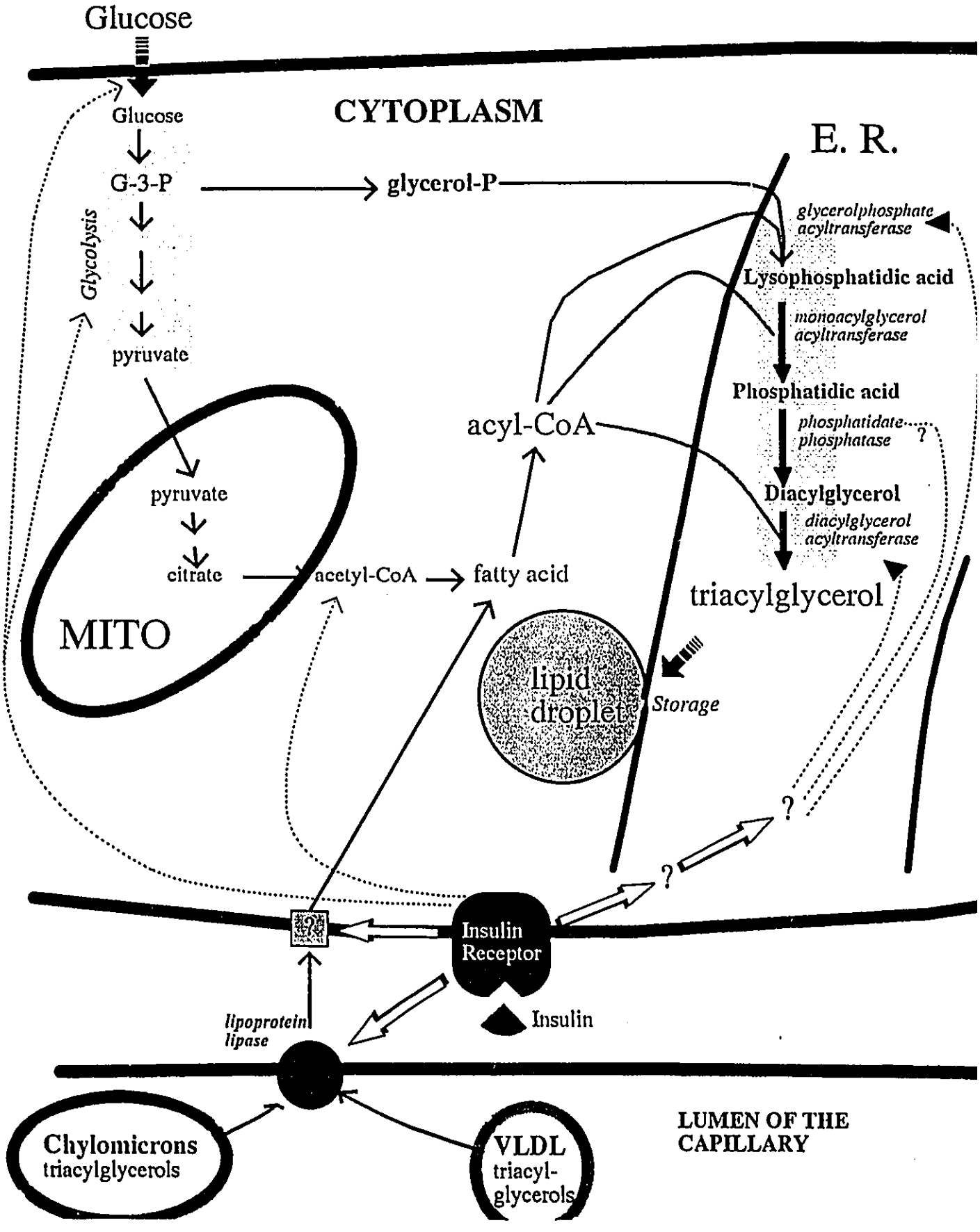
Unlike many other protein tyrosine kinases which have been identified, our kinase does not appear to be autophosphorylated since incubation of the kinase with ^{32}P-γ-ATP in the presence of Mg^{2+} does not result in labelling of the 68 kDa band (Figure 14). It is possible that the kinetics of autophosphorylation are such that dephosphorylation occurs during the incubation and running of the gels. However it is more likely that other kinases in the adipocyte phosphorylate our kinase since a 68 kDa band is radiolabelled in phosphorylation experiments with adipose cytosol alone (Figure 14).

To our knowledge adipose tissue GPAT and DGAT have not yet been purified; therefore we cannot assign any of the phosphorylated bands to either of these two enzymes. DGAT from soybean cotyledons has been found to consist of three different polypeptides of 40.8, 28.7 and 24.5 kDa [108]. However, studies by radiation inactivation of rat liver DGAT suggests that a unit of 68-76 kDa comprising one or more polypeptides is required for activity [109]. It is therefore interesting that one of the major substrates in microsomes of our purified kinase is a polypeptide of approximately 69 kDa. Furthermore, it has been suggested that a 54 kDa microsomal polypeptide which is labelled by radioactive iodoacetate but

is not phosphorylated by PKA, is a component of GPAT (86). This band may correspond to the other major microsomal substrate (53 kDa) of our kinase. However, *Escherichia coli* GPAT comprises a single polypeptide of 83 kDa (110) and murine mitochondrial GPAT has been recently identified as a 90 kDa polypeptide (111).

One of the effects of insulin is an increase in adipocyte triacylglycerol synthesis, which is not secondary to its effects on lipoprotein lipase, glucose uptake, or fatty acid synthesis (2) (see Figure 17). Although insulin action through its receptor tyrosine kinase activity is well established the search for physiologically relevant phosphorylation substrates and for intracellular cytosolic mediators of signal transduction continues. Experiments indicate that the *in vivo* phosphorylation of several proteins including pp185 (112) and perilipin from 3T3 adipocytes [113] are insulin dependent. *In vitro* studies also indicate that the insulin receptor and insulin like growth factor receptor stimulate soluble but not insoluble protein tyrosine phosphatase activity in a dose dependent fashion in rat skeletal muscle (114). Other studies suggests that diabetes appears to induce tissue specific changes in phosphoprotein phosphatase activites (resulting in significant alterations in the dephosphorylation of insulin receptor and glycogen synthase). There appears to be a differential regulation of protein tyrosine phosphatase (PTPase) and protein serine phosphatase (PSPase) activities in diabetes particularly,

Figure 17. Hypothetical regulation of TAG synthesis in adipose tissue



in the liver (115). Another study which investigated the effects of okadaic acid demonstrates that a complex cascade exists which involves PSPase activities in addition and independent of the PTPase activities (116). In the context of TAG synthesis and its regulation by insulin, Figure 17 shows a model for the multiple effects of insulin on glucose transport, glycolysis, fatty acid synthesis, lipoprotein lipase secretion and modulation of TAG synthetic enzyme activities. The mechanism of action of insulin is unclear but is believed to involve a cascade of kinases and phosphatases initiated by the insulin receptor tyrosine kinase. Whether a phosphatase or kinase is next in the signalling cascade remains to be determined. The remaining distal intermediates (i.e., kinases/phosphatases and their respective target enzymes) involved in insulin action (e.g., insulin effects of TAG synthesis) have yet to be elucidated. Furthermore the mechanism of regulation of adipocyte TAG synthesis by other hormones including growth hormone and leptin, likely involves a signalling pathway comprised of kinases and/or phosphatases acting on various target enzymes via a phosphorylation-dephosphorylation mechanism.

Only one other soluble protein tyrosine kinase has been purified from 3T3 cells. Its molecular weight was reported to be 53 kDa and has been suggested to be involved in insulin-independent regulation of glucose metabolism (102,103). Whether this kinase or our tyrosine kinase are involved in a phosphorylation cascade leading to DGAT inactivation remains to be established.

From a different perspective, as suggested by Sohling et al., [84]it is possible physiologically that DGAT, in certain tissues, may be regulated by a phosphorylation mechanism, and thereby play a role in the nuclear inositol signalling cascade through DGAT's effects on the accumulation of DAGs.

CONCLUDING REMARKS

Compared to nucleic acids and proteins, little attention has been given to the regulation of compartmentalization, packaging, transport, differential synthesis, intracellular movement of lipids both in the cell and in the body. This may be due to the relative difficulty in studying unstable membrane bound enzymes and the lipids which interact with them. However, some form of control over lipid metabolism must occur for a eukaryotic cell to have a compartmentalized nucleus, effective and viable plasma membranes and other organelles. Control must also occur for an organism to transport lipids to its component cells and meet their energy demands. The regulation of DGAT by a phosphorylation-dephosphorylation mechanism opens new avenues in the study of TAG, PL and lipoprotein synthesis as well as, of yet unidentified signal transduction pathways.

Possible future directions. Further studies described below should provide greater insight into the physiological relevance and the identification of substrates of the purified protein tyrosine kinase.

1) Induction in adipocytes. DGAT and other lipogenic enzymes are induced in the process of adipocyte differentiation. It would be of interest to ascertain if the soluble tyrosine kinase is present in preadipocytes.

2) Identification/Purification of DGAT and GPAT enzymes could be attempted by incubating washed microsomes with the purified kinase and ^{32}P-ATP. running on PAGE under non-denaturing conditions, electro-eluting the bands correponding to phosphorylation substrates of the purified kinase and search for DGAT or GPAT activities. Alternatively antiphosphotyrosine antibodies could be instrumental for the selective precipitation of solubiiized phospho-DGAT and phospho-GPAT and, in that way, identifying the polypeptide bands corresponding to these acyltransferases. Once the subtrates have been identified, tyrosine phosphorylation could be demonstrated by performing phosphoaminoacid analysis of microsomal phosphoproteins.

3) Purification of larger quantities of the purified kinase could allow the production of antibodies which could be used to screen libraries, in an attempt to clone the gene, or examine the presence of the kinase in different tissues and its role in the regulation of different enzymes including DGAT, GPAT, and ACAT, from various tissues.

4) Strong evidence for the physiological relevance of the phosphorylation/desphosphorylation of the synthetic enzymes could be obtained from studies of the hormonal effects on the kinase/phosphatase system. Adipocytes in primary cultures could be exposed to different hormones and endogenously formed ^{32}P-ATP. The homogenates could be fractionated to purify the kinase and the corresponding PTK activity (with the synthetic peptide), and its labelling could be studied on SDS-PAGE. Furthermore once DGAT and GPAT have been identified from adipose tissue their phosphorylation could be studied by examining the effect of hormone treatment on endogenous microsomal phosphoprotein labelling.

5) Purification and identification of the adipose protein tyrosine phosphatases which function antagonistically with the tyrosine kinase could be important in proving that regulation of the acyltransferases occurs. Since we have detected an activity that could be associated with the phosphatase during the first few steps of

the purification of the kinase, this would suggest a starting point for the fractionation of the adipose cytosol and the subsequent purification of the protein phosphatase.

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Appendix A

List of Abbreviations

The abbreviations used are listed in alphabetical order: **ATP**, adenosine 5'-triphosphate; **BSA**, bovine serum albumin; **cAMP**, cyclic adenosine monophosphate; **CoA**, coenzyme A; **DAG**, diacylglycerol; **DGAT**, diacylglycerol acyltransferase; **DTT**, DL-dithiothreitol; **EDTA**, ethylenediaminetetraacetate, **FA**, fatty acid; **FABP**, fatty acid binding protein; **GPAT**, glycerolphosphate acyltransferase; **HPLC**, high performance liquid chromatography; **HSL**, hormone sensitive lipase; **IR**, insulin, receptor; **LPA**, lysophosphatidic acid; **MGAT**, monoacylglycerol acyltransferase; **PAGE**, polyacrylamide gel electrophoresis; **PAP**, phosphatidic acid phosphatase; **PEG**, polyethylene glycol compound; **PL**, phospholipid; **PKA**, protein kinase A; **PKC**, protein kinase C; **PPase**, protein phosphatase; **PTK**, protein tyrosine kinase; **PSP**, protein serine phosphatase; **PTP**, protein tyrosine phosphatase; **TAG**, triacylglycerol.

Appendix B
CURRICULUM VITAE

Timothy E. Lau

Academic Address:
Department of Biochemistry
Faculty of Medicine
140 Louis Pasteur
Ottawa, Ont.,
K1N 6N5
(613) 564-3467

Home Address:
42 Whippoorwill Dr.,
Gloucester, Ont.,
K1J 7H9
(613) 745-8084

EDUCATION

1993-	Doctorate of Medicine (M.D.) University of Ottawa
1992-1994	Masters in Biochemistry (M.Sc.) (submitted) University of Ottawa
1988-1992	Bachelor of Science (BSc.(Hon.) University of Ottawa

ACADEMIC AWARDS

1992-1993	A+ Excellence Scholarship

PROFESSIONAL EXPERIENCE

1992-present	Masters research
1993	Teaching assistant/ Demonstrator
1992	Summer research project
1991-1992	Fourth year research projects
1991	Summer research project

PUBLICATIONS

1) Rodriguez, M.A., Dias, C., and Lau, T.E. (1992) Reversible ATP-dependent inactivation of adipose diacylglycerol acyltransferase. Lipids 27,577-581

2) Rodriguez, M.A., Lau, T.E., and Dias, C. (1992) *in Drugs Affecting Lipid Metabolism*. Regulation of triglyceride synthesis by a kinase-phosphatase system. Catapano, A.L., Gotto Jr., A.M., Smith, L.C., and Paoletti, R. Eds. Kluner academic publishers. Dordrecht, Netherlands, 149

3) Lau, T.E., and Rodriguez, M.A. (1993) Diacylglycerolacyltransferase kinase. FASEB J. 7(7),639

4) Rodriguez, M.A., Lau, T.E., and Dias, C. (1993) Effects of Antipsychotic Drugs on Lipid Synthesis. CFBS Proceedings.

5) Rodriguez, M.A., Dias, C., and Lau, T.E. (1994) Acyl-CoA delivery to microsomal acyltransferases. CFBS Proceedings, P556.

6) Rodriguez, M.A., and Lau, T.E. (1994) A protein tyrosine kinase associated with the ATP-dependent inactivation of diacylglycerol acyltransferase. Int. J. Obesity 18(2),245

7) Rodriguez, M.A., Lau, T.E., and Lapperiere, J. (1994) Inhibition of adipose and hepatic acyltransferases by antipsychotic drugs. Can. J. Physiol. Pharmacol. 72(1),444

PAPERS TO BE SUBMITTED

1) Rodriguez, M.A., Dias, C., and Lau, T.E. (1994) Acyl-CoA delivery to microsomal acyltransferases. Paper to be submitted to Can. J. Phys. Pharm.
2) Rodriguez, M.A., Lapperiere, J., and Lau, T.E. (1994) Inhibition of adipose and hepatic acyltransferases by antipsychotic drugs. Paper to be submitted to Biochemical Pharmacology.
3) Lau, T.E., and Rodriguez, M.A. (1994) A protein tyrosine kinase associated with the ATP-dependent inactivation of diacylglycerol acyltransferase. Paper to be submitted to Lipids.

ACADEMIC STUDENT SERVICE

1993	Vice President, Student affairs Graduate Students Federation (GSAED)
1993	Student representative to the University Committee for Campus security
1992-93	Graduate student representative to the departmental curriculum committee
1992-93	President/ Biochemistry graduate students association
1992	Biochemistry delegate for the GSAED council
1992	Student representative of the University Strategic Planning Committee. Support staff analysis (UPP)
1991-92	President and Founder of the Undergraduate Biochemistry Students Association (SFUO).
1990-91	Vice-President Social. Science Students Association (SSA)
1989-90	Biochemistry representative to the SSA