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LIPOPROTEIN BIOSYNTHESIS: EXAMINATION OF THE LIPIDATION PROCESS

by

Kimberly Joy Karnas

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Kimberly Joy Karnas

entitled Lipoprotein Biosynthesis:
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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dr. Michael A. Wells — Date 6/20/01

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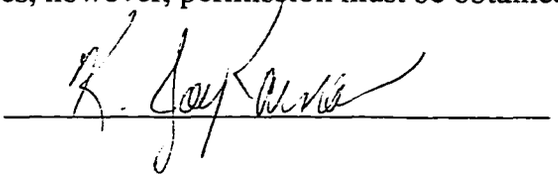
SIGNED: 

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DEDICATION

My family is the most important component of my life, and I would like to dedicate this document to them. Without the support of my family, I could not have survived the past six years. My parents, Claire and Tony Karnas, have always supported me in everything that I have done, including my cross-country journey to complete my education. My sister, Colleen Karnas, has also given wonderful support to me, our phone conversations and twice-annual shopping sprees helping to close the physical distance between us. As Mom always said, "your sister is your best friend," and never have those words been truer than during my Arizona years, where Colleen made every attempt to include me in her life.

Above all, the loving support of my husband, Brian Exton, kept me pushing forward in pursuit of my educational goals. Modifying his own plans, he joined me in the desert, enduring one triple-digit summer after another as I promised "just one more year of graduate school." Finally, I dedicate this dissertation to Jared Exton, who has shown me nothing is more precious than a child's love.

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ABSTRACT

Lipoproteins, protein-lipid complexes that have a polar exterior and a non-polar interior, have been found in many vertebrate and insect species, and their basic structure and function appear to be conserved. They facilitate the intercellular transport of hydrophobic lipids through aqueous media. Their synthesis requires an unusual process referred to as lipidation, whereby lipids are added to the protein component of the lipoprotein. Lipidation is thought to occur during or immediately following translation of these proteins, but how this process occurs is unknown. Of particular interest is the extent to which the protein sequence of the apoproteins drives lipidation and the level of involvement of other proteins in this process.

In this project, lipidation was studied by expressing the apolipoproteins from the tobacco hornworm, *Manduca sexta*, in two different expression systems. The first used budding yeast, *Sacchormyces cerevisiae*, to both determine the ability of unicellular organisms to produce lipoproteins and examine the role that the known secretory pathway for soluble proteins plays in lipoprotein biosynthesis. The second used *Drosophila* Schneider 2 cells to begin to examine the apolipoprotein sequence for regions that are crucial to lipoprotein biosynthesis. The yeast expression system revealed that unicellular organisms are capable of expressing, lipidating, and secreting *M. sexta* apolipoproteins. This is first demonstration of any apolipoprotein being expressed in a unicellular organism, and represents a major finding, as unicellular organisms have no need for a particle that functions in intercellular transport. A second major finding in this project is that lipophorin production occurs in the absence of the full apoLp-I sequence. This

finding was true for both expression systems, and indicates that the lipidation code resides within the first 45% of the precursor protein sequence. Furthermore, deletion analysis has revealed that removal of any portion of the apoLp-II sequence prevents expression of the apolipoprotein. Taken together, these experiments indicate that all of the information required to make a lipoprotein is included in the apoLp-II sequence.

INTRODUCTION

(1) Overview

To sustain life, multicellular animals obtain essential nutrients from their diet. Transport of most of these nutrients to various tissues occurs by directly dissolving them into the aqueous blood. The hydrophobic nature of lipids, however, poses a problem. Their transport requires assistance from lipoproteins, protein-lipid complexes that have a polar exterior and a non-polar interior. Little is known about the production of this complex, and this study uses the tobacco hornworm, *Manduca sexta*, as a model system for examining this process.

This project integrates several areas of biology: entomology, lipid biochemistry, and yeast molecular biology. This section is designed to give the reader a knowledge base for understanding the details and implications of this project. It begins by introducing the reader to some general concepts of insect biology, contrasting many features to the vertebrate system. The current understanding of insect lipoprotein biosynthesis is described, as is that of the vertebrate lipoprotein is most similar to the insect lipoproteins, mammalian apolipoprotein B. As the known secretory pathway for soluble proteins factors into this research, a general overview of the pathway found in both mammals and yeast is given. Finally, the purpose of this project will be discussed, focusing on the three main questions that were central to this research. (1) Is the capacity for lipoprotein production unique to multicellular organisms? (2) Is the pathway elucidated for soluble secretory proteins involved in lipoprotein production or is there an

alternate pathway? (3) Which portions of the apolipoprotein sequence are crucial for lipoprotein production?

(2) Insect Biology

Insects are the most dominant form of animal life on the planet, surpassing all other terrestrial animals in numbers. Nearly one million insect species have been described, and it has been estimated that another 80-100 million remain undescribed. They live practically everywhere, and have done so for about 350 million years (compared to about 2 million for man). They are highly adapted, displaying many unusual features and a wide variety of colors. While many insects are thought of as pests, destroying crops and spreading disease, many are extremely valuable to man. By pollinating plants, insects assist the production of fruits, nuts, clovers, vegetables, etc. They also produce several products that are of commercial value, such as honey, wax, and silk. Insects are fairly easy to rear and have a relatively short life cycle, making them extremely useful in scientific research, especially when you consider that basic physiological processes and biological phenomena are similar for all animals.

A. Insect Phylogeny

A main characteristic shared by all insects is the existence of two postcephalic segments, the thorax and abdomen. The thorax is further divided into three segments, each containing a pair of legs. The second and third thoracic segments often house wings. The abdomen is also subdivided, usually into 11 or fewer segments. Insects can be grouped into three basic categories, based on the extent of change at metamorphosis.

Ametabolous insects completely lack metamorphosis, and newly hatched larvae are essentially a small form of the adult. Insects in which larva resemble the adult, but lack wings and genitalia, are considered hemimetabolous. Holometabolous insects exhibit complete metamorphosis where the larvae are generally quite different from the adult, and there is an intervening pupae stage.

Butterflies and moths are holometabolous insects (Superorder Holometabola) belonging to the order Lepidoptera. Their life cycle begins with the hatching of a worm-like larva, containing three pairs of thoracic legs and a pair of prolegs on segments 3-6 of their abdominal segments. They feed on green plants, storing energy for molting and metamorphosis. Adults usually have two pairs of membranous and often colorfully scaled wings. They primarily feed on nectar, and play a crucial role in pollination.

The lepidopteran *Manduca sexta*, commonly known as the sphynx moth or tobacco hornworm, is appropriately named. The species name, *sexta*, refers to the six orange-yellow spots along each side of the abdomen of the adult. The genus, *Manduca*, means "glutton," and refers to the larvae's voracious appetite. It is well adapted for feeding and devours various plant members of the family *Solanaceae,* such as tobacco, tomato, potato, pepper, and nightshade. They do not feed continuously, however. Just prior to each larval molt, the insect voids its gut, and must rely on stored lipids for energy.

B. Insect Anatomy

Insects are anatomically quite different from humans. They appear to be made upside-down and inside-out, having a ventral nerve cord and an exoskeleton. They lack

lungs, and breathe through openings in their body wall called spiracles. Air passes through large, chitin-lined tubes called tracheae, and into smaller tracheoles, which extend to the surface of nearly every cell. Thus, the heart and blood play only a minor role in gas exchange.

Insects have open circulatory systems, and there is no distinction between blood and interstitial fluid. The general body fluid, called hemolymph, functions to transport nutrients, hormones, and wastes. A dorsal heart pumps hemolymph through vessels and out into sinuses, interconnected spaces surrounding the organs. Chemical exchange between hemolymph and body tissues occurs in these spaces. Body movements of the insect squeeze the sinuses to help circulate the hemolymph. As the heart relaxes, hemolymph is drawn back into the circulatory system through pores called ostia.

Insects have a complete digestive tract and nutrients are obtained in a manner similar to that of vertebrates. Food is ingested at the mouth, hydrolyzed extracellularly, and nutrients absorbed across the lining of the digestive tract into the blood. Most insects do not eat as regularly as vertebrates throughout their life history. Thus, nutrients obtained during the larval stage must be stored for later use during pupation and adult activities, such as flight and egg production. This storage occurs in an organ called the fat body.

The fat body consists of ribbons of tissue, two cells thick, found immediately beneath the body wall. All fat body cells are in immediate contact with the hemolymph, facilitating nutrient exchange. The fat body is essentially analogous to the vertebrate liver and adipose tissue, functioning in both protein synthesis and nutrient storage. Most

hemolymph proteins are synthesized in this tissue, and when protein synthesis stops as the insect prepares for pupation, proteins are removed from the hemolymph and stored as granules in the fat body. Carbohydrates are stored in the fat body as glycogen and are also used in lipogenesis, the process of lipid synthesis. The fat body is the principle site of lipogenesis, whereby fatty acids are synthesized and then incorporated into triacylglycerol (TAG). This contributes to the large proportion of lipids in most insects. More than 70% of the dry weight of the fat body is stored TAG. The amount varies with the developmental stage of the insect, increasing during times of active feeding and decreasing when feeding stops or when energy use is high, such as during flight or oogenesis.

(3) Insect Lipoproteins

The digestive system of insects is subdivided into three main regions: foregut, midgut, and hindgut. Absorption mainly occurs in the midgut. As is true for all animals, absorbed nutrients are transported via the blood to sites of use or storage. Most nutrients, including sugars, electrolytes, vitamins, and minerals, are directly absorbed into the bloodstream. The hydrophobicity of lipids and sterols, however, poses a definite transport problem. A specialized system capable of sequestering the nonpolar molecules away from the aqueous hemolymph must be used for their transport. Lipid-bearing proteins called lipoproteins have a polar exterior and a non-polar interior, and are able to act as vehicles that transport lipids in both vertebrates and insects.

Lipoproteins vary in their composition and thus their buoyant densities--the more lipid in the particle, the lower the density. High-density lipoproteins (HDLs) contain relatively little lipid, as compared to low-density lipoproteins (LDLs). Additionally, a very-high-density lipoprotein (VHDL) exists; the most common insect VHDLs are vitellogenins, egg proteins. These categories are not discrete due to the variation of lipid content of particles and the fact that lipoproteins of different densities may be present in the blood at the same time.

Three main differences exist between vertebrate and insect lipoproteins. The first difference is semantics: insect lipoproteins are generally referred to as lipophorins. The main lipophorin used in lipid storage is a high-density lipophorin (HDLp). Secondly, most insects use lipophorin to transport diacylglycerol (DAG), whereas vertebrate lipoproteins are mostly used for the transport of TAG (Canavoso *et al* 2001). Third, lipophorins are not internalized during lipid delivery, and thus act as a reusable shuttle for lipid transport (Arrese *et al* 2001, Chino 1985, Soulages and Wells 1994).

A. Lipid Transport in Insects

Hydrolysis of dietary TAGs in the midgut lumen of *Manduca sexta* results in free fatty acids that are absorbed by midgut cells (Figure 1) (Canavoso *et al* 2001). The absorbed fatty acids are then transformed into DAG using the phosphatidic acid pathway. If this DAG is not immediately exported to the hemolymph, it is converted to TAG. The TAG acts as a reservoir, as free fatty acids and DAG can be toxic to cells when present at high concentrations. TAG must be converted back to DAG for export from the midgut cells. Export involves three factors: HDLp, a lipophorin receptor, and the lipid transfer

particle. The lipid transport particle is a VHDLp whose physiological function is not completely understood, but it is thought to facilitate DAG transfer from midgut cells to HDLp, docked with its receptor on the hemolymph side of the midgut cell. Once filled with DAG, HDLp leaves its receptor at the midgut, and docks with a second receptor at the fat body. The lipid transfer particle is thought to mediate the transfer of lipid from HDLp to the fat body cells. HDLp is then free to recycle back to the midgut.

B. Lipophorin Composition

All insect lipophorins contain two proteins: apolipophorin I (apoLp-I), which has a molecular weight of 230,000-250,000, and apolipophorin II (apoLp-II), which has a molecular weight of 70,000-85,000 (Robbs *et al* 1985, Beenakkers *et al* 1988, Sundermeyer *et al* 1996). One molecule each of apoLp-I and apoLp-II are found in a lipophorin particle (Blacklock and Ryan 1994). It is believed that these two apolipoproteins, combined with a small amount of phospholipid, comprise the basic lipophorin particle (Blacklock and Ryan 1994). This particle has the capability to accept or donate lipid, giving rise to the variety of lipophorin subspecies seen in the different life stages of insects (Prasad *et al* 1986b, Shapiro *et al* 1988, Soulages and Brenner 1991, Blacklock and Ryan 1994).

A third apolipoprotein, apolipophorin-III (apoLp-III) has a molecular weight of 17,000-20,000 and is found free in the hemolymph as well as associated with the lipophorin of adult insects (Kawooya *et al* 1984, 1986, Beenakkers *et al* 1988). During normal metabolism, the additional lipid added to HDLp contributes only minor changes to the volume and density of the particle. However, when lipid demand is especially

high, such as during flight, so much lipid is added to the particle that it begins to lose its solubility in hemolymph. It is at these times that apoLp-III binds to the particle, resulting in the formation of low-density lipophorin (LDLp). This reversible association stabilizes the lipid-water interface of lipophorin allowing the molecule to carry great amounts of DAG (Kawooya *et al* 1986, Soulages and Wells 1994b).

C. Lipophorin Structure

The structure of lipophorin appears to be similar to mammalian lipoproteins, composed of a hydrophobic core of nonpolar lipid constituents encased in a monolayer of amphipathic phospholipids and apolipoproteins (Figure 2)(Edelstein *et al* 1979, Katagiri *et al* 1987, Shapiro *et al* 1988, Katagiri *et al* 1991, Soulages and Brenner 1991, Blacklock and Ryan 1994). Differential scanning calorimetry, small-angle x-ray scattering, and NMR have revealed that lipophorin is a spherical particle with three layers (Katagiri *et al* 1987, Katagiri *et al* 1991, Blacklock and Ryan 1994). The outer layer is proposed to contain apoLp-I, part of apoLp-II, and phospholipids. The remaining portion of apoLp-II is contained in the hydrophobic middle layer where the DAG is stored. This layer seems to vary in size and composition depending on the amount of DAG present. In the center of the particle, a hydrophobic core of hydrocarbon is found.

The structural localization of lipophorin components is supported by enzyme studies. Phospholipid is easily depleted from lipophorin molecules by exposure to phospholipase A_2 (Kawooya *et al* 1991, Gondim *et al* 1992) and apoLp-I is susceptible to proteolytic digestion (Pattnaik *et al* 1979), indicating that both of these molecules reside on the surface of lipophorin. ApoLp-II, however, is only partially susceptible to

proteolysis (Pattnaik *et al* 1979, Robbs *et al* 1985) suggestive of only a partial exposure to the outer layer of lipophorin. Varying the amount of neutral lipid content has an effect on apolipophorin susceptibility to proteolysis. As the lipid content of lipophorin decreases, apoLp-I and -II become more susceptible to digestion, with apoLp-I preferentially degraded (Ryan *et al* 1992). Additionally, Ryan *et al* (1992) found that as the lipid content of the lipophorin molecule decreases, fluorescence quenching of apoLp-I tryptophan residues by acrylamide increases. The data indicate that in high lipid-containing molecules, tryptophan residues are shielded by interactions with lipid, but as the lipid content of the molecule decreases there is more exposure of tryptophan residues to the solvent. Thus, in the intact particle, lipids shield the apolipoproteins from the external environment.

Further evidence for the localization of apoLp-I and -II comes from immunological studies. In the intact lipophorin molecule, there is a higher immunoreactivity to apoLp-I than apoLp-II, supporting the idea that apoLp-II is somehow sequestered away from the external aqueous environment (Shapiro *et al* 1984, Robbs *et al* 1985). Furthermore, apoLp-I and -II are believed to lie in close proximity (~11Å) as it is possible to cross-link them with the cross-linking reagents, dimethylsuberimidate and dimethyladipimidate (Kashiwazaki and Ikai 1985).

D. Apolipoprotein Synthesis

In insects, the fat body is the site of lipid storage (Fernando-Warnakulasuriya *et al* 1988) and several studies have shown that this is also the site of apolipoprotein synthesis (Prasad *et al* 1986a, Prasad *et al* 1987, Weers *et al* 1992, Shelby and Chippendale 1991).

Immunoblot studies have found that only homogenates from *M. sexta* fat body tissue, and not homogenates from midgut tissue, contain apoLp-I and -II (Prasad *et al* 1986a). Furthermore, *in vitro* incubation of *M. sexta* fat body with radiolabeled amino acids followed by analysis of the proteins secreted from the fat body, shows that apoLp-I and -II are synthesized by and secreted from the fat body (Prasad *et al* 1986a, Shelby and Chippendale 1991, Weers *et al* 1992, Weers *et al* 1993).

Pulse-labeling of fat body protein synthesis followed by SDS-PAGE analysis showed a 280kDa protein simultaneously disappears from homogenates as apoLp-I and apoLp-II appear (Weers *et al* 1993). This 280kDa protein is recognized by both apoLp-I and -II antibodies, indicating that this 280kDa protein is a precursor that is later cleaved into the two separate apolipoproteins. The cloning of the precursor cDNAs from *M. sexta* and *Drosophila melanogaster* confirmed this observation, demonstrating that the precursor is arranged with apoLp-II at its amino terminus and apoLp-I lies at the carboxy terminus. (Kutty *et al* 1996, Sundermeyer *et al* 1996). The *M. sexta* sequence contains a putative 23 amino acid signal sequence and an RGRR site where an insect convertase is believed to cleave the precursor protein into its two components, apoLp-II and apoLp-I.

Additionally, pulse-labeling experiments have found that translation on rough endoplasmic reticulum, transport to the Golgi complex, glycosylation, and secretion take about 45 minutes in the fat body of the southwestern corn borer, *Diatraea grandiosella* (Shelby and Chippendale 1991), and only about 35 minutes in the locust *L. migratoria* (Weers *et al* 1993). Both apoLp-I and -II are glycosylated, and tunicamycin, an inhibitor of protein glycolysis, neither prevents cleavage of the precursor (Weers *et al* 1993), nor

secretion of apoLp-I and -II (Shelby and Chippendale 1991, Weers *et al* 1993), yet both protein synthesis and vesicular transport inhibitors prevent the production of lipophorin particles (Shelby and Chippendale 1991). Taken together, these results suggest that the complete synthesis of the apoLp-I,II precursor is necessary for nascent lipophorin particle formation, but glycosylation is not.

E. Apolipoprotein Lipidation

Significant synthesis of lipophorin molecules occurs during the larval stage (Prasad *et al* 1986b, 1987), and, unlike in mammals, insect lipophorin is synthesized regardless of the amount of lipid in the diet (Fernando-Warnakulasuriya *et al* 1988). Excess fat in the diet results in increased lipid content of lipophorin, not increased number of lipophorin particles.

The mechanism for lipidation, the process whereby the apolipoproteins are packaged with lipids into a soluble lipoprotein particle is unknown. Several observations have indicated that this process occurs at the site of apolipoprotien synthesis, the fat body. (1) ApoLp-II is insoluble (Soulages and Wells 1994). (2) The synthesis, processing, assembly, and secretion of the lipophorin particle is quite rapid (Shelby and Chippendale 1991, Weers *et al* 1993). (3) No free apoLp-I or - II peptides are secreted, they are always associated with lipids (Weers *et al* 1992) and each other (Weers *et al* 1993), and even though apoLp-I is three times larger than apoLp-II, they are secreted in equimolar amounts (Shapiro *et al* 1984). (4) ApoLp-I and -II coimmunoprecipitate from fat body homogenates (Weers *et al* 1993). Combined, these observations suggest that lipidation occurs during or closely following the translation of the precursor protein

(Blacklock and Ryan 1994). The newly formed lipophorin particle is secreted as a nascent particle that contains only phospholipids and proteins; DAG is added to this nascent particle at the midgut (Prasad *et al* 1986a, Fernando-Warnakulasuriya *et al* 1988).

(4) A Mammalian Lipoprotein: Apolipoprotein B (ApoB)

A. ApoB Structure

Comparison of the entire deduced amino acid sequences of *M. sexta* and *D. melanogaster* precursor proteins shows an identity of approximately 21% (Van Heusden *et al* 1998). Sequence comparisons of the apoLp-II,I precursor protein from *Locusta migratoria*, *M. sexta*, and *D. melanogaster* with human apolipoprotein B (apoB) revealed contiguous conserved sequence motifs (Babin *et al* 1999). In mammals, secretion of apoB determines the plasma low-density lipoprotein levels (Davis 1990). ApoB is a large secretory protein that contains long amphipathic β-sheets and α-helices, as well as several hydrophobic regions (Knott *et al* 1986). These hydrophobic regions do not resemble transmembrane domains and are rather short, possibly too short to span the lipid bilayer (Bostrum *et al* 1988). This, combined with reports that show 1) the peptide is not fully integrated into the membrane, easily extracted by alkaline carbonate treatment (Chuck *et al* 1990, Pease *et al* 1991), 2) the length of the apoB peptide determines the size of the lipoprotein molecule (Spring *et al* 1992), and 3) trypsin-accessible and -inaccessible peptides are grouped in five non-random domains, has lead to the proposal that the nascent peptide is inserted into the inner leaflet of the ER.

B. ApoB Biosynthesis

Assembly of the lipoprotein particle occurs co-translationally, although additional lipids are added posttranslationally to give rise to the very low-density lipoprotein (VLDL) particle (Boren *et al* 1994). ApoB mRNA levels remain constant (Pullinger *et al* 1989, Leighton *et al* 1990, Davidson *et al* 1988) and secretion is believed to be regulated by several posttranslational factors that influence the amount of apoB that is posttranslationally degraded. One of these regulating factors is lipid availability. TAG is the major glyceride transported by the lipoprotein, and two pools of TAG exist in mammalian cells, one cytoplasmic and one microsomal (Wu *et al* 1996). While levels of TAG are generally higher in the cytoplasmic pool, it is the content of the microsomal pool that regulates apoB secretion. Oleate, one of the fatty acid chains of TAG, stimulates apoB secretion by decreasing the amount of apoB degraded intracellularly (Pullinger *et al* 1989, Dixon *et al* 1991, White *et al* 1992, Leiper *et al* 1994).

The current theory on a second aspect of regulation, apoB translocation, is that when apoB is incompletely translocated across the ER membrane, degradation occurs (Davis *et al* 1990, Thrift *et al* 1992, Du *et al* 1994, Bonnardel and Davis 1995). The apoB signal sequence appears to play a role in this determination of whether apoB is degraded or secreted (Sturley *et al* 1994). ApoB translocation, however, is a highly debated topic. Several experiments have determined that translocation is a discontinuous process, paused at discrete locations due to pause sites on the protein (Chuck *et al* 1990, Chuck and Lingappa 1992, 1993, Hegde and Lingappa 1996). The pause sites have been identified and a consensus sequence (KKTKNSEEFA) has been determined (Chuck and

Lingappa 1993). These pauses in translocation occur without a break in translation, resulting in a "back-up" of untranslocated protein in the cytosol that can be both degraded by proteases and recognized by antibodies (Chuck *et al* 1990, Chuck and Lingappa 1993, Du *et al* 1994, Hegde and Lingappa 1996). These translocation-blocked apoB peptides are then degraded by a protease that is inhibited by the thiol protease inhibitor *N*-actylleucylleucylnorleucinal (ALLN) (Thrift *et al* 1992, Du *et al* 1994, Leiper *et al* 1994).

An association of apoB with cytosolic Hsp70 has been observed, and it is believed that this chaperone assists translocation by maintaining the "backed-up" apoB in a translocationally competent state (Zhou *et al* 1995). Other labs have found ribosome pause sites, leading them to believe that translocational pausing is just an artifact of the uncoupling of translation and translocation (Leiper *et al* 1996). It has also been shown that the ribosomal pause sites could cause the formation of transmembrane forms of apoB *in vitro*, but these pause sites do not appear to affect translocation in intact cells (Pease *et al* 1995). Alternatively, it has been argued that apoB translocation occurs continuously, without these reported pauses (Ingram and Shelness 1996, Leiper *et al* 1996). In this case, it is believed that degradation is not a result of untranslocated apoB, but rather of a poorly assembled, lipid-deficient particle (Ingram and Shelness 1996).

Support for the predominant translocation-block theory comes from studies of an autosomal recessive disease called abetalipoproteinemia (ABL). In patients with ABL, the liver and intestine are unable to secrete apoB (Du *et al* 1996). The ABL phenotype was found to be caused by a mutation in a protein called microsomal triglyceride transfer protein (MTP) (Wetterau *et al* 1992, Sharp *et al* 1993, Ricci *et al* 1995). This protein

complex is composed of two subunits, protein disulfide isomerase (PDI) and a 88-97kDa protein (Wetterau *et al* 1991). A biopsy from one ABL patient revealed a mutation that caused a truncation in the 88-97kDa protein that prevented it from forming a stable complex with PDI, suggesting that the C-terminus of this protein is vital to complex formation (Ricci *et al* 1995). When this complex is stably assembled, it functions in the liver and intestine to transfer lipids to nascent apoB lipoprotein particles (Wetterau *et al* 1991, Sharp *et al* 1993). MTP is believed to stimulate the assembly and secretion of lipoprotein by transferring lipid to apoB (Gretch *et al* 1996, Wang *et al* 1996). Expression of both MTP and apoB is capable of stimulating secretion of TAG associated lipoprotein in cells which do not normally secrete TAG, indicating that these two components are sufficient for TAG-bound lipoprotein secretion (Gretch *et al* 1996). Expression of different portions of apoB indicate that the C-terminus is required for lipid binding, but not for lipoprotein particle formation (Yao *et al* 1991, Spring *et al* 1992, Du *et al* 1994), while the amino terminus is necessary but not sufficient for recognition by MTP (Gretch *et al* 1996). Cells that cannot express MTP are unable to translocate or secrete apoB (Gordon *et al* 1994, Leiper *et al* 1994). Such cells accumulate apoB situated with its N-terminus in the ER lumen and the C-terminus on the cytoplasmic surface of the ER (Du *et al* 1994). Proteolytic digestion of such translocation-arrested proteins results in lumenal apoB peptides that are secreted into the plasma. Use of protease inhibitors that block posttranslational degradation have determined that the proteolytic digestion most likely occurs during translation at a time when translation is blocked or paused (Wang *et al* 1996). Analyses of plasma from ABL patients reveal little

or no full-length apoB, but high levels of the truncated forms of apoB (Du *et al* 1996). This strongly implies that translocation blocks do occur for apoB. Furthermore, it suggests that MTP-facilitated lipidation of apoB may be required for complete apoB translocation. Variations in MTP function may be responsible for regulation of apoB secretion.

(5) The Secretory Pathway Elucidated for Soluble Proteins

A. Composition of the Translocon

Competition studies, cross-linking studies, and protein deletion studies demonstrate that membrane and secretory proteins use the same cellular machinery for translocation, with the only difference being that membrane proteins contain one or more transmembrane segments that become folded and integrated into the lipid bilayer (Borel and Simon 1996a, 1996b). Targeting of proteins to the endoplasmic reticulum generally occurs co-translationally, although a post-translational pathway has been decribed for yeast, *Saccharomyces cerevisiae* (Andrews and Johnson 1996). Nascent secretory and membrane proteins are selected during translation on free ribosomes by the binding of the signal recognition particle (SRP) to the signal sequence at the amino-terminus of a nascent peptide as it emerges from a free ribosome (Gilmore 1993). The binding of SRP to its target on the ER membrane targets the ribosome-nascent chain complex to the site of translocation, the translocon (Walter and Blobel 1981, Walter and Lingappa 1986, Rapoport *et al* 1996a, 1996b). SRP then dissociates from the signal sequence and translocation begins.

The translocon is a multi-layered structure. The signal and signal-anchor sequence are able to contact the phospholipid bilayer directly (Martoglio *et al* 1995) and transmembrane domains of the nascent protein are known to move past a transmembrane protein called Sec61p to sites adjacent to another protein called TRAM before entering the membrane bilayer (Do *et al* 1996). The majority of the translocon is believed to be formed by a complex designated the Sec61 complex that contains Sec61p and two other proteins. Purified mammalian and yeast Sec61p complexes form cylinders in detergent that are visible by electron microscopy (Hanein *et al* 1996). These structures are also found in freeze-fracture analyses of native rough ER membranes isolated from both mammals and yeast. These cylinders contain 3-4 heterotrimers and are about 50-60Å in length, have a diameter of about 85Å, and a central pore of about 20Å, and. It is unclear whether the central pore extends all of the way through the cylinder, or if the cylinder is enclosed on one end.

The translocon was first identified as an aqueous channel through the ER membrane when peptides in the process of translocation were found to be accessible to aqueous perturbants (Gilmore and Blobel 1985). The use of electrophysiological techniques reveals the presence of such channels in microsomal membranes (Simon and Blobel 1991). These channels are generally not able to conduct ions, yet when puromycin is used to uncouple nascent peptides from their ribosome-bound peptidyl-tRNA, conduction occurs. This indicates that the peptide was blocking the channel and removal of the peptide opened the channel. Furthermore, increasing the salt concentration in the experimental protocol causes the detachment of ribosomes from the

microsomal membranes, resulting in the closing of the ion-conducting channels. Thus, the ribosome seems to interact with components of the translocon to keep it in an open configuration.

Further evidence that the translocon is a proteinaceous channel comes from studies using fluorescent probes incorporated into signal sequence and nascent chains. The lifetime of the fluorescence during translocation demonstrates that secretory proteins are in an aqueous environment throughout translocation (Crowley *et al* 1993, 1994). In addition, collisional quenching of the fluorescence indicates that a lumenal "gate" protein blocks access to the translocon until after the nascent chain is greater than 70 amino acids long (Crowley *et al* 1994). Protease digestion studies following treatment with detergent to disrupt the membrane further confirm the inaccessibility of short nascent peptides to the lumen (Connolly *et al* 1989). Coincidently, the signal sequence-dependent binding of ribosomes to ER translocons can still occur in the absence of SRP and SRP receptor once the nascent chain is greater than 70 amino acids in length (Jungnickel and Rapoport 1995). Taken together, this indicates that interactions between the signal sequence and the translocon cause the gate to open (Andrews and Johnson 1996). This gate, combined with the tight binding of the ribosome to the surface of the endoplasmic reticulum, allows the membrane to maintain its role as a permeability barrier between the cytosol and the endoplasmic reticulum lumen.

It is theorized that the translocon is a passive pore and the nascent chain is free to move bidirectionally through the ER membrane; forward (trans) movement is driven by protein synthesis (Ooi and Weiss 1992). Processes such as glycolsylation, folding,

disulfide bond formation, or association with chaperones prevent retrograde movement of proteins through the translocon (Ooi and Weiss 1992, Nicchitta and Blobel 1993, Simon 1993). Furthermore, translation termination causes a dissociation between the ribosome and integral membrane proteins, closing the channel and preventing retrograde movement of the nascent peptide (Ooi and Weiss 1992).

B. The Structure of the Mammalian Translocon

The translocon consists of integral and peripheral membrane proteins on the endoplasmic reticulum. The mammalian translocon is minimally composed of five proteins, Sec61α, Sec61β, Sec61γ, TRAM and the SRP receptor (Gorlich and Rapoport 1993, Andrews and Johnson 1996, Do *et al* 1996, Rapoport *et al* 1996a, 1996b). The mammalian sec61α protein is a 34-37kDa nonglycoprotein that is predicted to span the membrane 10 times and shares sequence homology with the yeast protein sec61 and the *E.coli* protein secY (Gorlich *et al* 1992b, Do *et al* 1996). It complexes with two additional low molecular weight polypeptides sec61β (14kDa) and sec61γ (8kDa) (Gorlich and Rapoport 1993). The sec61 complex binds directly to the ribosome in a nascent peptide independent manner, although the association is much tighter when the ribosome contains a peptide (Gorlich *et al* 1992b). Cross-linking studies have shown that nascent chains are adjacent to sec61α during most of their transit through the membrane (Mothes *et al* 1994). Interactions between the ribosomal complex, nascent chain, SRP, and SR can occur in the absence of Sec61, but Sec61 is necessary for protein translocation cross-linking of nascent peptides to TRAM (Gorlich and Rapoport 1993). TRAM and Sec61p appear to contact different regions of the signal sequence during

translocation (High *et al* 1993). Cross-linking analyses indicate that the amino terminal region of the signal sequence is adjacent to TRAM (Mothes *et al* 1994), while Sec61p is adjacent to the hydrophobic core and regions C-terminal of the signal sequence (High *et al* 1993).

C. The Structure of the *Saccharomyces cerevisiae* Translocon

The yeast translocon is very similar to that found in mammalian systems. Homologues have been found for all proteins except TRAM. Three particular proteins (Sec61p, Sec62p, and Sec63p) are crucial to protein translocation in yeast, as defects in alleles of their respective genes *sec6l, sec62, or sec63* result in a cytoplasmic accumulation of untranslocated proteins destined for the secretary pathway (Deshaies and Schekman 1989, Deshaies et al 1991). Sec61p forms a heterotrimeric complex with SS1p (homologue of mammalian Sec6lγ) and Sbhlp (homologue of mammalian Sec61β) that functions in both cotranslational and posttranslational translocation (Panzer *et al* 1995, Siegel 1995). Translocating peptides can be cross-linked to Sec61p, Sec62p, (Musch *et al* 1992, Sanders *et al* 1992) and a lumenal heat shock 70 protein, Kar2p (Sanders *et al* 1992). The cross-link with Sec6lp reveals that it is most likely in continuous contact with the peptide, whereas Sec62p appears to only be involved in translocation transiently (Musch *et al* 1992).

Kar2p, a lumenal heat shock 70 protein that is homologous to the mammalian protein BiP, is an essential component of the translocon (Brodsky *et al* 1995). Certain *kar2* mutants can prevent translocation completely, resulting in cytoplasmic accumulations of protein precursors that still contain their signal sequences (Vogel 1990).

Recent data suggests that Kar2p functions in both posttranslational insertion of precursor proteins into the translocon and in an ATP-dependent manner to pull the precursor protein out of the pore and into the ER lumen (Lyman and Schekman 1997). Although it has been demonstrated that the mammalian Kar2p homologue, Bip, is not required for protein translation in mammalian ER (Gorlich and Rapoport 1993), mammalian microsomes do require lumenal proteins for unidirectional translocation (Niechitta and Blobel 1993, Simon 1993).

(6) Purpose

The purpose of this project was to determine the minimal requirements for lipoprotein production, lipidation, and secretion. Using an apolipoprotein cloned from the tobacco hornworm *Manduca sexta* and two *in vivo* expression systems, budding yeast *Sacchoromyces cerevisiae* and S2 *Drosophila* cells, three questions were addressed:

(1) Is the capacity for lipoprotein production unique to multicellular organisms? Lipoproteins function in the intercellular transport of lipids. Unicellular organisms have no need for such a particle, and no such particle has ever been found to be produced by these cells. Lipoprotein production has been suggested to occur by the insertion of the apolipoprotein into the ER bilayer (Chuck *et al* 1990, Pease *et al* 1991, Spring *et al* 1992). If lipoproteins require an unusual pathway for production, single-celled organisms will not be capable of lipidating apolipoproteins. Furthermore, if single-celled organisms are incapable of lipidating apolipoproteins, it should be expected

that the apolipoproteins will be degraded as is observed for mishandled apoB (Davis *et al* 1990, Thrift *et al* 1992, Du *et al* 1994, Bonnardel and Davis 1995). It was determined whether yeast cells were capable of synthesizing, lipidating, and secreting apolipoproteins.

(2) **Is the pathway elucidated for soluble secretory proteins involved in lipoprotein production or is there an alternative pathway?** Homologous secretory pathways have been described for all examined organisms--bacteria, yeast, and vertebrate cells. To date, all secreted proteins examined have been found to use this secretory pathway. Lipidation of apolipoproteins is thought to be associated with the translation of the precursor protein, and it is reasonable that this process should be compatible with secretion. Photocrosslinking studies have shown that translocating proteins do have access to ER phospholipids (Nilsson *et al* 1994, Martoglio *et al* 1995, Rapoport *et al* 1996), so the insertion of the apolipoprotein into the ER bilayer could occur during tranlocation. Using yeast secretory mutants, the requirement for the secretory pathway in the production of lipophorin was investigated. Additionally, attempts were made to develop a system for determining which accessory proteins are required for apolipoprotein synthesis, lipidation, and secretion.

(3) **Which portions of the apoLp-II sequence are crucial for lipoprotein production?** ApoLp-II is translated before apoLp-I and becomes sequestered away from the external aqueous environment in the intact lipophorin particle,

thus the protein sequence of apoLp-II is likely crucial to lipidation. The proper folding of apoB, particularly the formation of disulfide bonds has been demonstrated to be required for lipidation (Tran *et al* 1998, Burch and Herscovitz 2000). Therefore, both deletion analysis and alanine-scanning mutagenesis were used to begin to explore the protein sequence for portions that are key to apolipoprotien lipidation. Based on apoB research and their abundance in the first 130 amino acids of the apoLp-II sequence, cysteine and proline residues were specifically targeted.

METHODS AND MATERIALS

This work conducted in support of this project involved the development of two expression systems. This section details the procedures involved in using these systems (cloning, transformation, protein expression, etc.), beginning with the yeast expression system and following with the *Drosophila* S2 expression system. General procedures used in both systems, such as mutagenesis, His-tag purification, and western analysis, are described at the end of this section.

(1) The Yeast Expression System

A. Cloning

A cDNA clone containing a portion (1470 amino acids) of the apoLp-II,I precursor was altered such that silent mutations were made to remove a native BamHI restriction endonuclease site (at 1336bp) (Figure 3) and insert new BamHI restriction sites to either end of the sequence (at -10bp and 4413bp) (Figure 3) such that the 5' untranslated region of the precursor fragment and all of the plasmid DNA would be removed upon excision. An additional adenine base was placed just prior to the start codon (just inside the BamHI site), resulting in an AAAAATG start sequence (Figure 3), and an AvrII site including an in-frame false stop codon was placed at the end of the fragmented sequence (4409bp, just inside the BamHI site) (Figure 3). Using the new BamHI sites, the apoLp-II,I precursor fragment was cloned into a centromere-based yeast plasmid, pAAB85 (Figure 4) (Adams *et al* 1995), between the galactose promoter and actin terminator. The plasmid was then transformed into a wild type yeast strain (S150-

2B, Dieckmann Lab, The University of Arizona, Tucson, AZ), as described below. The plasmid was also transformed into five temperature sensitive mutants acquired from the laboratory of Randy Schekman (The University of California, Berkeley, CA): RSY528RDM50-76D (*Sec62*), RSY1132/CSY150 (*Sec61*, Stirling *et al* 1992), RSY457/CSY128 (*Sec65*, Stirling *et al* 1992), RSY153/JRM163 (*Sec63-1*, Rothblatt *et al* 1989), and RSY529/RDM50-94C (*Sec62*, Deshaies and Schekman 1989). Two non-temperature sensitive mutants, MS137 (*Kar2-137*) and JR89 (*sec62*, Deshaies and Schekman 1989) were tested against their wild type strains, MS10 (Rose *et al* 1989) and JR81 (Deshaies and Schekman 1989). These four strains were obtained from The Dieckmann Lab (The University of Arizona, Tucson, AZ)

Carboxy-Terminal Addition of GFP

An Xba I restriction endonuclease site was inserted just prior to the green fluorescent protein (GFP) sequence (at 612bp) on pEGFP-N1 (Clontech, Palo Alto, California). Using this Xba I site and one already present on the plasmid, GFP was removed from the pEGFP-N1 plasmid and inserted on pAAB85 (Figure 5). An apoLp-II,I fragment containing all of the aforementioned BamHI restriction site changes (Figure 3) but lacking the AvrII/false stop codon was inserted in-frame, in front of GFP using the BamHI restriction sites on pAAB85 and GFP. The plasmid was then transformed into a wild type yeast strain (S150-2B, Dieckmann Lab, The University of Arizona) as described below.

Carboxy-Terminal Addition of His Tag

An AflII restriction endonuclease site was placed just prior to the false stop codon in the modified apoLp-II,I fragment described above, and this apoLp-II,I fragment was inserted into pAAB85 using the BamHI restriction sites (as described above). A double-stranded DNA fragment containing ten histidine codons (CAT) and a stop codon (TAG) flanked by sticky ends compatible to the AflII cut sites was ligated into the AflII-treated apoLp-II,I fragment.

B. Transformation

Plasmids were transformed into yeast strains as described by Gietz and Schiestl (1995) and plated on solid minimal media (lacking uracil) as described in Ausubel *et al* (1992). Selected colonies were grown in liquid minimal media (lacking uracil) as described in Ausubel *et al* (1992).

C. Expression

All yeast strains were grown in liquid minimal media (lacking uracil). Wild type strains were grown in a 30°C shaking incubator and all temperature sensitive mutant strains were grown in shaking incubators at 16 °C or 36 °C. Expression of the apoLp-II,I fragment was induced in both strains by the presence of galactose in the media, and repressed by the presence of dextrose.

D. Isolation of Proteins

First, to degrade the cell wall and produce spheroplasts, a procedure modified from Ausubel *et* al (1992) was used. Yeast cells were rinsed with sterile water and incubated in 1 volume (1mg cells = 1ml buffer) zymolase buffer (50mM Tris-Cl, pH 7.5, 10mM $MgCl_2$, 1M sorbitol) supplemented with 30mM DTT for 15 minutes at room

temperature. Cells were pelleted (1500g) and the supernatant discarded. Cells were then incubated in 1 volume zymolase buffer supplemented with 10mM DTT and 5 U/µl lyticase (Sigma, St. Louis, Missouri) for 1hr at 30 °C. Cells were pelleted (1500g) and resuspended in 1 volume extraction buffer (0.1M Na_2CO_3, pH 9). Following a 15 minute incubation at room temperature, samples were immediately subjected to 8% SDS-PAGE.

E. Fractionation

Lipid-associated apoLp-II,I fragments were isolated from the supernatant by single spin density gradient ultracentrifugation in a Beckman VC53 vertical rotor, using a procedure modified from Shapiro *et al* (1984). KBr was added to a final concentration of 40% KBr of solution. 20ml of this mixture was placed in Beckman 40ml QuickSeal tubes and overlayered with 20ml of a 30% KBr, 0.9% NaCl solution. Tubes were sealed and centrifuged at 50,000rpm for 16hr at 4 °C. 1ml fractions were collected, dialyzed, and loaded on to 8% SDS-PAGE gels.

F. Isolation By Antibodies

The IgG of antibodies raised in rabbits (Cocalico, Reamstown, PA) against native apoLp-II or native apoLp-I was incubated with protein A matrix/250µm acrylic beads (Sigma, St. Louis, Missouri) in binding buffer (20mM $NaPO_4$, pH 7.0) for two hours at 4 °C. Beads were then incubated overnight at 4 °C with dialyzed sample that was purified on a 20/44 KBr gradiant. Protein was eluted from beads with 0.1M glycine buffer, pH 3.0 and neutralized with 1M Tris-HCl, pH 9.0. Sample was immediately subjected to 8% SDS-PAGE.

(2) The *Drosophila* S2 Expression System

A. Cloning

Using the Xho I and EcoRI sites flanking an unmodified version of the same apoLp-II,I fragment clone described above (Figure 3), the entire clone (1484 amino acids, including the 5' untranslated region and minus the false stop codon) was placed into a Drosophila Expression System vector, pAc5.1/V5-HisA, containing an in-frame C-terminal V5 epitope tag followed by an in-frame C-terminal polyhistidine purification tag (Figure 6) (Invitrogen, Carlsbad, California). S2 *Drosophila* cells (Exelixis, San Francisco, CA) were then transiently transfected with the plasmid as described below.

AMINO ACID CHANGES AND DNA DELETION MUTANTS

Alanine-scanning mutagenesis was performed, individually substituting alanine residues for cysteine and proline residues at the following sites: C81A, C102A, C123A, P95A, P101A, P103A, P109A, and P120A (Figures 3, 7). Transient transfections were made as described below.

Deletion mutants were made by the insertion of Afl II restriction endonuclease sites at various locations on the cDNA (two adjacent sites per deletion) (Figures 3, 8). The plasmids were cut using Afl II endonuclease, purified away from the small 200-300 bp fragment, and re-ligated using T4 ligase. Transient transfections were made as described below.

CARBOXY-TERMINAL ADDITION OF GFP

An AvrII restriction site was placed just after the polyhistidine tag on the pAc5.1/V5-HisA vector containing my apoLp-II,I fragment cDNA in such a way as to

remove the stop codon. Using the two Xba I sites on pEGFP-N1 (Clontech, Palo Alto, California) as described above and the complimentary Avr II cut site on pAc5.1/V5-His A, the entire GFP sequence (including its stop codon) was placed in-frame behind the polyhistidine tag (Figure 9). Transient transfections were made as described below.

B. Transient Transfection and Protein Expression

S2 cells were transfected using a calcium phosphate procedure modified from Invitrogen, Carlsbad, California. S2 cells were grown in 3ml S2 media (Gibco) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum overnight in 35mm plates and transfected at a 19:1 recombinant:selection plasmid DNA ratio. Calcium phosphate solution was incubated with cells 18-20 hours, and replaced with fresh S2 media supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. At 5 days posttransfection, media was collected from cells by centrifugation (1000g), dialyzed in binding buffer (4M NaCl, 40mM imidazol, 160mM Tris, pH 7.9), His-tag purified (see below), and analyzed by western blot (see below).

(3) Other General Procedures

A. Apolipoprotein Isolation from Fat Body and Hemolymph

Fat body was isolated from 5th instar larvae and homogenized in a homogenization solution (1mM DFP, 5mM Benzamidine, 1mM PMSF, 5mM EDTA, 5mM glutathione) at a concentration of 5ml/1g of tissue. HDLP was purified from *Manduca sexta* hemolymph on a 1-40% potassium bromide gradiant. Homogenate and purified HDLP were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

(4-15% gradient gels). The proteins were transferred to nitrocellulose and immunobloted with polyclonal antibodies to the apoLp-I or apoLp-II protein.

B. Mutagenesis

All alterations to DNA sequences in both the *Saccharomyces* and *Drosophila* systems were done using the Quickchange Site-Directed Mutagenesis (Stratagene, La Jolla, California) which uses Pfu to make base pair changes to plasmid DNA.

C. Isolation of Proteins By Nickel-Charged Resin

His-tag purification of the apolipoproteins was done in batch using His-Bind Resin (Novagen, Madison, WI). The resin was incubated with distilled water and separated by centrifugation (1000g). The resin was charged by incubation with nickel buffer (400mM $NiSO_4$) for twenty minutes followed by a rinse with binding buffer (4M NaCl, 40mM imidazol, 160mM Tris, pH 7.9). The charged resin was incubated with the dialyzed media for twenty minutes, rinsed with binding buffer, and incubated with stripping buffer (4M imidazol, 2M NaCl, 80mM Tris, pH 7.9) for twenty minutes. The stripping buffer was separated from the resin by centrifugation, mixed with SDS-PAGE buffer, and loaded onto 8% SDS-PAGE gels.

D. Antibodies

HDLP was purified from *Manduca sexta* hemolymph by gradiant ultracentrifugation and the apolipoproteins were isolated by SDS-PAGE and used to generate polyclonal anti-apoLp-II and anti-apoLpI antibodies in rabbits by Cocolico. Monoclonal anti-green fluorescent protein (GFP) antibodies raised in mice were

purchased from Novagen (Madison, WI). Horseradish peroxidase-conjugated goat-anti rabbit and goat anti-mouse antibodies were purchased from BioRad (Hercules, CA).

E. Western Analysis

Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8% gels). The proteins were transferred to nitrocellulose. The nitrocellulose was blocked with 4% milk and incubated at 4°C overnight in 1:1000 dilutions of primary antibody anti-apoLp-II, anti-apoLp-I, or anti-GFP. The nitrocellulose was then incubated at room temperature for one hour with: 1:5000 goat-anti-rabbit for apoLp-II and apoLpI blots and 1:3000 goat-anti-mouse for GFP blots. The blots were then developed using phosphate-buffered saline (PBS, 50mM Na_2PO_4, 150mM NaCl, pH 7.2) containing 0.5mg/ml chloronapthol, and 0.0125% hydrogen peroxide. Molecular markers are shown at left.

F. Thin-Layer Chromatography

Lipids were isolated from the fractions by Folch Extraction with methanol and chloroform (Folch *et al* 1957, Bligh and Dyer 1959). Any neutral lipids in the samples were separated by thin-layer chromatography (TLC) on silica gel G plates using hexane-ether-acetic acid 60:40:1 (v/v/v) (modified from Mangold 1969). The neutral lipids (monoacylglycerol diacylglycerol, free fatty acid, and triacylglycerol) as well as the material that remained at the origin, the phospholipid fraction (PL), were visualized with I_2 vapor. PLs in the fractions were separated on silica gel G plates using dichloromethane-methanol-water 65:25:1 (v/v/v) and visualized with I_2 vapor.

RESULTS

(1) ApoLp-II and ApoLp-I Production in *Manduca sexta*

To confirm that the *M. sexta* precursor protein is cleaved into the two distinct proteins prior to its release into the hemolymph of the insect, fat body and hemolymph proteins were compared by western blot (Figure 10). Antibodies raised against apoLp-I recognized two proteins (~330kDa and ~250kDa) in the fat body homogenate, but only one protein (~250kDa) in the hemolymph. The same high molecular weight protein (~330kDa) from the fat body homogenate was recognized by antibodies raised against apoLp-II, as was a lower molecular weight protein (~70kDa). In the hemolymph, only the lower molecular weight protein (~70kDa) was recognized by antibodies raised against apoLp-II. Thus, the apoLp-II,I precursor protein was fully translated in the fat body and cleaved into two distinct proteins (apoLp-II and apoLp-I) prior to the release of these resulting proteins into the hemolymph.

(2) ApoLp-II and ApoLp-I Biosynthesis in Expression Systems

Further examination of lipoprotein biosynthesis required development of an expression system that was easily manipulated. Two such systems, a yeast expression system and a *Drosophila* S2 expression system, were developed for this purpose. Both systems were used to express apoLp-I and apoLp-II from *M. sexta*. These two proteins are expressed as a single precursor protein with apoLp-II at the amino terminus. A full-length clone is not available for the precursor, so in both systems a cDNA that encoded about 45% of the precursor protein was used. This fragment of the precursor includes all

of the deduced signal sequence and apoLp-II, but only about 40% of apoLp-I. Expression of this precursor protein fragment in these two systems made it possible to address three specific questions about lipoprotein biosynthesis. (1) Is the capacity for lipoprotein production unique to multicellular organisms? (2) Is the pathway elucidated for soluble secretory proteins involved in lipoprotein production or is there an alternate pathway? (3) Which portions of the apolipoprotein sequence are crucial for lipoprotein production?

A. Investigation of Lipoprotein Biosynthesis in Unicellular Organisms

Lipoprotein biosynthesis requires that three separate processes occur: (1) synthesis of the apolipoproteins, (2) lipidation of the lipoproteins, and (3) secretion of the resulting particle. Unicellular organisms are known to both synthesize and secrete proteins, but have never been shown to lipidate any protein. To address whether this is possible, yeast cells were examined to see if they could (1) synthesize apoLp-I and apoLp-II, (2) secrete apoLp-I and apoLp-II, and (3) lipidate apoLp-I and apoLp-II. Development of the yeast system involved the cloning of a 4.5kb portion of the 5' end of the precursor cDNA onto a yeast shuttle plasmid behind a GAL 10-10 promoter (Figure 4). This plasmid was then transformed into wild type *Saccharomyces cerevisiae* yeast cells. Expression of the apoLp-II,I cDNA was controled by the GAL 1-10 promoter--when yeast cells were grown in the presence of galactose, expression was induced. Conversely, when cells were grown in the presence of dextrose, expression was repressed.

To address whether yeast cells are capable of *expressing* apolipoproteins, cells were grown in the presence of either galactose or dextrose and both cell homogenate and cell media were compared by western blot. A 160kDa protein that was recognized by antibodies to the apoLp-II protein appears in the cell homogenate (Figure 11). The size of this protein indicated that apoLp-II was not cleaved away from the apoLp-I portion of the precursor. This protein was not released from cells, as was not found in the liquid media in which these cells were grown. This protein was not found in either the media or the homogenate of cells grown in the presence of dextrose. Thus, it was demonstrated that it is possible to *express* apolipoproteins in a unicellular organism.

Next, the ability of the yeast cells to *secrete* an apolipoprotein was examined. It had already been determined that the aopLp-II,I precursor protein fragment was not in the media. Yeast cells, however, are enclosed by a cell wall. It is possible that apoLp-II,I precursor protein fragment was secreted outside the plasma membrane but was unable to cross the cell wall. To address this, yeast cells expressing the apoLp-II,I precursor protein fragment were exposed to lyticase, an enzyme that disrupts the cell wall of yeast cells, forming spheroplasts. These cells were isolated by centrifugation at 1700g. The resulting supernatant contained any material found between the cell wall and plasma membrane of the cells. The 160kDa protein recognized by antibodies to apoLp-II was found in this supernatant (Figure 12), suggesting that the protein was secreted from the cells, but unable to pass through the yeast cell wall.

In order to determine the length of time required for synthesis and secretion of the apoLp-II,I precursor protein fragment, cells were grown overnight in media containing

dextrose. The cells were then rinsed in water and placed in media containing galactose. Samples were removed at particular time points and placed on ice. After the final time point, cells were exposed to lyticase and isolated by centrifugation. The resulting supernatant was analyzed by western blot. It took about two hours for the precursor protein fragment to appear outside the plasma membrane, and the amount of apoLp-II,I found in this space increased with time (Figure 13). Thus, the yeast cells were able to *express* and *secrete* apoporoteins, and this process takes about two hours.

The expressed protein, however was not as stable as HDLp isolated from *M. sexta*. When stored at 4°C, -20°C, or -80°C prior to SDS-PAGE, little or no protein was found following western blot. It is unlikely that this instability was a result of degradation, as addition of protease inhibitors did not prevent the disappearance of the protein. A more plausible explanation is that the particle is somewhat hydrophobic due to the reduced protein content (recall that only 45% of the total precursor protein was expressed) and bound to the tube. Addition of 15% ammonium sulfate or 22% potassium bromide to the spheroplast supernatant resulted in a stabilization of the particle, allowing for long-term storage. Rinsing the spheroplasts in either of these buffers also resulted in a greater yield of protein from the spheroplasts. Use of 0.1M sodium bicarbonate as the extraction buffer resulted in the best protein yield from spheroplasts, and allowed the sample to be directly analyzed by SDS-PAGE, without an additional dialysis step. However, the sample was still somewhat unstable in this buffer, indicating it was not as ideal for long-term storage. Whether this instability in low salt buffers was due to the folding of the protein or the lack of the terminal 60% of the sequence is unknown.

To address the final part of the first question, whether the apoLp-II,I precursor protein fragment produced by yeast cells was packaged with lipids into a lipophorin-like particle, density gradient ultracentrifugation was performed on the spheroplast supernatant. The gradient was analyzed by western blot, and it was found that the apoLp-II precursor protein fragment protein migrated to the top part of this gradient (Figure 14), at a density of approximately 1.24-1.27g/ml (non-lipidated proteins generally have a density of approximately 1.33mg/ml). The calculated lipid content of this particle is 20-30%, which is comparable to that of *M. sexta* HDLp (Prasad *et al* 1986a). Thin layer chromatography was attempted to determine the lipid content of the particle, however, it was not possible to obtain a pure enough sample of the particle for this study. The density of the particle found in the interstitial space infers that the yeast cells were capable of *expressing*, *secreting*, and *lipidating* apolipoproteins.

To further explore the structure of the particle produced by yeast cells, material from between the cell wall and plasma membrane was exposed to apoLp-II and apoLp-I antibodies immobilized by agarose beads. These antibodies had been raised against denatured *M. sexta* apoLp-II and apoLp-I and both recognized the SDS-PAGE denatured apolipoprotein precursor protein fragment produced by the yeast cells. However, the native form of this expressed precursor protein was incapable of being recognized by these antibodies (Figures 15, 16). Additionally, a polyhistidine tag added to the carboxy terminus of the precursor protein was also incapable of binding to a nickel affinity column, and isolation of the fusion protein by this method was not possible (Figure 17). Taken together, these results indicate that the proteins were folded in such a way that the

antigenic portions were sequestered away from the external environment. This is consistent with the aforementioned results, which indicate that the proteins are packaged with some amount of lipid. Furthermore, native lipophorin purified from *M. sexta* hemolymph was not recognized by these antibodies, indicating that the sequestration of the antigenic portions of the apolipoproteins was similar in both the yeast-expressed particle and the naturally expressed *M. sexta* particle.

In an attempt to visualize lipophorin expression in the yeast cells, immuno-electron microscopy was done using both galactose-induced and dextrose-repressed yeast cells. The primary antibody was unable to bind the expressed lipoproteins, even following attempts to denature the particle. Thus, sections from both the dextrose-repressed and galactose-induced yeast cells had equivalent amounts of gold beads bound. A second visualization technique was employed using fluorescence microscopy. The green fluorescent protein (GFP) was placed on the amino terminus of the apoLp-II,I precursor protein fragment and expressed in yeast cells. While the GFP-tagged precursor protein was expressed, as indicated by western blot using both anti-apoLp-II and anti-GFP antibodies (Figure 18), this fusion protein did not fluoresce. This is consistent with previously mentioned results that indicated that the precursor protein fragment is folded with some amount of lipid. It is likely that the GFP portion was denatured on the surface of the particle.

B. Investigation of the Secretory Pathway

The second aim of this project was to determine the role that the secretory pathway plays in the translation, lipidation, and secretion of apolipoproteins. To address

this, the apoLp-II,I precursor protein fragment was expressed in various temperature sensitive secretory mutants (Sec61p, Sec62p, Sec63p, Sec65p, and Kar2p). These particular mutants had no effect on expression, lipidation, or secretion as similar amounts of protein are found outside the plasma membrane at 15°C and 35°C (Figure 19). This indicated that either these proteins were not essential to lipophorin biosynthesis or the mutations incurred by these proteins did not effect the role that they play in lipophorin biosynthesis.

In order to determine whether any other proteins are involved in lipoprotein production, a screen needed to be developed, whereby yeast cells incapable of producing lipoproteins could be isolated and analyzed. Yeast cells expressing the apoLp-II,I precursor fragment would be mutagenized such that each cell would incur a single mutation in a single gene. Following mutagenesis, any cells incapable of producing a lipoprotein would be analyzed to determine which gene was affected. To facilitate this screen, GFP was added to the carboxy terminus of the apoLp-II,I precursor protein fragment. As the resulting fusion protein was unable to fluoresce, it was not possible to proceed with this screen.

A second methodology was developed to determine whether any accessory proteins are involved in the production of lipoproteins. This methodology uses a new technology called RNAi in which genes are post-transcriptionally silenced by the addition of double stranded RNA molecules. RNAi has been shown to work in *Drosophila* S2 cells, so a second expression system was developed (see below). This

approach was to be done in collaboration with Exelixis. Unfortunately, collaboration with this company was terminated before the completion of this project.

C. Investigation of the Apolipoprotein Sequence

The third aim of this project was to determine the role that the primary apolipoprotein sequence plays in expression, secretion, and lipidation. The yeast expression system proved difficult to manipulate for many reasons. First, site-directed mutagenesis could not be done directly on the yeast shuttle plasmid. This was most likely due to the large size of the plasmid. Secondly, isolation of the apoLp-II,I precursor protein fragment was a long and involved process, and some amount of degradation inevitable occurs. This was most likely due to contaminants in the lyticase enzyme. Finally, as previously mentioned, the apoLp-II,I precursor protein fragment was somewhat unstable unless stored in high salt solutions. Therefore, the third aim of this project was addressed by using the second expression system, the *Drosophila* S2 expression system.

First, the S2 *Drosophila* cells needed to be examined to determine whether they were capable of producing lipoproteins. As in the yeast system, this was studied in three parts: (1) expression of apoLp-I and apoLp-II, (2) secretion of apoLp-I and apoLp-II, and (3) lipidation of apoLp-I and apoLp-II. The same 4.5kb portion of the 5' end of the precursor cDNA that was used in the yeast system was cloned onto an S2 expression vector that constitutively produced protein with a carboxyl V5 epitope tag followed by a polyhistidine tag (Figure 6).

In order to determine whether *Drosophila* S2 cells were capable of *expressing* apolipoproteins, media and cell homogenate were examined by western blot from both control cells and cells transfected with plasmid DNA. A minute amount of protein was detected in both the cell homogenate and the media. When the media was purified by a high affinity nickel column and the column eluted in a smaller total volume, a large amount of protein was found. Expressed apolipoprotein was found in the media, but unlike the protein produced in the yeast expression system, this expressed protein was cleaved into two distinct polypeptides (Figure 20). The 70 kD polypeptide appeared to be the same size as apoLp-II isolated from *M. sexta* hemolymph and was recognized by anti-apoLp-II antibodies. The second polypeptide was approximately 80 kD and was recognized by anti-apoLp-I antibodies. The size of this protein was as expected for expression of the first 40% of apoLp-I. Thus, not only were the *Drosophila* S2 cells capable of *expressing* the apolipoprotein, but they were also capable of *cleaving* the precursor and *secreting* the resulting proteins.

Next, the ability of the *Drosophila* S2 cells to lipidate the apolipoproteins needed to be addressed. It was the polyhistidine tag, fused to apoLp-I downstream of the V5 epitope, that made possible the isolation of the two proteins by use of nickel charged resin (Figure 20). The two proteins co-purify, indicating that apoLp-II is associated with apoLp-I and that the intact particle was isolated. Attempts were made to analyze the lipid content of the particle via thin layer chromatography, however, it was not possible to purify enough protein for this purpose. Using density gradiant ultracentrifugation, it was possible to determine that the density of the particle was approximately 1.24-1.27mg/ml

and a calculated lipid content of 20-30%, as was determined for the particle produced in *S. cerevisiae*. An interesting note was that this particle was more stable than the one produced by *S. cerevisiae*, as samples can be collected from cells that was several days old and may be stored at 4°C without loss of protein sample. Thus, not only are *Drosophila* S2 cells capable of *expressing*, *secreting*, and *lipidating* the apolipoproteins, but the resulting particle is more similar to that produced by *M. sexta*, as cleavage of the precursor protein occurs and the resulting particle is more stable than the one produced by *S. cerevisiae*.

In an attempt to visualize expression of the lipoprotein in the *Drosophila* S2 cells, a fusion protein was made with GFP at the carboxy terminus of the apoLp-I fragment (Figure 9). The fusion protein was expressed in *Drosophila* S2 cells, and the media was purified by a high affinity nickel column. As was found in the yeast expression system, it was possible to express and secrete the GFP fusion protein (data not shown), but the product did not fluoresce.

The third aim of this project was to examine the apolipoprotein sequence to determine its role in expression, lipidation, and secretion. Two approaches were taken for this study. First, alanine-scanning mutagenesis was done, changing particular cysteines and prolines in the first 130 amino acids of apoLp-II to alanines (Figure 7). The individual sequences were expressed in the *Drosophila* S2 cells and it was found that the apoLp-II protein produced co-purified with the apoLp-I protein from media in a manner identical to that of the unaltered sequence (Figure 21). Thus, the particular residues that were chosen are not crucial to expression, lipidation, and secretion.

The second approach that was used to examine the apolipoprotein sequence involved deletion of 70-100 amino acids of the apoLp-II sequence (Figure 8). Expression of these altered sequences in *Drosophila* S2 cells revealed that decreasing the length of apoLp-II prevented apolipoprotein expression, as no apoLp-II was found in cell homogenates or in the media (Figure 22). Thus, it appears that the apoLp-II sequence is crucial to lipophorin biosynthesis. Whether there is an actual "lipidation code" or whether it is the length of the apolipoprotein that is important has not yet been determined.

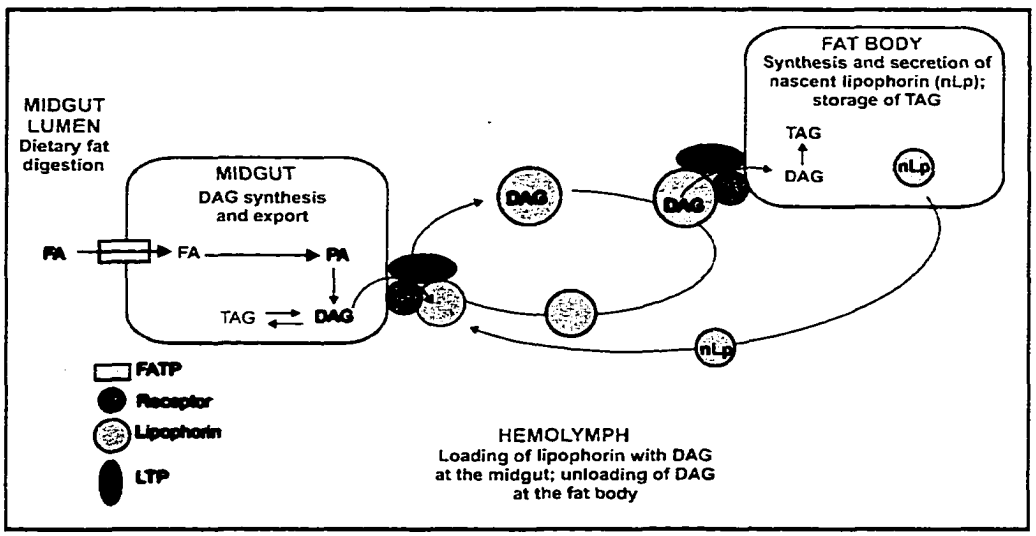


FIGURE 1: LIPOPROTEIN BIOSYNTHESIS AND LIPID TRANSPORT.

Lipophorin particles are produced by fat body cells and released as empty, nascent lipophorin (nLp) particles. These particles dock with their receptor at the midgut and where they are filled with diacylglycerol (DAG). This process is mediated by the lipid transfer particle (LTP). Lipophorin transports DAG to fat body cells and then returns to the midgut as a recycled particle. FA, fatty acid; FATP, fatty acid transporter; PA, phosphatidic acid; TAG triacylglycerol (from Canavosa *et al* 2001).

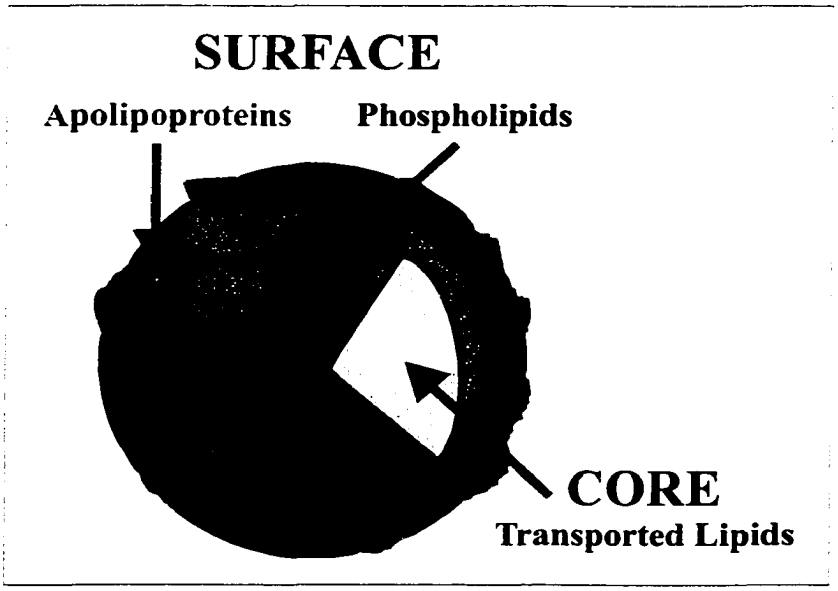


FIGURE 2: THE STRUCTURE OF LIPOPROTEINS.

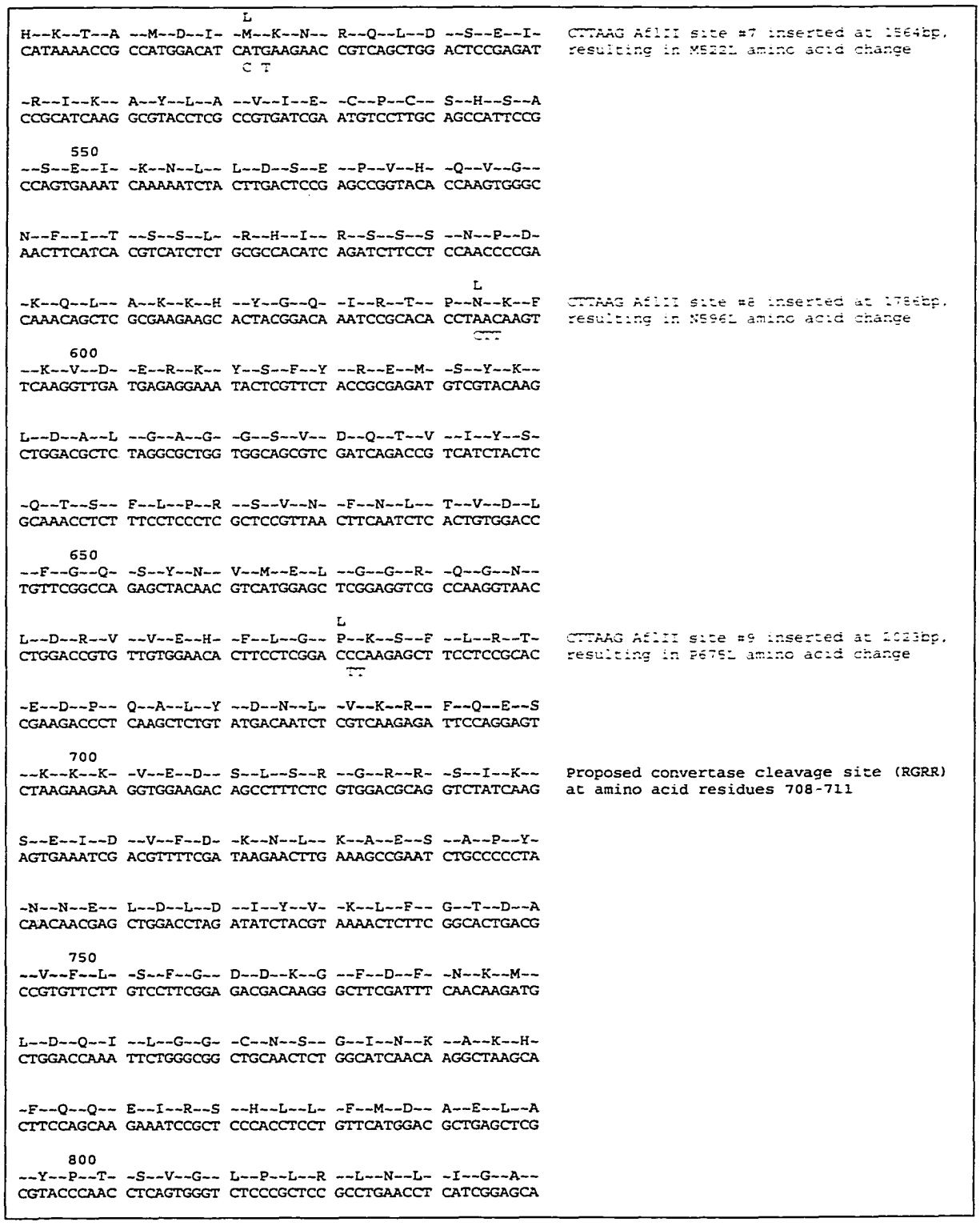
In general, lipoproteins have two main layers: a hydrophobic core in which transported lipids are found and a surface domain consisting of a monolayer of amphipathic phospholipids and apolipoproteins.

FIGURE 3: ANNOTATED APOLP-II,I SEQUENCE.

The protein sequence (top line) and DNA sequence (bottom line) for the amino-terminal portion of the *M. sexta* precursor protein used in the project. Alterations made to the sequence, as described in the Methods and Materials Section, are given in blue letters above (amino acids) or red letters below (nucleotides). Descriptions of the alterations are given in red in the column to the right of the sequence. Numbers above the sequence refer to amino acid number, beginning with the start methionine.

```
                                                  GAATTC   ApoLII,I cloned with GAATTC EcoRI site
                                                           just prior to the 5' UTR

TTAAAGCTAA AAAGACTGAA TCATTTATAA TAAGTGTCCC ACTTTTACCC
                                                     GG
        1
        M- -G--K--S-- N--R--L--L --S--V--L- -F--V--I--   GGATCC BamHI site inserted prior to
CTGTCAAAAT GGGGAAAAGT AATAGATTAT TAAGTGTGCT ATTTGTAATT   start codon
A CCA
                                                    L
S--V--L--W --K--A--A- -Y--G--N-- G--K--C--Q --I--A--C-   CTTAAG AflII site #1 inserted at 91bp,
TCCGTTTTAT GGAAAGCAGC GTACGGGAAC GGCAAATGCC AGATAGCCTG   resulting in C31L amino acid change
                                              .     CT

-K--G--S-- S--S--P--S --F--A--A- -G--Q--K-- Y--N--Y--G
TAAAGGATCT TCAAGCCCAT CTTTCGCCGC CGGACAAAAA TACAACTATG
   G
    50                                        .
--V--E--G- -T--V--S-- V--Y--L--T --G--A--D- -N--Q--E--
GAGTGGAAGG CACCGTCAGT GTGTACCTCA CCGGAGCAGA CAACCAAGAG

                                                    A
T--S--L--K --M--L--G- -Q--A--S-- V--S--A--I --S--N--C-   C61A amino acid change
ACCAGCCTCA AGATGCTCGG GCAGGCGTCG GTGTCCGCGA TAAGCAACTG
                                                    GC
                                          A
-E--L--E-- L--S--V--H --N--M--V- -L--S--G-- P--D--G--K   P95A amino acid change
CGAGCTTGAA CTCTCCGTGC ACAACATGGT CCTCTCTGGA CCGGACGGCA
                                          GCG
     100A   A  A            L      A                   P101A, C102A, P103A, P109A amino acid
--K--Y--P- -C--P--Q-- G--I--E--K --P--V--R- -F--S--Y--   changes; CTTAAG AflII site #2 inserted
AAAAATACCC ATGTCCGCAA GGCATCGAAA AGCCAGTCAG GTTTTCCTAC   at 319bp, resulting in E107L amino acid
        G   GC G             CTT    G                   change
                  A          A
Q--D--G--R --V--G--P- -E--I--C-- A--A--E--D --D--S--R-   P120A and C123A amino acid changes
CAGGACGGCA GAGTCGGGCC GGAGATCTGT GCTGCGGAGG ACGACAGCCG
                  G          GC

-R--S--L-- N--I--K--R --A--I--I- -S--L--L-- Q--A--E--Q
TCGCTCGCTG AACATAAAGA GGGCTATCAT CTCTTTACTC CAAGCCGAGC

    150
--K--P--S- -V--Q--V-- D--V--F--G --V--C--P- -T--E--V--
AGAAGCCTTC CGTACAGGTG GACGTGTTCG GCGTGTGTCC CACGGAGGTG


S--S--S--Q --E--G--G- -A--V--L-- L--H--R--S --R--D--L-
TCGTCCTCTC AAGAGGGCGG CGCCGTTCTG CTGCACCGCT CCCGAGACCT


-S--R--C-- A--H--R--E --Q--G--R- -N--D--F-- V--N--S--I
CTCGCGCTGC GCTCACCGCG AACAAGGACG CAACGACTTC GTCAATTCCA

    200             L
--A--N--P- -D--A--G-- I--K--D--L --Q--V--L- -Q--S--M--   CTTAAG AflII site #3 inserted at 613bp,
TCGCCAACCC TGACGCCGGA ATCAAGGATT TGCAAGTGCT GCAATCCATG   resulting in I205L amino acid change
                      C T
```

```
L--N--V--E --S--K--V- -N--N--G-- V--P--E--K --V--S--A-
TTGAACGTGG AGTCGAAAGT GAACAACGGA GTGCCAGAGA AAGTGTCCGC


-I--E--E-- Y--L--Y--K --P--F--S- -V--G--E-- N--G--A--R
CATTGAGGAG TACCTGTACA AACCCTTCTC AGTGGGAGAG AACGGCGCAA

     250
--A--K--V- -H--T--K-- L--T--L--S --G--K--G- -G--A--G--
GGGCAAAGGT CCACACCAAG CTGACCCTCT CCGGCAAGGG AGGCGCTGGT


G--G--N--A --H--C--T- -E--S--R-- S--I--I--F --D--V--P-
GGTGGTAACG CCCACTGCAC GGAGTCTCGC AGCATCATCT TCGACGTCCC

                          K
-H--G--T-- S--S--A--S --G--N--L- -N--S--V-- I--S--A--V   CTTAAG AflII site #4 inserted at 871bp,
CCACGGCACG TCCTCGGCGA GCGGTAACCT CAACTCCGTG ATCAGTGCTG   resulting in N291K amino acid change
                          T  G
     300
--K--E--T- -A--R--T-- V--A--N--D --A--S--S- -K--S--A--
TGAAGGAGAC CGCCAGGACC GTCGCCAATG ACGCTAGCTC CAAGTCTGCT


G--Q--F--A --Q--L--V- -R--I--M-- R--T--S--S --K--D--D-
GGACAGTTTG CACAACTTGT CAGGATCATG AGAACGTCAA GCAAAGATGA


-L--M--R-- I--Y--S--Q --V--K--A- -H--Q--L-- E--K--R--V
CTTGATGAGG ATTTACAGCC AAGTCAAGGC ACACCAACTG GAAAAACGCG

     350
--Y--L--D- -A--L--L-- R--A--G--T --G--E--S- -I--E--A--
TGTACCTGGA CGCCTTGCTT CGCGCCGGCA CTGGCGAGAG CATCGAAGCC


S--I--Q--I --L--K--S- -K--D--L-- S--Q--L--E --Q--H--L-  CTTAAG AflII site #5 inserted at 1108bp
TCCATCCAGA TCCTGAAGTC CAAGGACCTC AGCCAGCTGG AGCAGCACCT
             T

-V--F--L-- S--L--G--N --A--R--H- -V--N--N--- P--A--L--K
GGTGTTCCTG TCGCTCGGCA ACGCCAGACA CGTCAACAAC CCAGCTTTGA

     400
--A--A--A- -G--L--L-- D--M--P--N --L--P--K- -E--V--Y--
AGGCTGCTGC TGGTCTCTTG GACATGCCGA ATCTGCCCAA GGAGGTGTAC


L--G--A--G --A--L--G- -G--A--Y-- C--R--E--H --D--C--H-
CTCGGAGCCG GAGCCCTCGG CGGAGCGTAC TGCCGCGAAC ACGACTGCCA


-N--V--K-- P--E--G--I --V--A--L- -S--N--K-- L--G--S--K   BamHI site removed at 4004bp
CAATGTCAAG CCGGAGGGTA TCGTCGCTCT CAGCAACAAA CTCGGATCCA
                                                    T
     L  K
--L--Q--N- -C--R--P-- K--N--K--P --D--E--D- -V--V--V--   CTTAAG AflII site #6 inserted at 1348bp,
AGCTGCAGAA CTGCAGGCCC AAGAACAAGC CTGATGAGGA TGTGGTTGTC   resulting in Q450L and N451K amino acid
      TT   G                                             changes

A--I--L--K --G--I--R- -N--I--R-- H--L--E--D --S--L--I-
GCAATCCTCA AGGGAATCCG CAACATCCGC CACCTGGAGG ACTCTCTGAT


-D--K--L-- V--H--C--A --V--D--N- -N--V--K-- A--R--V--R
CGACAAGCTG GTGCACTGCG CTGTGGACAA CAATGTGAAG GCCAGGGTCA

     500
--A--V--A- -L--E--A-- F--H--A--D --P--C--S- -A--K--I--
GGGCCGTGGC TCTAGAAGCT TTCCATGCAG ACCCTTGCAG TGCTAAGATC
```



CTTAAG AflII site #7 inserted at 1564bp, resulting in M522L amino acid change

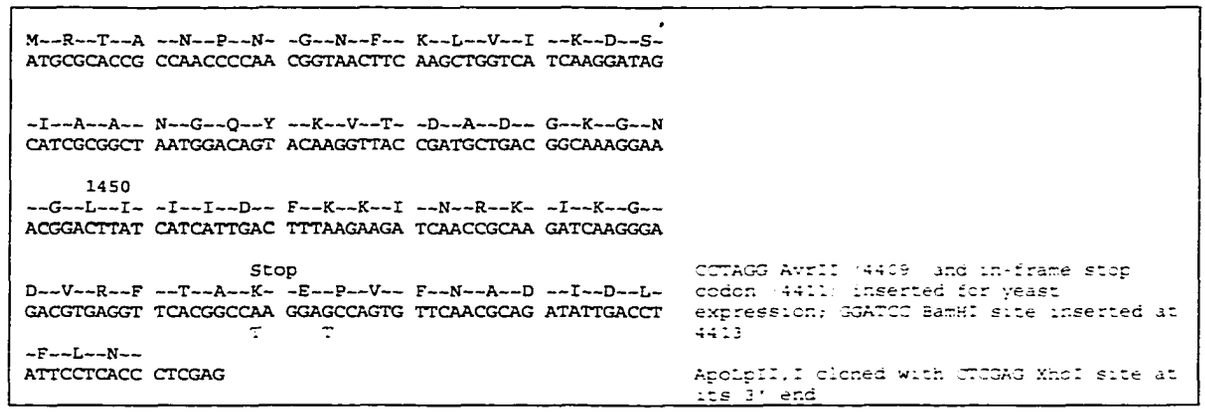
CTTAAG AflII site #8 inserted at 1784bp, resulting in N596L amino acid change

CTTAAG AflII site #9 inserted at 2023bp, resulting in P675L amino acid change

Proposed convertase cleavage site (RGRR) at amino acid residues 708-711

```
A--T--A--R --L--D--V- -A--T--N-- I--D--I--R --Q--I--F-
GCCACCGCGC GGCTCGACGT GGCCACCAAC ATCGACATTC GTCAGATCTT


-Q--S--P-- Q--N--A--K --A--D--I- -K--F--V-- P--S--T--D
CCAATCCCCC CAAAACGCTA AGGCCGACAT CAAATTCGTT CCCAGCACTG

     850
--F--E--I- -S--G--A-- F--I--I--D --A--D--A- -F--S--T--
ACTTTGAAAT CTCTGGAGCC TTCATCATAG ACGCTGATGC ATTCTCCACT


G--I--K--V --I--T--N- -L--H--S-- S--T--G--V --H--V--N-
GGAATCAAAG TGATCACCAA CTTGCACTCT TCCACTGGTG TACACGTCAA


-A--K--V-- L--E--N--G --R--G--I- -D--L--Q-- I--G--L--P
TGCTAAGGTC CTGGAGAATG GACGTGGAAT CGACCTTCAA ATCGGCTTAC

     900
--V--D--K- -Q--E--L-- I--A--A--S --S--D--L- -V--F--V--
CTGTTGACAA GCAAGAGCTG ATCGCCGCTA GCAGTGACTT GGTCTTCGTG


T--A--E--K --G--Q--K- -E--K--Q-- K--V--I--K --M--E--K-
ACCGCGGAAA AGGGACAGAA AGAGAAGCAG AAGGTGATCA AGATGGAGAA


-G--E--N-- E--Y--S--A --C--F--D- -Q--L--S-- G--P--L--G
AGGTGAAAAT GAGTACTCAG CCTGTTTCGA CCAGCTGTCT GGACCTCTGG

     950
--L--T--M- -C--Y--D-- M--V--L--P --F--P--I- -V--N--R--
GCTTGACCAT GTGCTACGAT ATGGTGCTGC CTTTCCCTAT TGTCAACCGC


N--D--K--L --D--S--I- -A--K--A-- M--G--K--W --P--L--S-
AACGACAAAT TGGACTCTAT TGCCAAGGCG ATGGGCAAAT GGCCTCTATC


-G--S--A-- K--F--K--L --F--L--E- -K--N--D-- L--R--G--Y
TGGCTCAGCT AAATTCAAAC TATTCCTCGA AAAGAACGAT CTTCGAGGCT

     1000
--H--I--K- -A--V--V-- K--E--D--K --D--A--G- -R--R--S--
ACCACATCAA GGCCGTTGTG AAGGAGGACA AGGATGCAGG CAGGAGGAGC


F--E--L--L --L--D--T- -E--G--A-- K--T--R--R --S--Q--L-
TTCGAACTGC TACTTGACAC TGAAGGTGCG AAGACCCGTC GCTCTCAACT


-T--G--E-- A--V--Y--N --E--N--E- -V--G--V-- K--L--G--L
GACTGGTGAA GCAGTTTACA ATGAGAATGA AGTTGGAGTC AAGCTTGGCC

     1050
--E--A--V- -G--K--V-- I--Y--G--H --I--W--A- -H--K--K--
TCGAAGCCGT CGGAAAAGTC ATCTACGGTC ACATCTGGGC ACACAAGAAA


P--N--E--L --V--A--S- -V--K--G-- K--L--D--D --I--E--Y-
CCCAACGAGC TCGTGGCATC CGTCAAAGGA AAGTTAGACG ACATCGAATA


-S--G--K-- L--G--F--S --V--Q--G- -N--E--H-- R--A--V--Y
CTCTGGAAAA CTCGGATTCT CCGTCCAAGG CAACGAGCAC CGTGCCGTCT

     1100
--K--P--I- -F--E--Y-- S--L--P--D --G--S--S- -P--G--S--
ACAAACCGAT CTTCGAATAC AGCTTGCCTG ATGGGTCGTC TCCTGGCAGC
```

```
K--K--Y--E --V--K--I- -D--G--Q-- V--I--R--E --C--D--G-
AAGAAGTACG AAGTCAAGAT CGACGGCCAA GTCATCAGAG AGTGTGATGG

-R--V--T-- K--Y--T--F --D--G--V- -H--V--N-- L--Q--N--A
AAGGGTTACG AAATACACAT TCGATGGCGT CCATGTAAAC CTCCAGAATG

     1150
--E--K--P- -L--E--I-- C--G--S--V --S--T--V- -A--Q--P--
CTGAGAAACC CTTGGAGATC TGTGGGTCTG TGTCTACAGT CGCACAACCG

R--E--V--E --F--D--V- -E--V--K-- H--Y--A--S --L--K--G-
AGGGAAGTGG AGTTCGATGT AGAGGTCAAG CATTACGCAT CCCTGAAGGG

-S--W--K-- G--S--D--V --V--L--A- -F--N--N-- Q--L--N--P
CTCGTGGAAG GGATCTGACG TCGTCTTAGC GTTCAACAAC CAACTGAACC

     1200
--K--I--N- -F--D--L-- K--G--K--F --E--N--T- -D--S--M--
CTAAAATTAA CTTTGACCTT AAAGGCAAAT TCGAGAACAC AGATTCTATG

H--N--E--L --D--I--H- -Y--G--P-- N--R--G--D --N--N--A-
CATAATGAAC TGGACATCCA CTACGGCCCC AACCGTGGAG ACAACAACGC

-R--I--T-- F--S--Q--I --L--K--Y- -H--V--E-- N--S--K--N
TAGGATCACC TTCTCGCAGA TTCTCAAGTA CCACGTCGAG AACTCTAAGA

     1250
--F--N--V- -I--T--K-- N--N--L--E --I--R--A- -V--P--F--
ACTTCAACGT CATTACCAAG AACAATTTGG AGATTCGCGC GGTACCATTC

K--L--V--A --N--A--D- -V--D--P-- K--K--I--D --I--D--I-
AAACTAGTGG CAAACGCTGA CGTAGACCCC AAGAAGATAG ACATCGACAT

-E--G--Q-- L--Q--D--K --S--A--G- -F--N--L-- D--A--R--T
CGAAGGTCAG CTTCAAGACA AGAGTGCTGG ATTCAACCTG GACGCTAGAA

     1300
--H--I--K- -K--E--G-- D--Y--S--I --K--V--K- -A--N--L--
CACACATCAA GAAGGAAGGA GACTACAGCA TCAAAGTCAA GGCTAATTTG

N--N--A--N --L--E--A- -F--S--R-- R--D--I--V --N--A--E-
AACAACGCCA ACCTTGAAGC CTTCTCTAGA AGGGATATTG TAAATGCTGA

-K--S--N-- V--E--N--Y --I--D--M- -K--G--V-- G--R--Y--E
AAAGTCTAAC GTGGAGAACT ATATAGACAT GAAGGGAGTT GGAAGGTACG

     1350
--L--S--G- -F--V--L-- H--K--T--K --P--N--D- -V--N--V--
AGCTATCTGG TTTCGTTCTC CATAAGACGA AGCCCAATGA CGTGAACGTT

G--F--I--G --H--L--K- -I--N--G-- G--G--K--N --E--D--F-
GGTTTCATTG GACACTTGAA GATCAACGGT GGCGGCAAGA ATGAGGATTT

-K--I--N-- I--G--H--I --E--T--P- -A--V--F-- S--S--H--A
CAAAATCAAC ATCGGCCACA TCGAGACCCC GGCTGTGTTC TCGTCCCACG

     1400
--T--I--S- -G--S--R-- G--D--I--I --D--Y--L- -L--K--I--
CCACCATCTC CGGCAGCAGG GGCGACATCA TCGATTACCT GCTGAAGATT
```


M--R--T--A --N--P--N- -G--N--F-- K--L--V--I --K--D--S-
ATGCGCACCG CCAACCCCAA CGGTAACTTC AAGCTGGTCA TCAAGGATAG
-I--A--A-- N--G--Q--Y --K--V--T- -D--A--D-- G--K--G--N
CATCGCGGCT AATGGACAGT ACAAGGTTAC CGATGCTGAC GGCAAAGGAA
1450
--G--L--I- -I--I--D-- F--K--K--I --N--R--K- -I--K--G--
ACGGACTTAT CATCATTGAC TTTAAGAAGA TCAACCGCAA GATCAAGGGA
Stop
D--V--R--F --T--A--K- -E--P--V-- F--N--A--D --I--D--L-
GACGTGAGGT TCACGGCCAA GGAGCCAGTG TTCAACGCAG ATATTGACCT
T T
-F--L--N--
ATTCCTCACC CTCGAG
CCTAGG AvrII (4409) and in-frame stop
codon (4411) inserted for yeast
expression; GGATCC BamHI site inserted at
4413
ApoLpII,I cloned with CTCGAG XhoI site at
its 3' end

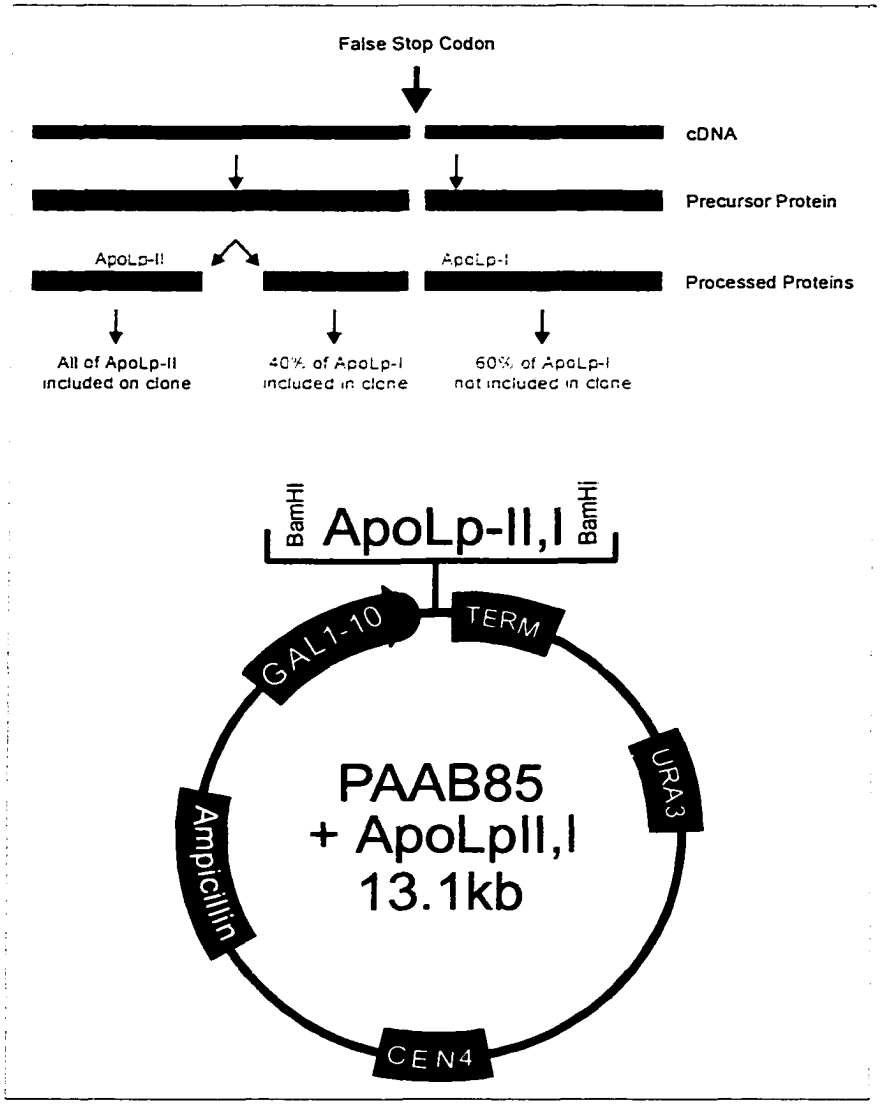


FIGURE 4: CLONING APOLP-II,I ONTO THE YEAST SHUTTLE PLASMID.

A 4.5kb portion of the 5' end of the apoLp-II,I precursor cDNA, approximately 45% of the entire precursor, was inserted onto a yeast shuttle plasmid, pAAB85. This centromere-based (CEN4) plasmid contains a galactose promoter (GAL1-10) and an actin terminator (TERM) for initiation and termination of transcription. A false stop codon was inserted at the 3' end of the cDNA sequence for translation termination. Additional features of the plasmid include the URA3 gene and genes for ampicillin resistance, allowing for the selection of both yeast and bacterial transfectants.

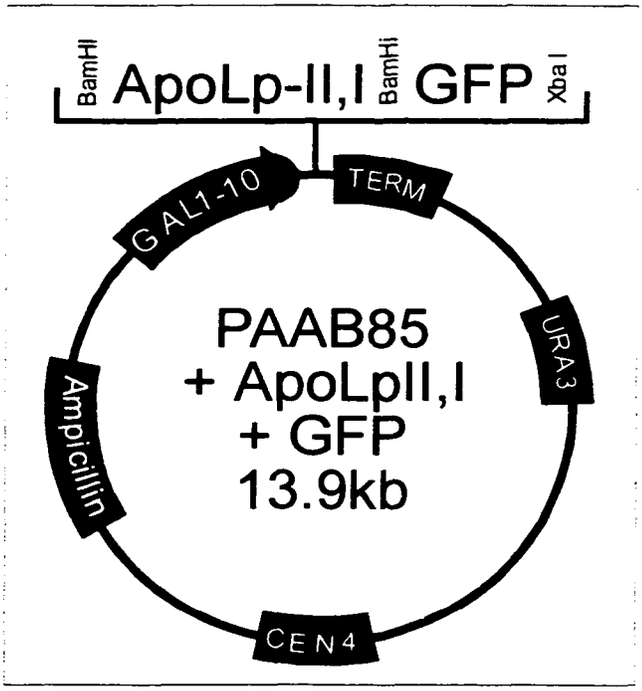


FIGURE 5: CLONING THE APOLP-II,I/GFP FUSION PROTEIN ONTO THE YEAST SHUTTLE PLASMID.

The green fluorescent protein (GFP) was inserted onto a yeast shuttle plasmid, pAAB85. This centromere-based (CEN4) plasmid contains a galactose promoter (GAL1-10) and an actin terminator (TERM) for initiation and termination of transcription. A 4.5kb portion of the 5' end of the apoLp-II,I precursor cDNA, approximately 45% of the entire precursor protein, was inserted inframe on the amino-terminal end of GFP. Additional features of the plasmid include the URA3 gene and genes for ampicillin resistance, allowing for the selection of both yeast and bacterial transfectants.

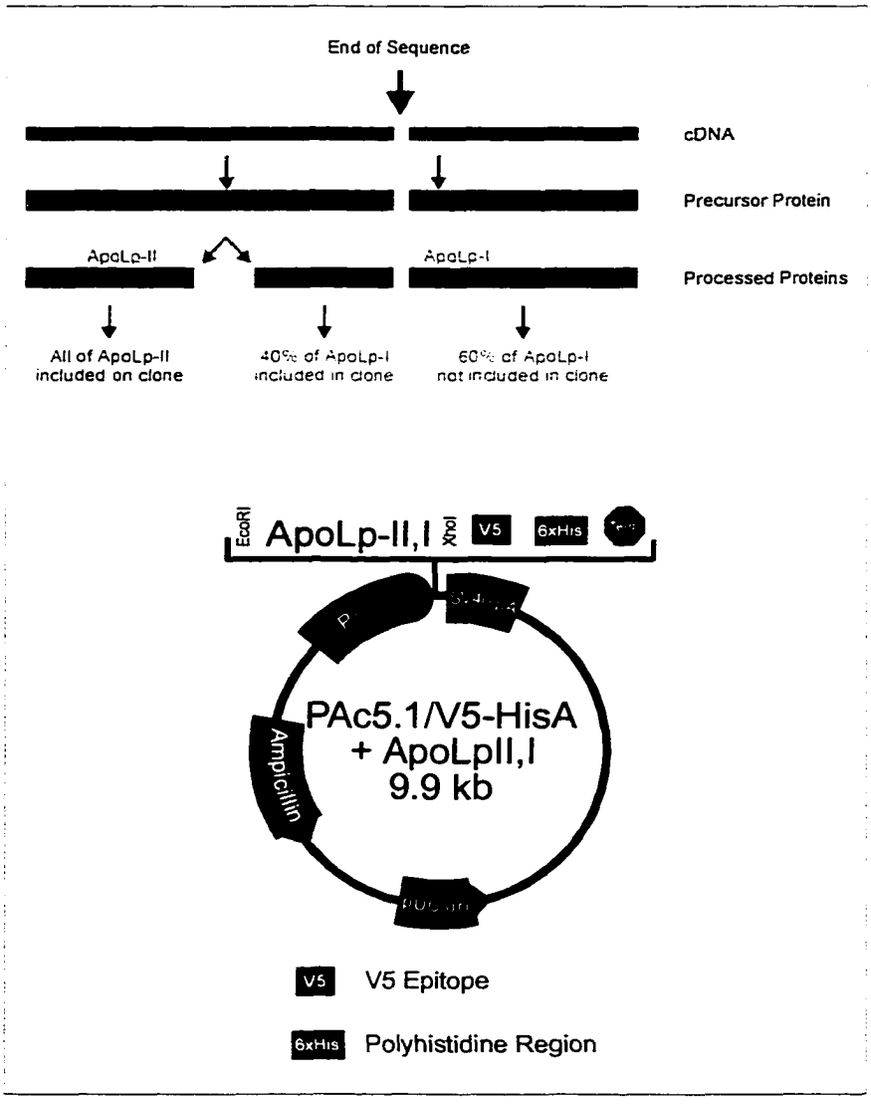


FIGURE 6: CLONING APOLP-II,I ONTO THE *DROSOPHILA* S2 PLASMID.

A 4.5kb portion of the 5' end of the apoLp-II,I precursor cDNA, approximately 45% of the entire precursor, was inserted on the S2 expression plasmid in front of a V5 epitope (V5), a histidine tag (6x His), and a stop codon (Term). An additional feature of the plasmid is the gene for ampicillin resistance, allowing for the selection of bacterial transfectants.

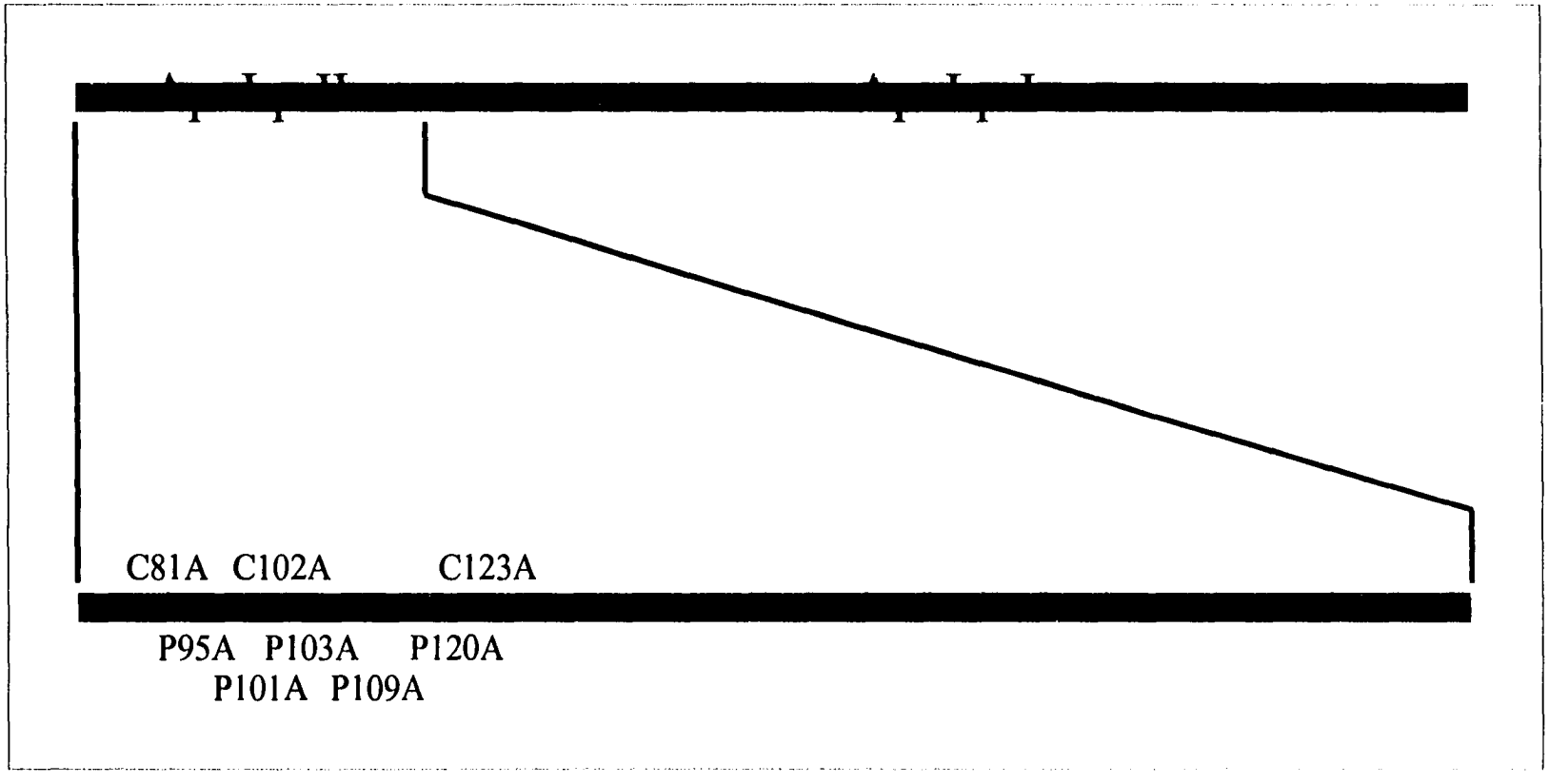


FIGURE 7: ALANINE-SCANNING MUTAGENESIS AMINO ACID CHANGES.

A schematic illustrating the proline to alanine and cysteine to alanine amino acid changes (X) to the apoLp-II sequence on the S2 plasmid. Numbers refer to position of amino acid in the sequence (C81A: cysteine to alanine at amino acid 81; P95A: proline to alanine at amino acid 95; etc.).

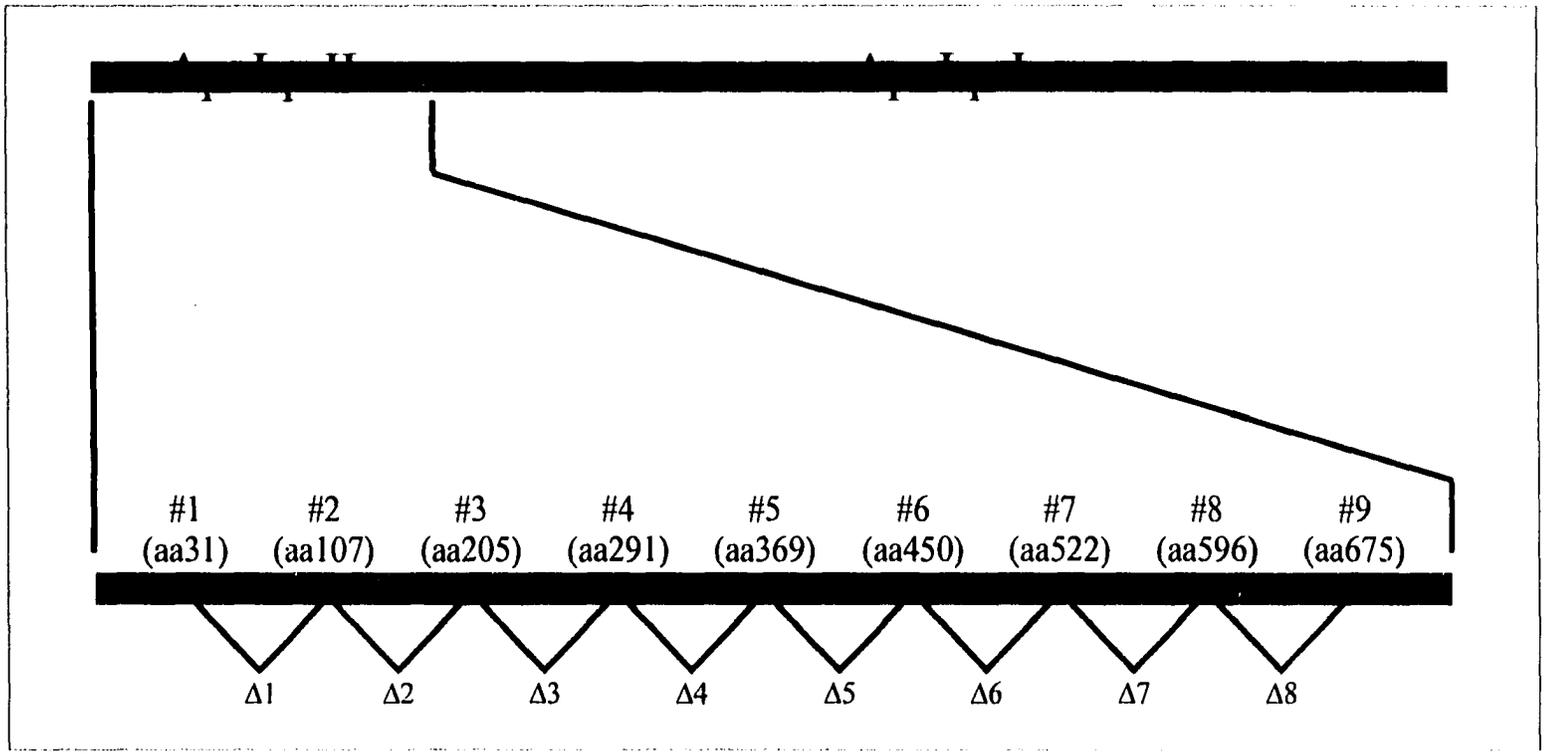


FIGURE 8: AMINO ACID DELETIONS.

A schematic illustrating positions of nine AflII endonuclease cut sites (X) inserted into the apoLp-II sequence on the S2 plasmid. Two consecutive sites were inserted into each of eight clones (Δ1 received both cut site #1 at amino acid 31 and cut site #2 at amino acid 107; Δ2 received both cut site #2 at amino acid 107 and cut site #3 at amino acid 205; etc.). The plasmid DNA was cut with the restriction endonuclease AflII, and religated to itself. This results in a 70-100 amino acid deletion (Δ1 lost 76 amino acids between 31 and 107; Δ2 lost 98 amino acids between 107 and 205; etc.).

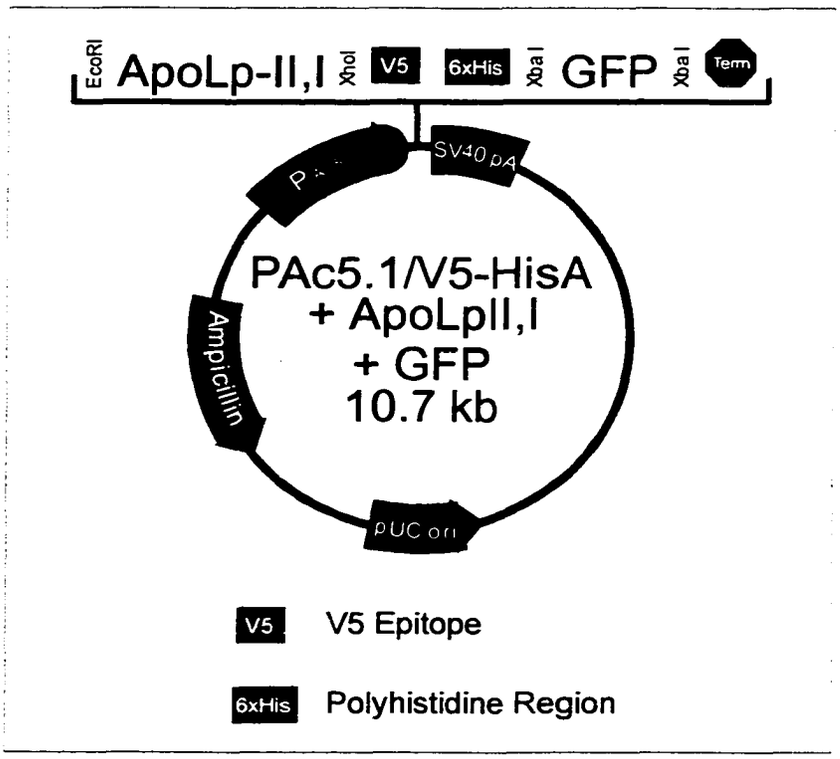


FIGURE 9: CLONING THE APOLP-II,I/GFP FUSION PROTEIN ONTO THE *DROSOPHILA* S2 PLASMID.

A 4.5kb portion of the 5' end of the apoLp-II,I precursor cDNA, approximately 45% of the entire precursor, was inserted on the S2 expression plasmid 5' of a V5 epitope (V5), a histidine tag (6x His), and a stop codon (Term). An additional feature of the plasmid is the gene for ampicillin resistance, allowing for the selection of bacterial transfectants.

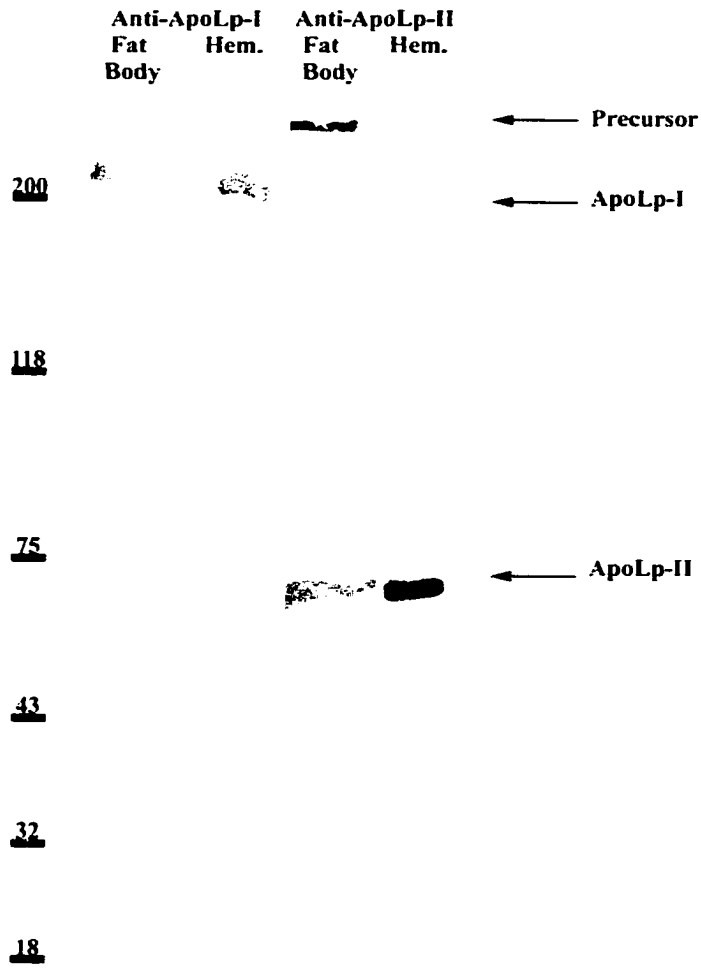


FIGURE 10: PROCESSING OF THE APOLP-II,I PRECURSOR PROTEIN.

Western analysis using antibodies specific for either apoLp-I (lanes 1 and 2) or apoLp-II (lanes 3 and 4) illustrates that the apoLp-I, II precursor protein is present only in *M. sexta* fat body homogenate (Hem., lanes 2 and 4). The cleavage products, apoLp-I and apoLp-II are present in both fat body homogenate (lane 1 and 3) and hemolymph (lanes 2 and 4). Precursor cleavage occurs prior to the release of apoLp-I and apoLp-II into the hemolymph. Numbers refer to marker bands in kilodaltons.

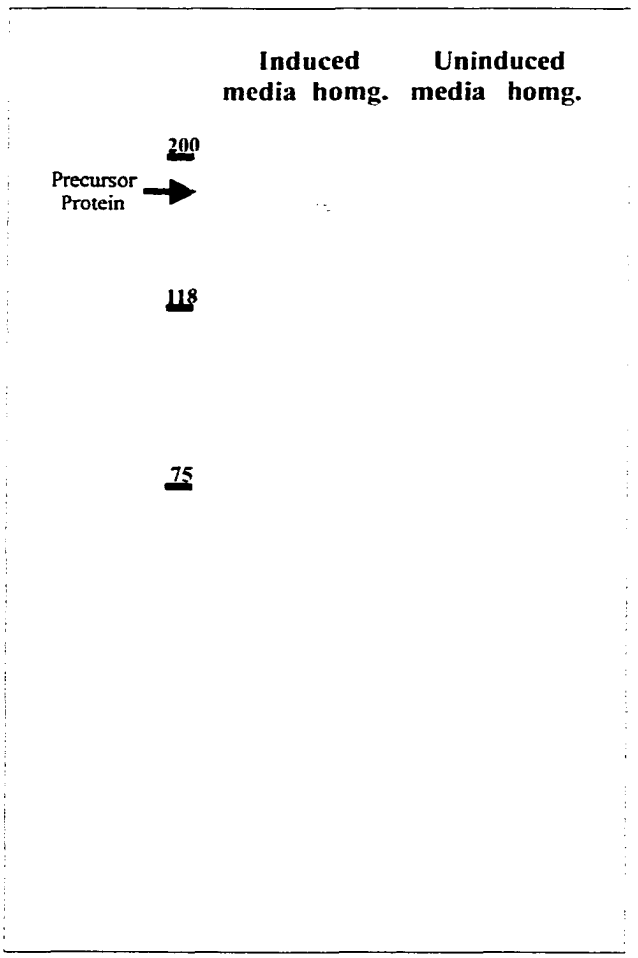


FIGURE 11: INDUCTION OF APOLP-II,I EXPRESSION IN *SACCHAROMYCES CEREVISIAE*.

A galactose promoter (induced by galactose, repressed by dextrose) controls transcription of apoLp-II,I cDNA. Translation results in a ~160kDa protein that includes all of apoLp-II and about 40% of apoLp-I. Degradation is evident in this crude cell homogenate, but the majority of the protein does not appear to be cleaved into apoLp-II and apoLp-I. This protein is found only in the homogenate (homg.) of cells induced by galactose (lane 2). Numbers refer to marker bands in kilodaltons.

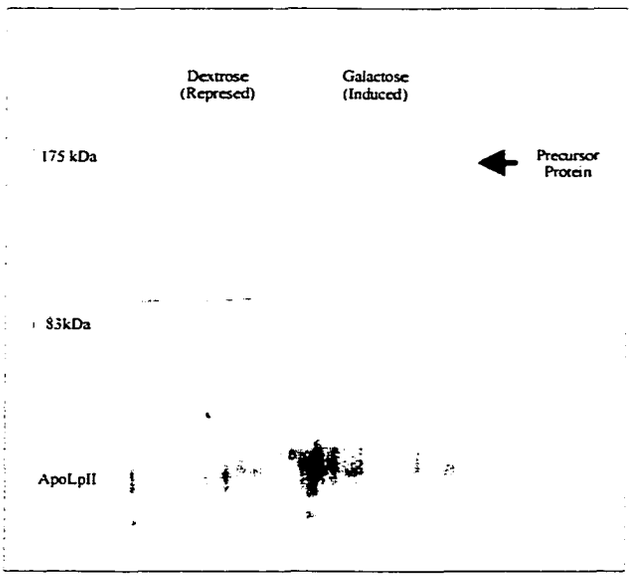


FIGURE 12: LOCALIZATION OF APOLP-II,I EXPRESSED IN *SACCHAROMYCES CEREVISIAE*.

Creation of speroplasts allows for the localization of the expressed apoLp-II,I precursor protein fragment in the periplasmic space between the yeast cell wall and plasma membrane. The expressed protein is only found in galactose-induced cells (lane 2). A second background protein band (~85kDa) as well as diffuse proteins (~60kDa) are routinely recognized by the polyclonal anti-apoLp-II antibody in both dextrose repressed and galactose induced cells. Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

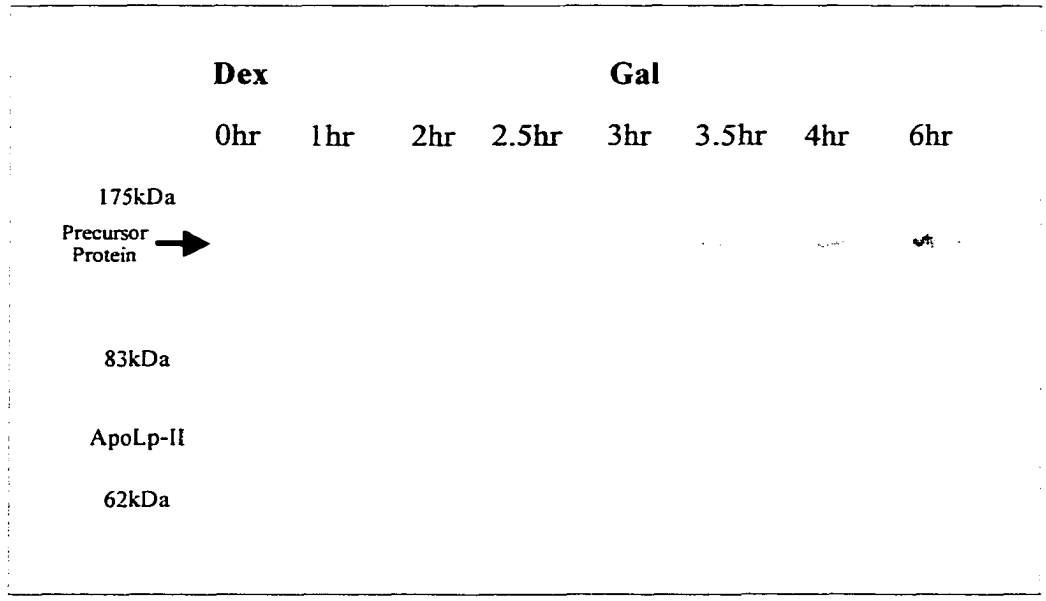


FIGURE 13: TIME COURSE OF APOLP-II,I EXPRESSION IN *SACCHAROMYCES CEREVISIAE*.

Cells were grown in dextrose overnight to repress expression of the plasmid. Cells were collected by centrifugation, rinsed in water, and resuspended in media containing galactose at time 0hr (lane 1). Samples were taken at specific time points and analyzed by western blot. Protein is visible in the periplasmic space by two hours (lane 3). Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

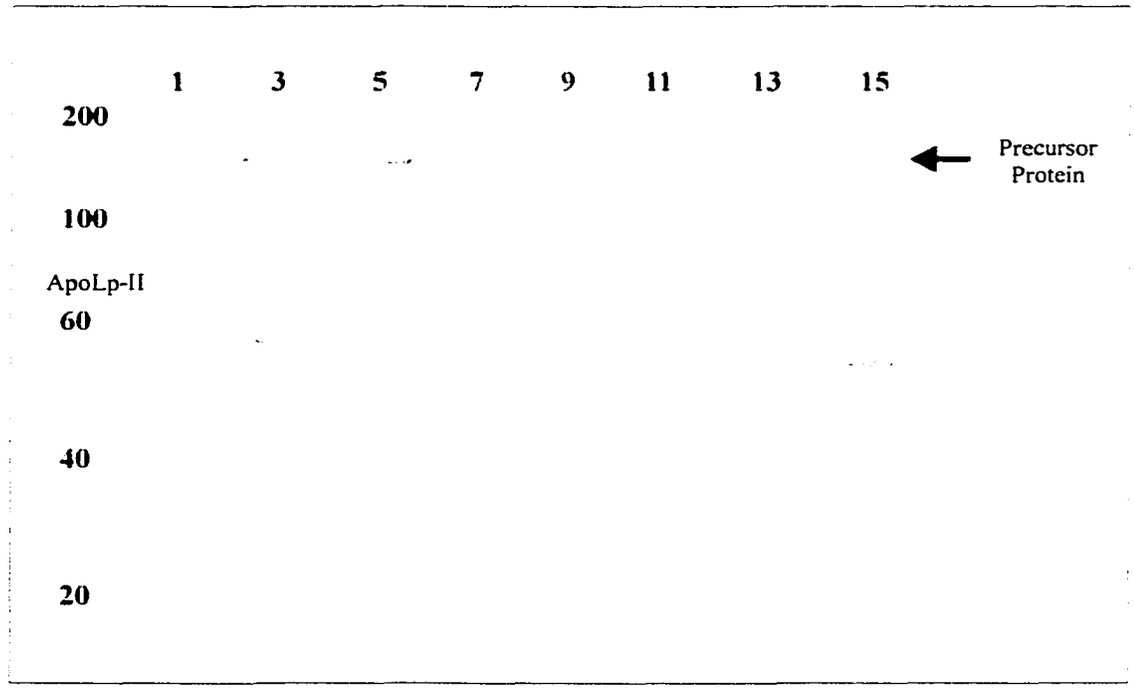


FIGURE 14: DENSITY GRADIENT ULTRACENTRIFUGATION OF APOLP-II,I FROM *SACCHAROMYCES CEREVISIAE*.

Material from between the cell wall and plasma membrane of cells grown in the presence of galactose was separated on a ¾ KBr gradient. This gradient was fractionated into forty 1ml samples that were then dialyzed and analyzed by Western analysis with antibodies against apoLp-II. Shown are odd fractions 1-15. The 160kDa protein was only detected in the top portion of the gradient for the galactose-induced cells (lanes 1-5), indicating a density of approximately 1.24-1.27 mg/ml. Diffuse proteins (~60kDa) are routinely recognized by the polyclonal anti-apoLp-II antibody in both dextrose repressed and galactose induced cells. Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

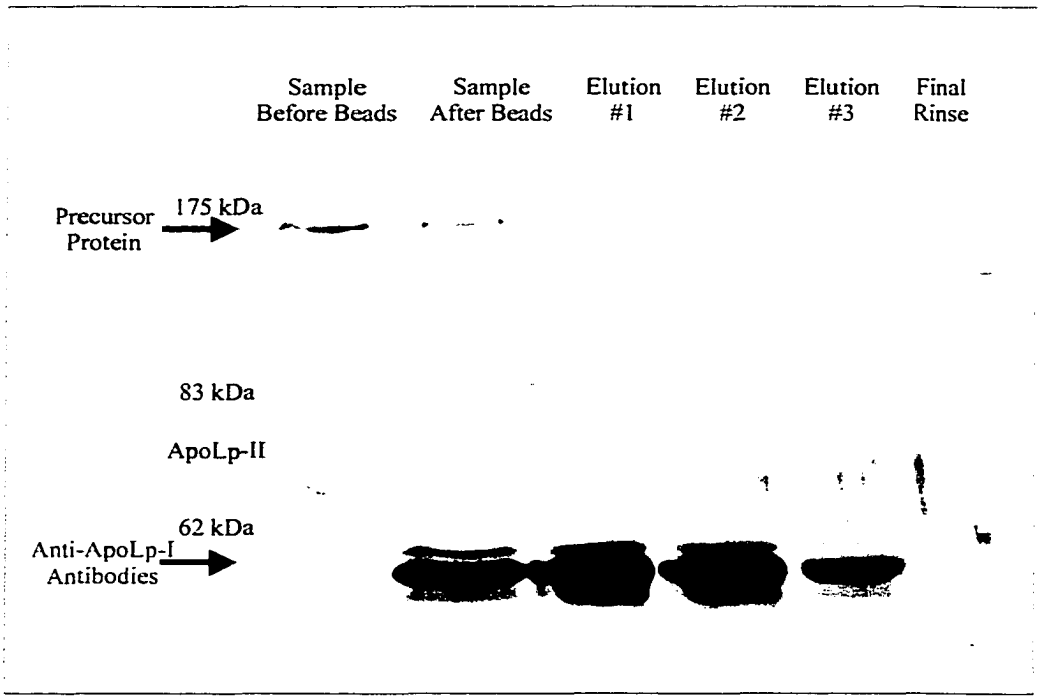


FIGURE 15: APOLP-I ANTIBODY PROTEIN PURIFICATION OF APOLP-II,I FROM *SACCHAROMYCES CEREVISIAE*.

ApoLp-I antibodies were conjugated to protein A agarose beads and used to column purify apoLp-II,I collected from the periplasmic space of galactose-induced *S. cerevisiae* cells. Samples were analyzed before (lane 1) and after (lane 2) exposure to the column, and the apoLp-II,I precursor protein fragment was found in both. No apoLp-II,I precursor protein fragment was found to elute from the column (lanes 3-6), indicating that the nascent particle was unable to bind to the column. ApoLp-I antibodies are visible in column elutions (lanes 3-6). Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

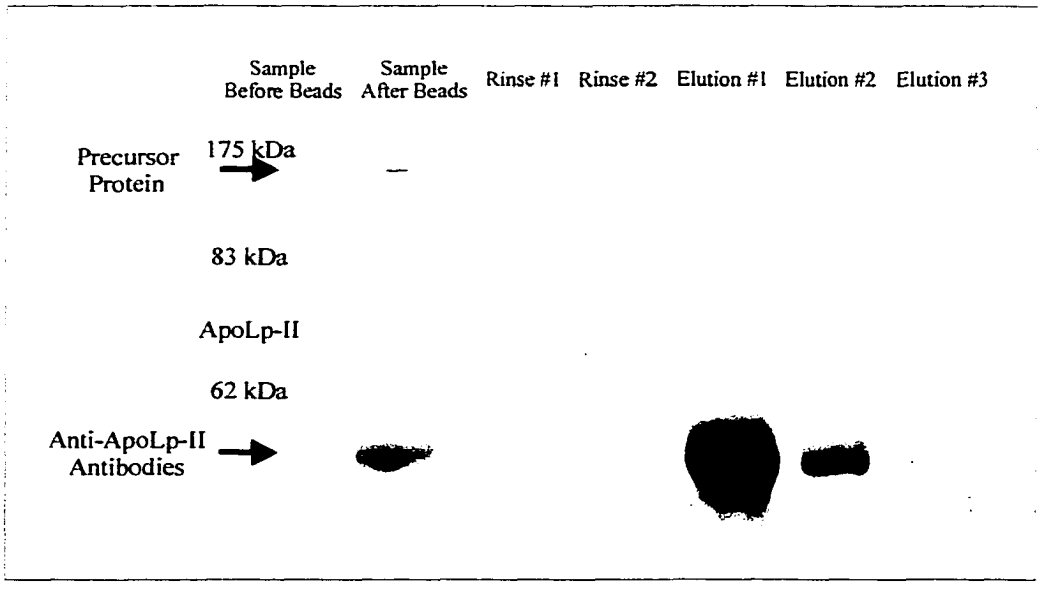


FIGURE 16: APOLP-II ANTIBODY PROTEIN PURIFICATION OF APOLP-II,I FROM *SACCHAROMYCES CEREVISIAE*.

ApoLp-II antibodies were conjugated to protein A agarose beads and used to column purify apoLp-II,I collected from the periplasmic space of galactose-induced *S. cerevisiae* cells. Samples were analyzed before (lane 1) and after (lane 2) exposure to the column, and the apoLp-II,I precursor protein fragment was found in both. No amount of the apoLp-II,I precursor protein fragment was found to elute from the column (lanes 3-7), indicating that the nascent particle was unable to bind to the column. ApoLp-II antibodies are visible in column elutions (lanes 5 and 6). Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

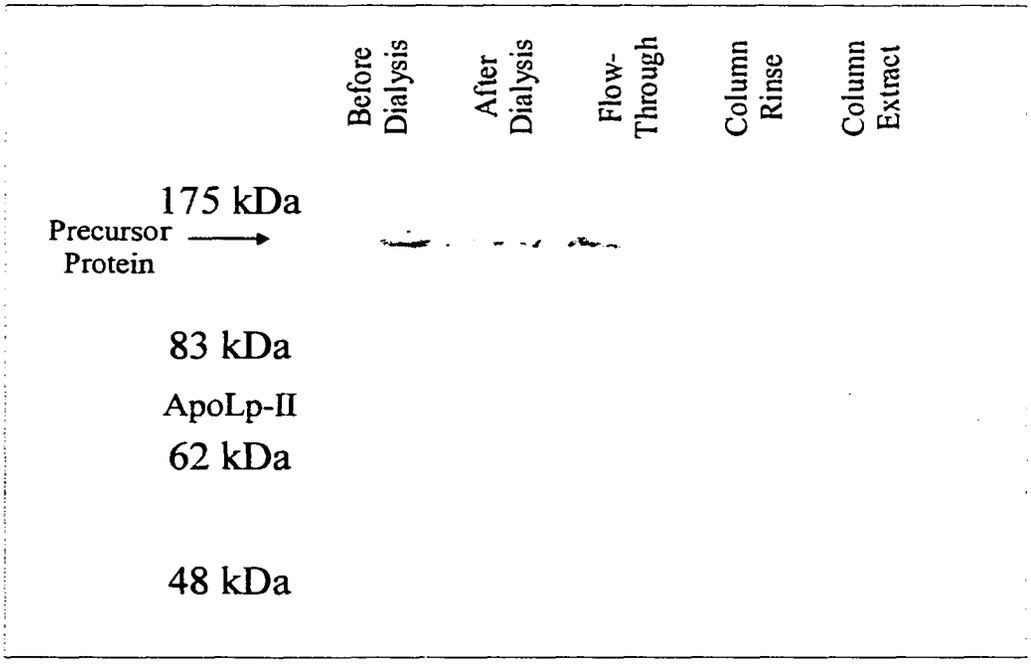


FIGURE 17: HISTIDINE TAG PURIFICATION BY HIGH AFFINITY NICKEL COLUMN.

Sample (lane 1) was dialyzed before it was run on the column (lane 2). All of the precursor protein was found in the flow-through (lane 3), indicating that the histidine tag was unable to bind the nickel column. Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

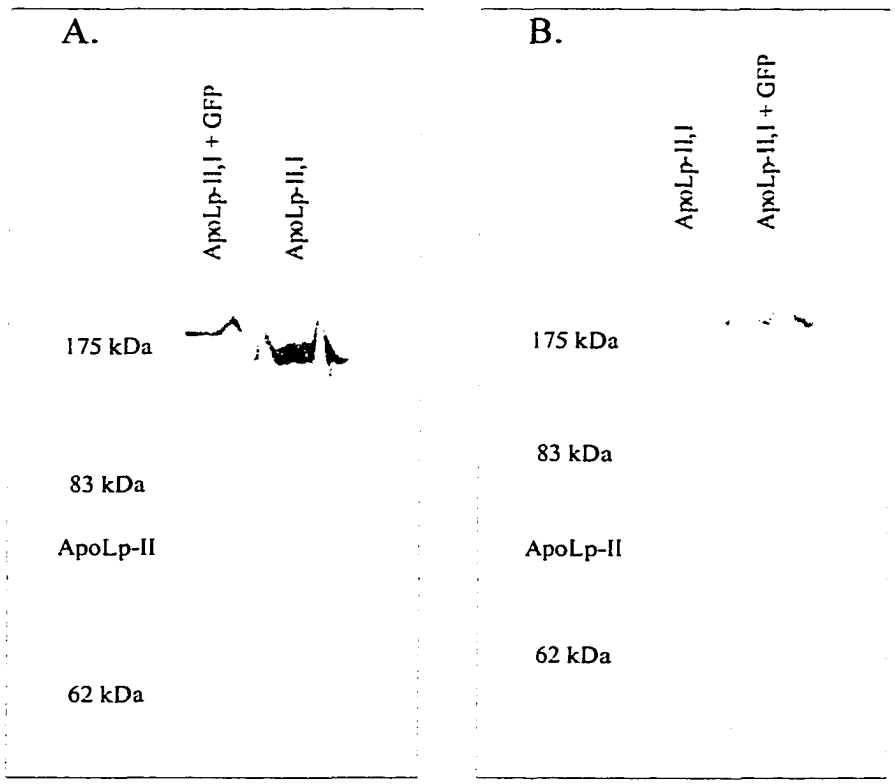


FIGURE 18: EXPRESSION OF THE APOLP-II,I/GFP FUSION PROTEIN.

The green florescent protein (GFP) was added to the carboxy terminus of the apoLp-II,I precursor protein fragment and expressed in *Saccharomyces cerevisiae* cells. Proteins secreted to the outside of the yeast cell plasma membrane were analyzed by western blot using either anti-apoLp-II (A) or anti-GFP antibodies (B). The addition of GFP to the apoLp-II,I precursor protein fragment increased the size of the expressed protein (compare lanes A1 and A2). The precursor protein fragment was only recognized by GFP antibodies when GFP was present (compare lanes B1 and B2). Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lanes indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in each SDS-PAGE gel.

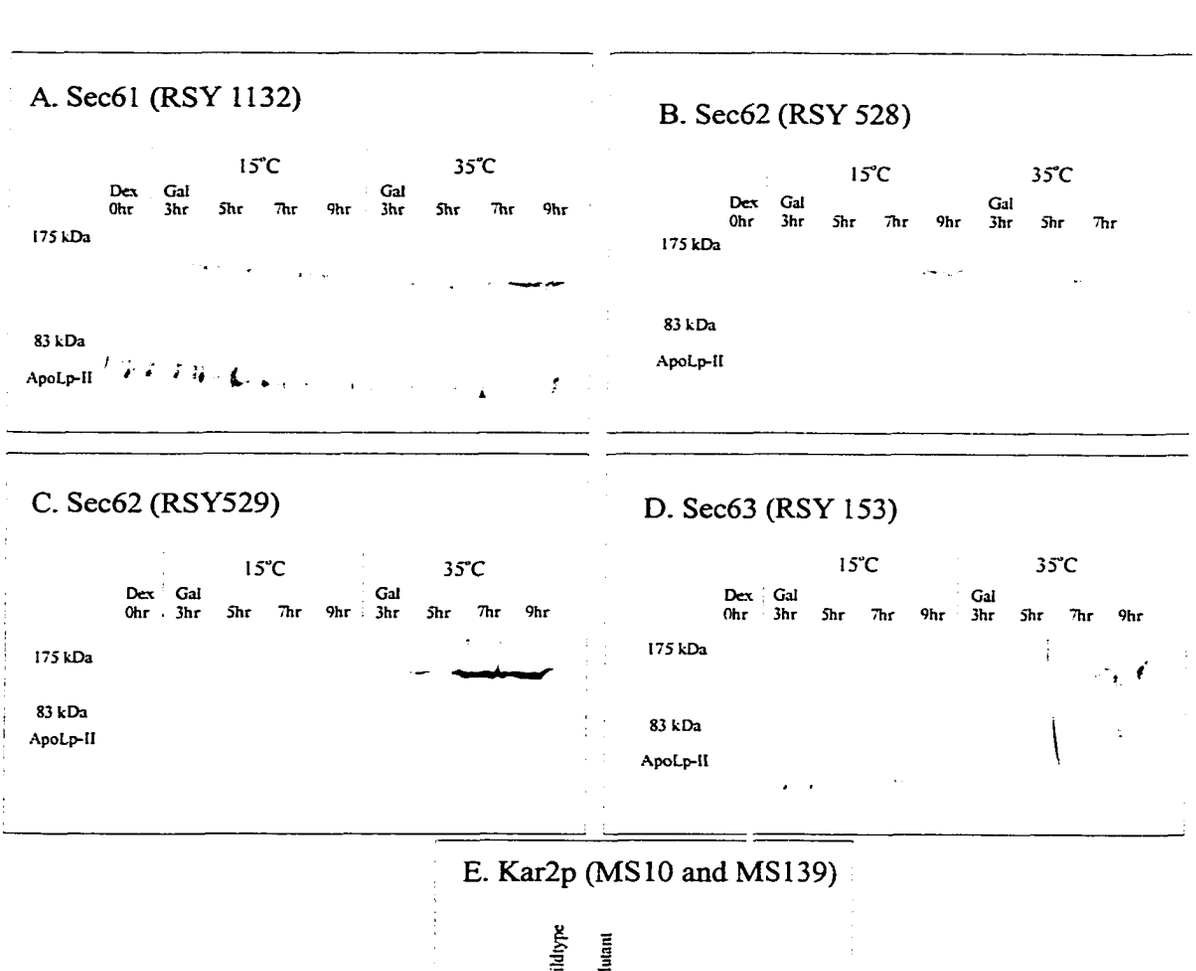


FIGURE 19: EXPRESSION OF THE APOLP-II,I PRECURSOR PROTEIN IN YEAST SECRETORY MUTANTS.

The precursor protein fragment was expressed in temperature sensitive yeast secretory mutants (A-D) and in one heat shock protein mutant (E). Expression of the precursor protein fragment occurs regardless of whether the mutation is induced by temperature shift from 15°C to 35°C. Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lanes indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in each SDS-PAGE gel.

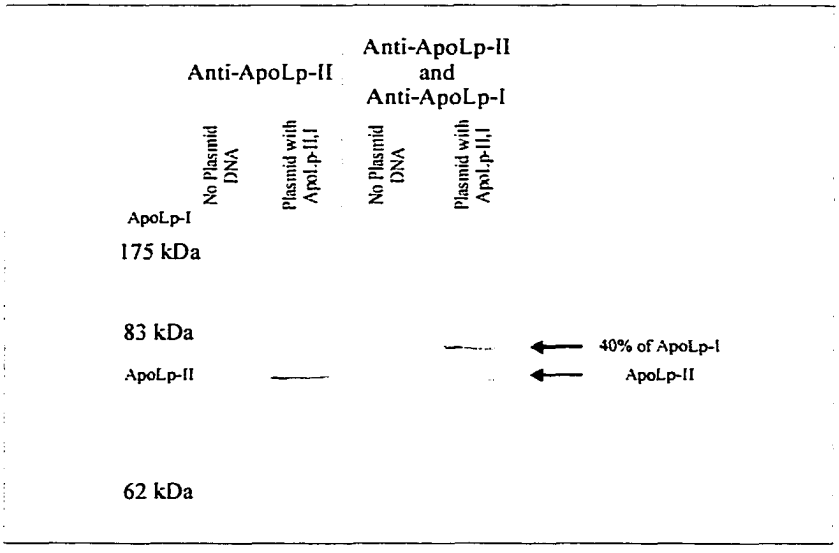


FIGURE 20: APOLP-II,I EXPRESSION IN *DROSOPHILA* S2 CELLS.

Non-transfected *Drosophila* S2 cells (lanes 1 and 3) and *Drosophila* S2 cells transfected with a apoLp-II,I-containing plasmid (lanes 2 and 4) were grown for five days at 25°C. Media was collected, purified on a high affinity nickel column, and analyzed by western blot using either apoLp-II antibodies (lanes 1 and 2) or both apoLp-I and apoLp-II antibodies (lanes 3 and 4). The apoLp-II antibody recognizes a protein that runs similar to *M. sexta* apoLp-II. The apoLp-I antibody recognizes a protein that is the size expected for 40% of apoLp-I. Numbers refer to marker bands in kilodaltons, and apoLp-I and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

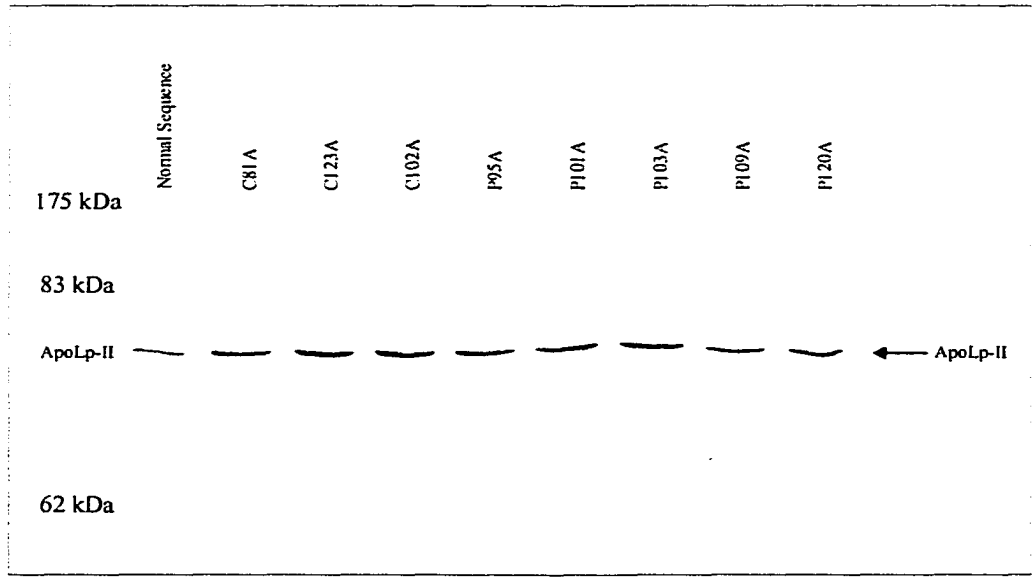


FIGURE 21: ALANINE-SCANNING MUTAGENESIS APOLP-II,I EXPRESSION IN *DROSOPHILA* S2 CELLS.

Drosophila S2 cells were grown for five days at 25°C following tranfection with plasmid containing either the normal apoLp-II,I sequence or a sequence containing an amino acid alteration (C81A indicates a cysteine to alanine change at amino acid #81; P95A indicates a proline to alanine change at amino acid #95; etc.). Expressed protein was isolated from media by high affinity nickel column and analyzed by western blot using apoLp-II antibodies. Alteration of amino acid sequence had no affect on protein expression, lipidation, or secretion, as apoLp-II was still found to be associated with apoLp-I in the media. Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

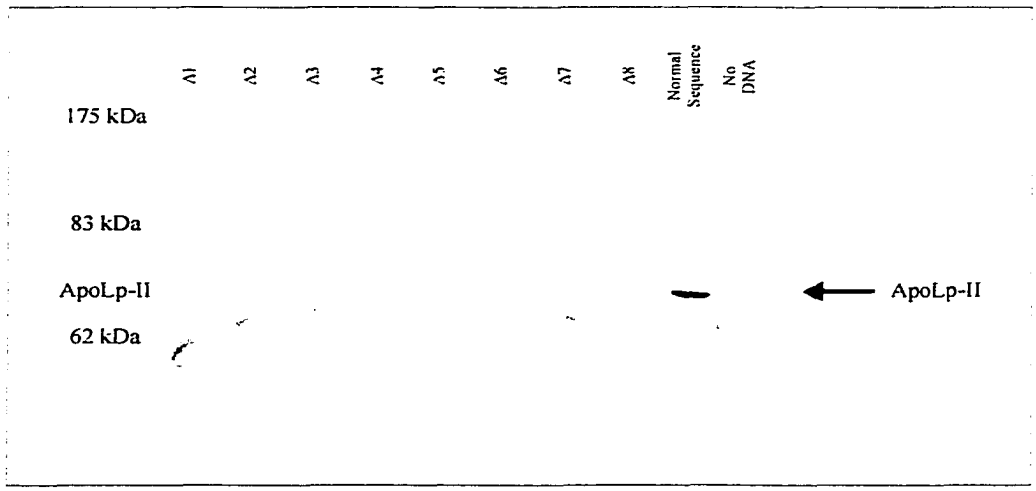


FIGURE 22: AMINO ACID DELETIONS OF CONSECUTIVE PORTIONS OF APOLP-II,I.

Drosophila S2 cells were grown for five days at 25°C following tranfection with plasmid containing either the normal apoLp-II,I sequence or a sequence containing an amino acid deletion (see Figure 9 for description of deletions). Expressed protein was isolated from media by high affinity nickel column and analyzed by western blot using apoLp-II antibodies. Diffuse proteins (~60kDa) are routinely recognized by the polyclonal anti-apoLp-II antibody in the media. Deletion of any portion of the apoLp-II amino acid sequence resulted in loss of protein expression, as apoLp-II was not found in the media or in the cell homogenate (not pictured). Numbers refer to marker bands in kilodaltons, and apoLp-II in the marker lane indicates where apoLp-II, purified from *M. sexta* hemolymph, ran in this SDS-PAGE gel.

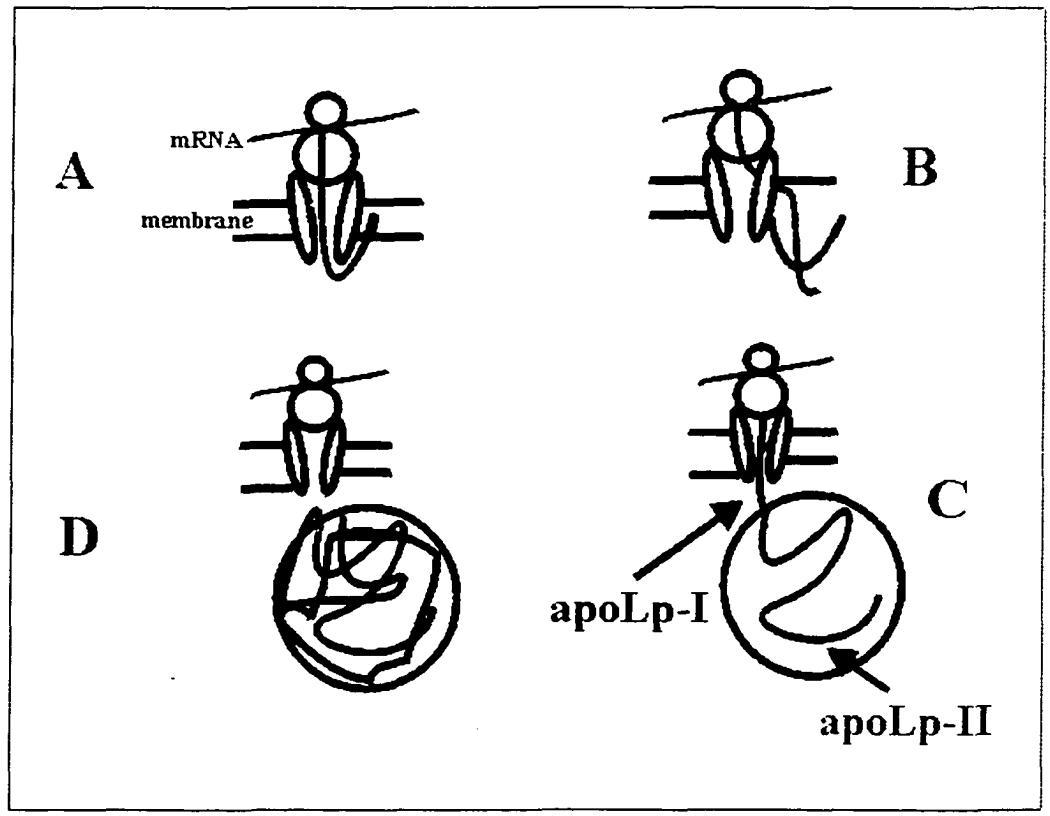


FIGURE 23: MODEL FOR LIPOPROTEIN BIOSYNTHESIS.

Liprotein biosynthesis begins with the translation of apoLp-II,I precursor mRNA on free ribosomes (not pictured). The amino-terminal signal sequence targets the nascent protein to the endoplasmic reticulum (A). The signal sequence is cleaved from the nascent protein and, as co-translational translocation proceeds, the apoLp-II portion of the precursor protein begins to integrate into the endoplasmic reticulum membrane. This process causes a bulging of the inner leaflet of the membrane (B). The increases length of the apoLp-II portion of the apoprotein precursor causes the bulge to enlarge until it finally pinches off of the membrane, forming the phospholipid monolayer of the lipoprotein (C). The apoLp-I portion of the precursor is translated and then associates with the outside surface of the particle (D).

DISCUSSION

While vertebrates and insects vary vastly in their size, shape, and distribution, many of their basic physiological processes are analogous. Given that insects are easily reared in a laboratory setting, reproduce in great number, and have a short life cycle, they have proven themselves valuable model systems for scientific research. One particular area of insect research centers on the molecular mechanisms of obtaining and storing dietary lipid.

Both vertebrates and insects obtain nutrients from their diet. Most of these nutrients are easily dissolved in their bloodstream for transport to sites of use or storage. Lipids, however, are hydrophobic, and their transport requires assistance from lipoproteins, protein-lipid complexes that have a polar exterior and a non-polar interior (Figure 2). The transported lipids are found in the core of this particle, and phospholipids and apolipoprotein(s) are found on its surface. Insect lipoproteins generally contain two apolipoproteins, apolipophorin-I (apoLp-I) and apolipophorin-II (apoLp-II). These two proteins are expressed as a single precursor protein in the fat body, an organ analogous to vertebrate liver and adipose tissue (Figure 10). The precursor protein is cleaved by endogenous convertase enzyme(s) and the apolipoproteins are packaged together with phospholipids prior to their secretion into the insect hemolymph. Therefore, it is believed that the apolipoproteins play a vital role in the process of lipoprotein biosynthesis.

This study uses the apolipoproteins from the tobacco hornworm, *Manduca sexta*, to investigate their role in the biosynthesis of lipoproteins. In general, three basic

questions were addressed: (1) Is the capacity for lipoprotein production unique to multicellular organisms? (2) Is the pathway elucidated for non-lipidated secretory proteins involved in lipoprotein production or is there an alternate pathway? (3) Which portions of the apolipoprotein sequence are crucial for lipoprotein production? Two expression systems have been developed, a yeast expression system and a *Drosophila* S2 expression system, and the same amino-terminal 45% of the *M. sexta* precursor was expressed in both. This portion of the precursor protein includes all of the signal sequence and all of apoLp-II, but only approximately 40% of apoLp-I. This portion of the apolipoprotein precursor was chosen because it was the largest clone available that included the entire amino-terminal portion of the sequence.

(1) Is the capacity for lipoprotein production unique to multicellular organisms?

Lipoproteins are used for the intercellular transport of lipids in multicellular organisms. Their production involves three processes: expression of apolipoprotein(s), lipidation of this apolipoprotein(s), and secretion of the particle. These three processes are intimately linked, and it has been shown that expressed vertebrate apolipoproteins are degraded if they are not immediately lipidated. Unicellular organisms have no need for lipoproteins, and, while they are capable of expressing and secreting proteins, they have never been shown to lipidate any protein. Lipoprotein biosynthesis may require a unique pathway or specific accessory proteins, such as MTP, an accessory protein that was found to be necessary for apoB production. Should additional proteins be required for

apolipoprotein lipidation, unicellular organisms would not be expected to have them. Therefore, even if they were capable of expressing apolipoproteins, it would be expected that these proteins would not be lipidated or secreted.

A. Apolipoprotein Expression

In order to explore lipoprotein biosynthesis in a unicellular organism, the three processes—expression, lipidation, and secretion—were examined individually in *S. cerevisiae*. First, expression of the apolipoprotein was addressed. The amino-terminal 45% portion of the *M. sexta* precursor protein was cloned onto a yeast shuttle plasmid and transfected into *S. cerevisiae* cells. When these cells were grown in the presence of galactose to induce expression, the entire cDNA was expressed, resulting in a ~160kDa protein (Figures 11, 12, and 13). This expressed protein was recognized by both apoLp-I and apoLp-II antibodies, in a similar fashion to the full length precursor protein produced by the fat body cells of *M. sexta* (Figure 10). This demonstrates that it is possible to *express* apolipoproteins in unicellular organisms and that their expression does not require a unique pathway.

One major difference with the protein expressed in the yeast system is that the precursor protein was not cleaved into the apolipoprotein subunits as is found in the insect. In *M. sexta*, a convertase in the Golgi apparatus is believed to cleave the precursor protein at the RGRR cleavage site (amino acids 708-711). The substrate specificity of the homologous enzyme in yeast, kexin, must prevent it from recognizing the cleavage site. When this same protein fragment is expressed in the *Drosophila* S2

cell system (see below), two distinct proteins are found on western blot. In this case, the cleavage site is recognized by the endogenous *Drosophila* convertase enzyme(s), further suggesting a substrate specificity problem with the yeast kexins.

B. Apolipoprotein Secretion

The second process that was addressed was the secretion of the apolipoprotein. In nature, *M. sexta* lipophorin is secreted out of fat body cells into the hemolymph. If this secretion requires a unique pathway, a unicellular organism, such as *S. cerevisiae* would not be able to secrete it. To investigate whether a unicellular organism is capable of secreting apolipoproteins, the precursor protein fragment was expressed in yeast cells. Then, western blot was used to examine two areas for the presence of apoLp-II: the liquid media in which the cells were grown and the area between the yeast cell wall and plasma membrane (Figure 11 and 12). No apoLp-II was detected in the media. It was, however, found trapped between the plasma membrane and the cell wall. Presumably, the particle produced by the yeast cells was too large to pass through pores in the cell wall of the yeast cell. The fact that the apolipoproteins are not able to cross the yeast cell wall is not surprising as this obstacle is not ever encountered in insect cells. The data indicates that not only are unicellular yeast cells capable of *expressing* apolipoproteins, but they are also capable of *secreting* a nascent particle. Furthermore, by examining the space between the plasma membrane and the cell wall at various time points following induction of apolipoprotein expression, it was determined that about two hours were required for expression and secretion of the apolipoprotein (Figure 13).

C. Apolipoprotein Lipidation

Finally, the lipidation of apolipoproteins by yeast cells was examined. Using density gradient ultracentrifugation, the density of the particle secreted into the space between the yeast cell wall and plasma membrane was determined to be 1.24-1.27mg/ml (Figure 14). Non-lipidated proteins generally have a density of about 1.33mg/ml; thus this density is what would be expected for a nascent particle consisting of only protein and phospholipid. The calculated lipid content of this particle was 20-30%, which is comparable to that of the nascent particle isolated from *M. sexta* (Prasad *et al* 1986a). Lipid analysis would be required to further characterize the lipid associated with this particle. Thus, not only are unicellular organisms capable of *expressing* and *secreting* apolipoproteins, but they can also *lipidate* them. The process of lipidation must occur via a universal pathway that is found in all cell types.

When attempts were made to column purify the particle using antibodies, it was found that the antigenic components of both apoLp-II and apoLp-I are not recognized by antibodies when the particle is in its native state (Figures 15 and 16). These proteins are either folded in such a way that all antigenic portions are unrecognizable, or, more likely, are covered by phospholipid. The antibodies used in this project were raised against denatured *M. sexta* apoLp-II or apoLp-I. They do not recognize intact HDLp naturally produced by *M. sexta*, so it is not surprising that the intact particle produced by *S. cerevisiae* is not recognized by these antibodies. It is possible that not only are the yeast

cells lipidating the apolipoproteins, but they fold and/or lipidate these proteins just like insect cells do.

The aforementioned data demonstrate that unicellular organisms are capable of *expressing*, *lipidating*, and *secreting* apolipoproteins. A major finding of this project is that the particle produced in the yeast expression system is folded and lipidated in a manner similar to that of the HDLp particle produced by *M. sexta* in nature. This is the first time this has ever been demonstrated, and it indicates that lipidation occurs via a universal pathway. If any accessory proteins are required for this process, they are found in the yeast genome.

D. Carboxy-Terminal Additions to the Apolipoprotein

In an attempt to visualize lipophorin expression in *S.* cerevisiae, a fusion protein was made with the green fluorescent protein (GFP), and the entire protein was expressed in the yeast system. The full-length fusion protein was expressed and secreted as indicated by western blot (Figure 18). While anti-GFP antibodies were able to recognize GFP in this gel-denatured state, they were unable to assist in column purification of the particle. The antigenic portion of GFP was inaccessible to these antibodies when GFP was in its native state. Furthermore, the yeast cells did not fluoresce when expressing the GFP fusion protein. Apparently, the GFP portion of this protein was not able to form the TIM barrel motif required for chromophore binding and fluorescence. Presumably, the phospholipid component of the particle disrupts the folding of GFP, and the GFP portion is denatured on the surface of the intact particle.

In a final attempt to column purify the lipophorin produced by yeast cells, a carboxy-terminal histidine tag was placed just prior to the false stop codon of the precursor protein fragment. This tag was incapable of binding to a nickel affinity column (Figure 17). Taken together with the GFP experiment, these data suggest that the carboxy terminal portion of the expressed protein is inaccessible, possibly folded into the particle.

E. Summary of Apolipoprotein Expression in Yeast

In summary, by transfecting *S. cerevisiae* cells with a portion of the *M. sexta* precursor apolipoprotein, it has been demonstrated that a unicellular organism is capable of *expressing* apolipoproteins. The resulting apolipoprotein is not cleaved into its two subunits, and antibodies raised against each of these subunits recognize the synthesized apolipoprotein. The expressed protein is found trapped between the yeast cell wall and plasma membrane, indicating that it is *secreted* out of the cell. Furthermore, the secreted protein has a buoyant density of 1.24-1.27mg/ml and is not recognized by antibodies when it is in its native state. This indicates that the secreted protein has been packaged with some amount of lipid, sequestering the antigenic portions of the apolipoproteins in a manner similar to the production of HDLp in nature. This observation is further corroborated by the inability of carboxy-terminally-added items, such as GFP and a histadine tag, to function properly. Therefore, unicellular organisms are capable of *expressing*, *lipidating*, and *secreting* lipoproteins. Lipoprotein biosynthesis does *not*

require a unique pathway and if any accessory proteins are required for this process, they are found in yeast.

(2) Is the pathway elucidated for non-lipidated secretory proteins involved in lipoprotein production or is there an alternate pathway?

A. Examination of the Secretory Pathway

Apolipoproteins are secreted proteins and their DNA sequences code for amino-terminal signal sequences. Therefore, it would be expected that the secretion of apolipoproteins involves the secretory pathway. The secretory pathway has been extensively studied in *S. cerevisiae*, and several yeast secretory mutants exist. It has been established that wildtype *S. cerevisiae* cells are capable of producing lipoproteins. To examine the involvement of the secretory pathway in this process, yeast secretory mutants were examined to determine whether they are capable of lipoprotein biosynthesis. Should a particular mutant be incapable of secreting the apolipoprotein, the altered gene product would be suspect for its involvement in the secretion of apolipoproteins.

In the yeast expression system, it was shown that expression and secretion of apoLp-II,I occurs even in cells containing mutations in crucial secretory genes (*sec61*, *sec62, sec63,*and *sec65*) (Figure 19). Additionally, expression occurs in yeast cells containing mutations in the protein kar2p, a heat shock protein thought to drive the translocation of nascent peptides into the endoplasmic reticulum. This is surprising since

apoB expression has been shown to be defective in the absence of the kar2p hsp70 homolog (Zhou *et al* 1995). These data suggest two possibilities. (1) None of the secretory proteins examined were involved in lipoprotein biosynthesis. However, this does not mean that there are *no* accessory proteins involved in lipoprotein biosynthesis, it simply implies that these *particular* proteins are not essential to the process, even though they have been shown to be required for the secretion of soluble proteins. A general screen for accessory proteins needs to be developed to explore other candidate proteins. (2) The secretory proteins that were examined are involved in lipoprotein biosynthesis, but the particular mutations incurred by these secretory proteins did not affect their ability to function in lipoprotein biosynthesis. Apolipoproteins are not common proteins. Unicellular organisms are not known to produce any similar protein. Therefore, it should not be surprising that they utilize different regions of the secretory pathway proteins, than those used by non-lipidated proteins. Regardless of which of these two possibilities is correct, this research implies a novel pathway for the expression, lipidation, and secretion of apolipoproteins.

B. A Model for Lipoprotein Biosynthesis

Both mammalian and insect apolipoproteins are associated with a phospholipid monolayer. This association occurs prior to the release of the proteins from the fat body, most likely early in the secretory pathway. Translation of the most homologous mammalian apoprotein, apoB, has been shown to be correlated to microsomal lipid levels. Additionally, it has been shown that proteins do make contact with phospholipids

during translocation (Nilsson *et al* 1994, Martoglio *et al* 1995). Based on this information, a model for the lipidation of apoLp-II and apoLp-I can be proposed (Figure 23). The signal sequence on the amino-terminus of apoLp-II first directed the ribosome/mRNA complex to the endoplasmic reticulum. Following signal cleavage, the amino-terminal portion of apoLp-II begins to associate with ER phospholipids, in a manner similar to that seen for production of transmembrane proteins. Instead of integrating into the ER membrane, however, apoLp-II remains between the lipid bilayer, forcing the inner leaflet of the membrane to bulge into the lumen of the ER. This bulge increases in size as more apoLp-II is translated into the translocon. Eventually, the bulge pinches off from the ER membrane, resulting in apoLp-II being buried inside a phospholipid monolayer in the ER lumen. ApoLp-I is then translated and translocated directly into the ER lumen where it can associate with the surface of the particle.

Evidence for this pathway is derived from several studies. (1) The precursor protein sequence includes a putative signal sequence, which would be expected to target it to the endoplasmic reticulum. (2) It is possible to produce a lipoprotein in the absence of the carboxy terminal 40% of apoLp-I. ApoLp-I can not be the driving force in the lipidation of the apolipoprotein. (3) ApoLp-II is on the amino-terminus of the precursor protein, and is thus translated first. It would be expected to drive the formation of the lipoprotein particle. (4) When delipidated, the apoLp-II protein is insoluble in an aqueous medium. Thus, this protein must be lipidated during or immediately following translation. (5) In the intact lipophorin particle, apoLp-II becomes sequestered away

from the external aqueous environment, while apoLp-I remains associated with the surface. This suggests that lipidation of apoLp-II must occur prior to lipidation of apoLp-I, and that the addition of lipids to apoLp-II is likely the driving force for lipidation. (6) Deletion of any portion of the apoLp-II sequence results in the loss of lipoprotein biosynthesis (see below). A novel pathway likely exists for lipoprotein biosynthesis, and apoLp-II is likely the driving force for lipidation. Whether additional accessory proteins are involved in this process and how the apoLp-II protein sequence drives this process is unknown.

C. Investigation of a Novel Pathway

If the known secretory proteins normally involved in the secretion of soluble proteins are not the major players in lipoprotein biosynthesis, other proteins must be involved. As *S. cerevisiae* is capable of expressing, lipidating, and secreting apolipoproteins, these accessory proteins must be present in yeast. In order to identify these proteins, a screen was devised involving the mutagenesis of *S. cerevisiae* cells such that each cell incurs one mutation in one gene in its genome. Expression of apoLp-II,I would then be induced in the mutant cells, and any cells that were incapable of secreting the precursor protein fragment would be identified and characterized. In order to conduct the screen of these mutant cells, GFP was attached to the carboxy terminus of the apoLp-II,I precursor protein fragment. Ideally, any cells that are unable to secrete the lipophorin particle could be identified by their lack of fluorescence. Unfortunately, the GFP fusion protein was incapable of fluorescing, and this procedure was abandoned.

A second method for identifying accessory proteins was devised involving a new gene silencing technology called RNAi. This technique has been shown to work successfully in *Drosophila* S2 cells. The precursor protein fragment was expressed in *Drosophila* S2 cells (described below), and cells were to be treated with double stranded RNA synthesized separately against each gene in the *Drosophila* genome to posttranscriptionally silence those genes. Should the dsRNA prevent apoLp-II,I expression, identification and characterization of that gene could begin to shed light on the pathway for lipidation and the proteins involved in this process. Unfortunately, this approach was to be done in collaboration with Exelixis, but due to funding situations, collaboration has been terminated.

(3) Which portions of the apolipoprotein sequence are crucial for lipoprotein production?

Even though the entire *M. sexta* apolipoprotien sequence is known, it reveals little information about the protein's structure. It is likely that regions of the protein are crucial to lipophorin function (i.e. receptor binding) and biosynthesis (i.e. secretion and lipidation). Where these regions are, however, remains a mystery. To solve this mystery, a new expression system was developed using *Drosophila* S2 cells. This system has several advantages over the yeast system. First, the plasmid DNA used in this system was easier to manipulate than the plasmid in the yeast system. Site directed mutagenesis could be performed directly on the plasmid, reducing the number of cloning steps.

Secondly, unlike yeast cells, *Drosophila* cells to not have a cell wall, making secreted proteins easier to obtain. Finally, stably transfected S2 cells can be used in RNAi experiments (see above).

A. Developing the *Drosophila* S2 Cell System

The same 45% amino-terminal portion of the *M. sexta* apolipoprotein sequence that was expressed in *S. cerevisiae* cells was cloned into a *Drosophila* S2 cell plasmid that was tranfected into S2 cells. As in the yeast system, the cells had to be examined to determine whether they were capable of producing a lipoprotein. Again, three processes were studied--expression of the apolipoproteins, lipidation of the lipoproteins, and secretion of the lipoprotein particle. Just as in the yeast system, it was found that all three of these processes occurred, and a particle with a density of approximately 1.24-1.27mg/ml was produced, demonstrating that the *Drosophila* S2 cells were capable of liporpotein biosynthesis.

However, the protein produced in the *Drosophila* S2 system differed from that produced in the yeast system in several ways. (1) Protein expression is constitutive. (2) As previously mentioned, S2 cells do not have a cell wall, so the expressed protein is secreted directly into the media. (3) The precursor protein is cleaved into two distinct proteins. When examined by western blot (Figure 20), one protein (~70kDa) was found to run identically to *M. sexta* apoLp-II and was recognized by apoLp-II antibodies. The second protein was recognized by apoLp-I antibodies and was approximately the size expected for the amino 40% of apoLp-I (~80kDa). Thus, the *Drosophila* S2 cells must

contain endogenous convertase enzyme(s) that recognize the precursor protein cleavage site. (4) The particle produced by the *Drosophila* S2 cells was more stable that the particle produced by yeast cells. Long-term storage of the particle was possible without the addition of salts to the buffer. Whether this was due to the cleavage of the precursor, or a difference in the lipidation process is unknown. (5) Due to the design of the plasmid, the carboxy terminal portion of the apoLp-I fragment contained a histidine tag. This tag was functional and the two proteins copurified on a nickel affinity column. This is likely due to the intervening V5 epitope sequence, which allowed the his-tag to be distanced from the lipoprotein. The association of these two proteins with one another after cleavage and secretion suggests the *Drosophila* S2 cells produced a nascent lipophorin particle.

In summary, the *Drosophila* S2 cell system was capable of *expressing*, *lipidating*, and *secreting* a nascent lipoprotein particle. The particle produced in this system is more similar to nascent HDLp produced in insects in that it contains a cleaved precursor protein and is more stable for long-term storage. Thus, this expression system provides an excellent means for the examination apoLp-II to determine which portion of its protein sequence drives lipidation.

B. Deletion Studies

The sequencing of insect apolipoproteins has revealed that apoLp-II is translated before apoLp-I. Previous research has shown that apoLp-II becomes sequestered away from the external aqueous environment in the intact lipophorin particle. Both the yeast

expression system and the *Drosophila* S2 systems demonstrate that it is possible to produce lipoproteins in the absence of the terminal 60% of the apoLp-I sequence. Taken together, these data infer that the first half of the precursor protein sequence, most likely apoLp-II, is responsible for apolipoprotein lipidation. To begin exploration of the apoLp-II sequence, consecutive deletions of 70-100 amino acids were made along the apoLp-II sequence (Figure 8). These mutant sequences were expressed in S2 cells, and it was determined that none were expressed, or if they were expressed, they were immediately degraded (Figure 22). Whether this reflects a critical length for the apoLp-II sequence or a need for an intact sequence is yet to be determined. Further research to insert random amino acids into these deleted regions to recover the length of apoLp-II is necessary.

C. Alanine Scanning Mutagenesis

Some studies have been conducted examining the protein sequence of mammalian apoB. It has been determined that the proper folding of apoB, particularly the formation of disulfide bonds, is required for lipidation (Tran *et al* 1988, Burch and Herscovitz 2000). Based on these studies and the abundance of cysteine and proline residues in the first 130 amino acids of the apoLp-II sequence, alanine-scanning mutagenesis was done, targeting three cysteine and five proline residues (Figure 7). The altered apolipoprotein sequences were expressed in S2 cells and media was examined for the presence of apoLp-II. ApoLp-II was found to in all eight of the examined sequences (Figure 21). Furthermore, apoLp-II copurifies with apoLp-I on a nickel affinity column in a manner similar to that of the wildtype sequence. Even though it is clear that the apoLp-II

sequence is crucial to lipoprotein biosynthesis, alteration of these particular amino acids does not alter the expression, lipidation, or secretion of the apolipoprotein by S2 cells.

Even though these results were unable to reveal the lipidation code, a system for discovery has been developed. Further examination of the protein sequence is necessary to determine which portion of the apoLp-II sequence provides the code for apolipoprotein lipidation. This methodology, used in combination with the deletion analysis described above, is ideal for investigation of the lipidation code.

CONCLUSIONS AND FUTURE DIRECTIONS

Cardiovascular disease is the number one killer in the United States, and the accumulation of cholesterol is a significant cause. High-density lipoproiten (HDL) has been the focus of intensive study because it can remove cholesterol from arteries, yet details of how this molecule is synthesized remain sketchy. Additionally, there are human diseases in which HDL synthesis appears to be disrupted by defective genes that code for proteins involved in lipid transport. Examples of such diseases are Tangier disease, in which the defective gene codes for a cholesterol transporter, the ABC1 transporter protein, and abetalipoproteinemia (ABL), caused by a mutation in a protein called microsomal triglyceride transfer protein (MTP). In both of these diseases, HDL synthesis is effected. Without a clear understanding of how HDL biosynthesis normally occurs, it is difficult to understand the extent to which defects in proteins like the ABC1 transporter protein and MTP disrupt its synthesis. The focus of this project has been to elucidate details of lipoprotein biosynthesis. Does it use a novel pathway? Are there accessory proteins involved in the lipidation process? What portion of the apolipoprotein sequence codes for lipidation?

These projects have revealed that the unicellular organism, *S. cerevisiae*, is capable of expressing, lipidating, and secreting *M. sexta* apolipoproteins. The yeast cells produce an intact lipoprotein, a particle required for intercellular transport of lipids. This is a major finding--the first demonstration of any apolipoprotein being expressed in a unicellular organism. It is remarkable that the yeast cells are able to lipidate this protein,

and produce a particle that is so similar to *M. sexta* nascent lipophorin. The fact that yeast can produce this particle indicates that the expression, lipidation, and secretion of lipoproteins occurs via universal pathways--if any lipidation accessory or "helper" proteins are required, they are present in yeast. These accessory proteins may not be the same proteins that are used for the secretion of non-lipidated proteins, and further research is required for their identification.

A second major finding in this project is that lipophorin production occurs in the absence of the full apoLp-I sequence. This finding was true for both expression systems, and indicates that the lipidation code resides within the first 45% of the precursor protein sequence. Furthermore, deletion analysis has revealed that removal of any portion of the apoLp-II sequence prevents expression of the apolipoprotein. Therefore, it is possible to conclude that all of the information required to make a lipoprotein is included in the apoLp-II sequence. An excellent expression system, the Drosophila S2 expression system, has been developed, and can be used for the examination of the apoLp-II sequence. A closer examination of the apolipoprotein sequence through deletion analysis and alanine-scanning mutagenesis should provide insights into the code for lipidation included in the amino acid sequence of apoLp-II.

REFERENCES

Adams, A.E.M., Shen, W., Lin, C., Leavitt, J., and Matsudaira, P. (1995). Isoform-specific complementation of the yeast *sac6* null mutation by human fimbrin. Mol. Cell Biol. *15*, 69-75.

Andrews, D.W. and Johnson, A.E. (1996). The translocon: more than a hole in the ER membrane? TIBS *21*, 365-369.

Arrese, E.L., Canavoso, L.E., Jouni, Z.E., Pennington, J.E., Tsuchida, K., Wells, M.A. (2001). Lipid storage and mobilization in insects: current status and future directions. Insect Biochem Mol Biol. *31*, 7-17.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K, eds. (1992). Short Protocols in Molecular Biology. Green Publishing Associates and John Wiley and Sons: New York.

Babin, P.J., Bogerd, J., Kooiman, F.P., Van Marrewijk, W.J., Van der Horst, D.J. (1999). Apolipophorin II/I, apolipoprotein B, vitellogenin, and microsomal triglyceride transfer protein genes are derived from a common ancestor. J Mol Evol. *49,* 150-160.

Beenakkers, A.M.Th., Chino, H., and Law, J.H. (1988). Lipophorin nomenclature. Insect Biochem. *18*, 1-2.

Blacklock, B.J. and Ryan, R.O. (1994). Hemolymph lipid transport. Insect Biochem. Molec. Biol. *24*, 855-873.

Bligh, E.G., and Dyer, W.J. (1959). A rapid method of lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911-917.

Bonnardel, J.A. and Davis, R.A. (1995). In HepG2 cells, translocation, not degradation, determines the fate of the *de Novo* synthesized apolipoprotein B. J. Biol. Chem. *270*, 28892-28896.

Borel, A.C. and Simon, S.M. (1996a). Biogenesis of polytopic membrane proteins: membrane segments of P-glycoprotein sequentially translocate to span the ER membrane. Biochemistry *35*, 10587-10594.

Borel, A.C. and Simon, S.M. (1996b). Biogenesis of polytopic membrane proteins: membrane segments assemble within translocation channels prior to membrane integration. Cell *85*, 379-389.

Boren, J., Rustaeus, S., and Olofsson, S. (1994). Studies on the assembly of apolipoprotein B-100- and B-48-containing very low density apolipoproteins in McA-RH7777 cells. J. Biol. Chem. *269*, 25879-25888.

Bostrum, K., Boren, J., Wettesten, M., Sjoberg, A., Bondjers, G., Wiklund, O., Carlsson, P., and Olofsson, S. (1988). Studies on the assembly of apo B-100-containing lipoproteins in HepG2cells. J. Biol. Chem. *263*, 4434-4442.

Brodsky, J.L., Goeckeler, J., and Schekman, R. (1995). BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum. Proc. Natl. Acad. Sci. USA *92*, 9643-9446.

Burch, W.L., Herscovitz, H. (2000). Disulfide bonds are required for folding and secretion of apolipoprotein B regardless of its lipidation state. J Biol Chem. 2000 *275*, 16267-16274.

Canavoso, L.E., Jouni, Z.E., Karnas, K.J., Pennington, J.E., Wells, M.A. (2001). Fat metabolism in insects. Annu. Rev. Nutr. *21*, 23-46.

Chino, H. (1985). Lipid transport: Biochemistry of hemolymph lipophorin. In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, ed. G.A. Kerkut, L.I. Gilbert. 10:115-135. Oxford:Pergamon.

Chuck, S.L., and Lingappa, V.R. (1992). Pause transfer: a topogenic sequence in apolipoprotein B mediates stopping and restarting of translocation. Cell *68*, 9-21.

Chuck, S.L. and Lingappa, V.R. (1993). Analysis of a pause transfer sequence from apolipoprotein B. J. Biol. Chem. *268*, 22794-22801.

Chuck, S.L., Yao, Z., Blackhart, B.D., McCarthy, B.J., Lingappa, V.R. (1990). New variation on the translocation of proteins during early biogenesis of apolipoprotein B. Nature. *26,* 382-385.

Connolly, T., Collins, P., and Gilmore, R. (1989). Access of proteinase K to partially translocated nascent polypeptides in intact and detergent-solubilized membranes. J. Cell Biol. *108*, 299-307.

Crowley, K.S., Reinhart, G.D., and Johnson, A.E. (1993). The signal sequence moves through a ribosomal tunnel into a noncytoplasmic aqueous environment at the ER membrane early in translocation. Cell *73*, 1101-1115.

Crowley, K.S., Liao, S., Worrell, V.E., Reinhart, G.D., and Johnson, A.E. (1994).

Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore. Cell *78*, 461-471.

Davidson, N.O., Carlos, R.C., Drewek, M.J., and Parmer, T.G. (1988). Apolipoprotein gene expression in the rat is regulated in a tissue-specific manner by thyroid hormone. J. Lipid Res. *29*, 1511-1522.

Davis, R.A., Thrift, R.N., Wu, C.C., and Howell, K.E. (1990). Apolipoprotein B is both integrated into and translocated across the ER membrane. J. Biol. Chem. *265*, 10005-10011.

Deshaies, R.J., and Schekman, R. (1989). SEC62 encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum. J. Cell Biol. *109*, 2653-2664.

Deshaies, R.J., Sanders, S.L., Feldheim, D.A., and Schekman, R. (1991). Assembly of yeast Sec proteins involved in trnaslocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. Nature *349*, 806-808.

Do, H., Falcone, D., Lin, J., Andrews, D.W., and Johnson, A.E. (1996). The cotranslational integration of membrane proteins into the phospholipid bilayer is a multistep process. Cell *85*, 369-378.

Du, E.Z., Kurth, J., Wang, S., Humiston, P., and Davis, R.A. (1994). Proteolysis-coupled secretion of the Nterminus of apolipoprotein B: Characterization of a transient, translocation arrested intermediate. J. Biol. Chem. *39*, 24169-24176.

Du, E.Z., Wang, S., Kayden, H.J., Sokol, R., Curtiss, L.K., and Davis, R.A. (1996). Translocation of apoprotein B across the endoplasmic reticulum is blocked in abetalipoproteinemia. J. Lipid Res. *37*, 1309-1315.

Edelstein, C., Kezdy, F.J., Scanu, A.M., and Shen, B.W. (1979). Apolipoproteins and the structural organization of plasma lipoproteins: human plasma high density lipoprotein-3. J. Lipid Res. *20*, 143-153.

Fernando-Warnakulasuriya, G.J.P., Tsuchida, K., and Wells, M.A. (1988). Effect of dietary lipid content on lipid transport and storage during larval development of *Manduca sexta*. Insect Biochem. *18*, 211-214.

Folch, J.M., Lees, M., Sloane Stanley, G.H. (1957). A simple method for isolation oand purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497-509.

Gietz, R.D., Schiestl, R.H., Willems, A.R., Woods, R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast. *11*, 355-360.

Gilmore, R. (1993). Protein translocation across the endoplasmic reticulum: a tunnel with toll booths at entry and exit. Cell *75*, 589-592.

Gilmore, R. and Blobel, G. (1985). Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. Cell *42*, 497-505.

Gondim, K.C., Atella, G.C., Kawooya, J.K., and Masuda, H. (1992). Role of phospholipids in the lipophorin particles of *Rodnius prolixus*. Arch. Insect Biochem. Physiol. *20*, 303-314.

Gordon, D.A., Jamil, H., Sharp, D., Mullaney, D., Yao, Z., Gregg, R.E. (1994). Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availablility. Proc. Natl. Acad. Sci. *91*, 7628-7632.

Gorlich, D., Prehn, S., Hartmann, E., Kalies, K., and Rapoport, T.A. (1992). A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. Cell *71*, 489-503.

Gorlich, D. and Rapoport, T.A. (1993). Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. Cell, *75*, 615-630.

Gretch, D.G., Sturley, S.L., Wang, L., Lipton, B.A., Dunning, A., Grunwald, K.A.A., Wetterau, J.R., Yao, Z., Talmud, P., and Attie, A.D. (1996). The amino terminus of apolipoprotein B is necessary but not suffcient for microsomal triglyceride transfer protein responsiveness. J. Biol. Chem. *271*, 8682-8691.

Hanein, D., Matlack, K.E.S., Jungnickel, B., Plath, K., Kalies, K., Miller, K.R., Rapoport, T.A., and Akey, C.W. (1996). Oligomeric reings of the Sec61p complex induced by lignads required for protein translocation. Cell *87*, 721-732.

Hegde, R.S., and Lingappa, V.R. (1996). Sequence-specific alteration of the ribosome-membrane junction exposes nascent secretory proteins to the cytosol. Cell, *85*, 217-228.

High, S., Martoglio, B., Gorlich, D., Andersen, S.S.L., Ashford, A.J., Giner, A., Hartmann, E., Prehn, S., Rapoport, T.A., Dobberstein, B., and Brunner, J. (1993). Site-

specific photocross-linking reveals that Sec61p and TRAM contact different regions of a membrane-inserted signal sequence. J. Biol. Chem. *268*, 26745-26751.

Ingram, M.F. and Shelness, G.S. (1996). Apolipoprotein B-100 destined for lipoprotein assembly and intracellular degradation undergoes efficient translocation across the endoplasmic reticulum membrane. J. Lipid Res. *37*, 2202-2214.

Jungnickel, B. and Rapoport, T.A. (1995). A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. Cell, *82*, 261-270.

Kashiwazaki, Y. and Ikai, A. (1985). Structure of apoproteins in insect lipophorin. Archives of Biochemistry and Biophysics *237*, 160-169.

Katagiri, C., Sato, M., and Tanaka, N. (1987). Small-angle x-ray scattering study of insect lipophorin. J. Biol. Chem. *262*, 15857-15861.

Katagiri, C., Sato, M., de Kort, S., Katsube, Y. (1991). Structure of Colorado potato beetle lipophorin: Differential scanning calorimetric and small-angle x-ray scattering studies. Biochemistry *30*, 9675-9681.

Kawooya, J.K., van der Horst, D.J., van Heusden, M.C., Brigot, B.L.J., van Antwerpen, R., and Law, J.H. (1991). Lipophorin structure analyzed by in vitro treatment with lipases. J. Lipid Res. *32*, 1781-1788.

Knott, T.J., Pease, R.J., Powell, L.M., Wallis, S.C., Rall, S.C., Innerarity, T.L., Blackhart, B., Taylor, W.H., Marcel, Y., Milne, R., Johnson, D., Fuller, M., Lusis, A.J., McCarthy, B.J., Mahley, R.W., Levy-Wilson, B., and Scott, J. (1986). Complete protein sequence and identification of structural domains of human apolipoprotein B. Nature *323*, 734-738.

Kutty, R.K., Kutty, G., Kambadur, R., Duncan, T., Koonin, E.V., Rodriguez, I.R., Odenwald, W.F., and Wiggert, B. (1996). Molecular characterization and developmental expression of a retinoid- and fatty acid-binding glycoprotein from *Drosophila*. J. Biol. Chem. *271*, 20641-20649.

Leighton, J.K., Joyner, J., Zamarripa, J., Deines, M., and Davis, R.A. (1990). Fasting decreases apolipoprotein B mRNA editing and the secretion of small molecular weight apoB by rat hepatocytes: evidence that the total amount of apoB secreted is regulated post-transcriptionally. J. Lipid Res. *31*, 1663-1668.

Leiper, J.M., Bayliss, J.D., Pease, R.J., Brett, D.J., Scott, D. and Shoulders, C.C. (1994). Microsomal triglyceride transfer protein, the abetalipoproteinemia gene product,

mediates the secretion of apolipoprotein B-containing lipoproteins from heterologous cells. J. Biol. Chem. *269*, 21951-21954.

Leiper, J.M., Bayliss, J.D., Pease, R.J., Brett, D.J., Scott, D. and Shoulders, C.C. (1994). Microsomal triglyceride transfer protein, the abetalipoproteinemia gene product, mediates the secretion of apolipoprotein B-containing lipoproteins from heterologous cells. J. Biol. Chem. *269*, 21951-21954.

Lyman, S.K. and Schekman, R. (1997). Binding of secretory precursor polypeptides to a translocon subcomplex is regulated by BiP. Cell *88*, 85-96.

Mangold, H.K. (1969). Aliphatic lipids. *In* Thin-Layer Chromatography. E. Stahl, editor. Springer-Verlag, New York, NY. 363-421.

Martoglio, B., Hofmann, M.W., Brunner, J., and Dobberstein, B. (1995). The protein-conducting channel in the membrane of the endoplasmic reticulum is open laterally toward the lipid bilayer. Cell, *81*, 207-214.

Mothes, W., Prehn, S., and Rapoport, T.A. (1994). Systematic probing of the environment of a translocation secretory protein during translocation through the ER membrane. EMBO J. *13*, 3973-3982.

Musch, A., Wiedmann, M., and Rapoport, T.A. (1992). Yeast Sec proteins interact with polypeptides traversing the endoplasmic reticulum membrane. Cell *69*, 343-352.

Nicchitta, C.V. and Blobel, G. (1993). Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. Cell *73*, 989-998.

Nilsson, I., Whitley, P., and von Heijne, G. (1994). The COOH-terminal ends of internal signal and signal-anchor sequences are positioned differently in the ER translocase. J. Cell Biol. *126*, 1127-1132.

Ooi, C.E. and Weiss, J. (1992). Bidirectional movement of a nascent polypeptide across microsomal membranes reveals requirements for vectorial translocation of proteins. Cell, *71*, 87-96.

Panzner, S., Dreier, L., Hartmann, E., Kostka, S., and Rapoport, T.A. (1995). Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. Cell *81*, 561-570.

Pattnaik, N.M, Mundall, E.C., Trambusti, B.G., Law, J.H., and Kezdy, F.J. (1979). Isolation and characterization of a larval lipoprotein from the hemolymph of *Manduca*

sexta. Comp. Biochem. Physiol. *63B*, 469-476.

Pease, R.J., Harrison, G.B., and Scott, J. (1991). Cotranslocational insertion of apolipoprotein B into the inner leaflet of the endoplasmic reticulum. Nature *353*, 448-450.

Pease, R.J., Leiper, J.M., Harrison, G.B., ad Scott, J. (1995). Studies on the translocation of the amino terminus of apolipoprotein B into the endoplasmic reticulum. J. Biol. Chem. *270*, 7261-7271.

Prasad, S.V., Fernando-Warnakulasuriya, G.J.P., Sumida, M., Law, J.H., and Wells, M.A. (1986a). Lipoprotein biosynthesis in the larvae of the tobacco hornworm, *Manduca sexta*. J. Biol. Chem. *261*, 17174-17176.

Prasad, S.V., Ryan, R.O, Law, J.H., and Wells, M.A. (1986b). Changes in lipoprotein composition during larval-pupal metamorphosis of an insect, *Manduca sexta*. J. Biol. Chem. *261*, 558-562.

Prasad, S.V., Tsuchida, K., Cole, K.D., and Wells, M.A. (1987). Lipophorin biosyntheis during the life cycle of the tobacco hornworm, *Manduca sexta*. UCLA Symp. Mol. Cell. Biol. (NS) 49:267-273.

Pullinger, C.R., North, J.D., Teng, B., Rifici, V.A., Ronhild de Brito, A.E., and Scott, J. (1989). The apolipoprotein B gene is constitutively expressed in Hep G2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. J. Lipid Res. *30*, 1065-1077.

Rapoport, T.A., Jungnickel, B., and Kutay, U. (1996a). Protein Transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. Annu. Rev. Biochem. *65*, 271-303.

Rapoport, T.A., Rolls, M.M., and Jungnickel, B. (1996b). Approaching the mechanism of protein transport across the ER membrane. Curr. Opinion Cell Biol. *8*, 499-504.

Ricci, B., Sharp, D., O'Rourke, E., Kienzle, B., Blinderman, L., Gordon, D., Smith-Monroy, C., Robinson, G., Gregg, R.E., Rader, D.J., and Wetterau, J.R. (1995). A 30-amino acid truncation of the microsomal triglyceride transfer protein large subunit disrupts its interaction with protein disulfide-isomerase and causes abetalipoproteinemia. J. Biol. Chem. *270*, 14281-14285.

Robbs, S.L., Ryan, R.O., Schmidt, J.O., Keim, P.S., and Law, J.H. (1985). Lipophorin of the larval honeybee *Apis mellifera* L. J. Lipid Res. *26*, 241-247.

Rose, M.D., Misra, L.M., and Vogel, J.P. (1989). KAR2, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. Cell , *57*, 1211-1221.

Rothblatt, J.A., Deshaies, R.J., Sanders, S.L., Daum, G. and Schekman, R. (1989). Multiple Genes are Required for Proper Insertion of Secretory Proteins into the Endoplasmic Reticulum in Yeast. J. Cell Biol. *109*, 2641-2652.

Ryan, R.O., Kay, C.M., Oikawa, K., Liu, H., Bradley, R., and Scraba, D.G. (1992). Effect of particle lipid content on the structure of insect lipophorins. J. Lipid Res. *33*, 55-63.

Sanders, S.L., Whitfield, K.M., Vogel, J.P., Rose, M.D., and Schekman, R.W. (1992). Sec61p and BiP directly facilitate polypeptide translocation into the ER. Cell *69*, 353-365.

Shapiro, J.P., Keim, P.S., and Law, J.H. (1984). Structural studies on lipophorin, an insect lipoprotein. J. Biol. Chem. *259*, 3680-3685.

Shapiro, J.P., Law, J.H., and Wells, M.A. (1988). Lipid transport in insects. Ann. Rev. Entomol. *33*, 297-318.

Sharp, D. Blinderman, L., Combs, K.A., Kienzle, B., Ricci, B., Wager-Smith, K., Gil, C.M., Turck, C.W., Bouma, M., Rader, D.J., Aggerbeck, L.P., Gregg, R.E., Gordon, D.A., and Wetterau, J.R. (1993). Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia. Nature *365*, 65-69.

Shelby, K.S. and Chippendale, G.M. (1991). Assembly and secretion of lipophorin by the larval fat body of the southwestern corn borer, *Diatraea grandiosella*: An in vitro study. Arch. Insect Biochem. Physiol. *18*, 203-217.

Siegel, V. (1995). A second signal recognition event required for translocation into the endoplasmic reticulum. Cell *82*, 167-170.

Simon, S. (1993). Translocation of proteins across the endoplasmic reticulum. Curr. Opinion Cell Biol. *5*, 581-588.

Simon, S.M. and Blobel, G. (1991). A protein-conducting channel in the endoplasmic reticulum. Cell *65*, 371-380.

Soulages, J.L. and Brenner, R.R. (1991). Study on the composition-structure relationship of lipophorins. J. Lipid Res. *32*, 407-415.

Soulages, J.L. and Wells, M.A. (1994). Lipophorin: the structure of an insect lipoprotein

and its role in lipid transport in insects. Advances in Protein Chemistry *45*, 371-415.

Spring, D.J., Chen-Liu, L.W., Chatterton, J.E., Elovson, J., and Schumaker, V.N. (1992). Lipoprotein Assembly: apolipoprotein B size determines lipoprotein core circumference. *267*, 14839-14845.

Stirling, C.J., Rothblatt, J., Hosobuchi, M., Deshaies, R., and Schekman, R. (1992). Protein Translocation Mutants Defective in the Insertion of Integral Membrane Proteins into the Endoplasmic Reticulum. Molec. Biol. Cell *3*, 129-142.

Sturley, S.L., Talmud, P.J., Brasseur, R., Culbertson, M.R., Humphries, S.E., and Attie, A.D. (1994). Human apolipoprotein B signal sequence variants confer a secretion-defective phenotype when expressed in yeast. *269*, 21670-21675.

Sundermeyer, K., Hendricks, J.K, Prasad, S.V., and Wells, M.A. (1996). The precursor protein of the structural apolipoproteins of lipophorin: cDNA and deduced amino acid sequence. Insect Biochem. Molec. Biol. *26*, 735-738.

Thrift, R.N., Drisko, J., Dueland, S., Trawick, J.D., and Davis, R.A. (1992). Translocation of apolipoprotein B across the endoplasmic reticulum is blocked in a nonhepatic cell line. Proc. Natl. Acad. Sci. USA *89*, 9161-9165.

Tran, K., Boren, J., Macri, J., Wang, Y., McLeod, R., Avramoglu, R.K., Adeli, K., Yao, Z. (1998). Functional analysis of disulfide linkages clustered within the amino terminus of human apolipoprotein B. J Biol Chem. *273*,7244-7251.

Van Heusden, M.C., Thompson, F., Dennis, J. (1998). Biosynthesis of Aedes aegypti lipophorin and gene expression of its apolipoproteins. Insect Biochem Mol Biol. *28*, 733-738.

Vogel, J.P., Misra, L.M., and Rose, M.D. (1990). Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. J. Cell Biol. *110*, 1885-1895.

Walter, P. and Blobel, G. (1981a). Translocation of proteins across the endoplasmic reticulum II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of in-vitro-assembled polysomes synthesizing secretory protein. J. Cell Biol. *91*, 551-556.

Walter, P. and Lingappa, V.R. (1986). Mechanism of protein translocation across the endoplasmic reticulum membrane. Ann. Rev. Cell Biol. *2*, 499-516.

Wang, S., McLeod, R.S., Gordon, D.A., Yao, Z. (1996). The microsomal triglyceride

transfer protein facilitates assembly and secretion of apolipoprotein B-containing lipoproteins and decreases contranlational degradation of apolipoprotein B in transfected COS-7 cells. J. Biol. Chem. *271*, 14124-14133.

Weers, P.M.M., Van der Horst, D.J., Van Marrewijk, W.J.A., Van den Eijnden, M., Van Doorn, J.M., and Beenakkers, A.M.T. (1992). Biosynthesis and secretion of insect lipoprotein. J. Lipid Res. *33*, 485-491.

Weers, P.M.M., Van Marrewijk, W.J.A., Beenakkers, A.M.Th., and Van der Horst, D.J. (1993). Biosynthesis of locust lipophorin. J. Biol. Chem. *268*, 4300-4303.

Wetterau, J.R., Combs, K.A., McLean, L.R., Spinner, S.N., and Aggerbeck, L.P. (1991). Protein disulfide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. Biochemistry *30*, 9728-9735.

Wetterau, J.R., Aggerbeck, L.P., Bouma, M., Eisenberg, C., Munck, A., Hermier, M., Schmitz, J., Gay, G., Rader, D.J., and Gregg, R.E. (1992). Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Sciene *258*, 999-1001.

Wu, X., Shang, A., Jiang, H., and Ginsberg, H.N. (1996). Low rates of apoB secretion from HepG2 cells result from reduced delivery of newly synthesized triglyceride to a "secretion-coupled" pool. J. Lipid Res. *37*, 1198-1206.

Yao, Z., Blackhart, B.D, Linton, M.F., Taylor, S.M., Young, S.G., and McCarthy, B.J. (1991). Expression of carboxyl-terminally truncated forms of human apolipoprotein B in rat hepatoma cells. J. Biol. Chem. *266*, 3300-3308.

Zhou, M., Wu, X., Huang, L., and Ginsberg, H.N. (1995). Apoprotein B100, an inefficiently translocated secretory protein, is bound to the cytosolic chaperone, heat shock protein 70. J. Biol. Chem. *270*, 25220-25224.