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Purification of diacylglycerol:acyltransferase from rat liver to near homogeneity

Maria Andersson, Margit Wettesten, Jan Borén, Anita Magnusson, Anders Sjöberg, Sabina Rustaeus, and Sven-Olof Olofsson[1]

Department of Medical Biochemistry and the Wallenberg Laboratory, University of Göteborg, S-413 90 Göteborg, Sweden

Abstract A method to isolate a protein related to the diacylglycerol:acyltransferase (DGAT) activity in rat liver microsomes has been developed. The microsomes were treated with sodium deoxycholate (DOC; 0.1 mg/mg protein) at a concentration of 1 mM, i.e., below the critical micellar concentration (CMC), to remove luminal and loosely bound proteins. Three percent of the DGAT activity and all of the acylCoA hydrolyse activity were present in the supernatant, i.e., among the extracted loosely bound proteins. The insoluble material, recovered as a pellet, was suspended in DOC (1.6 mg/ml and mg protein in the original microsomes), and subjected to multiple, short (1–2 sec) sonications. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 5 mg/ml) was then added, and the sonication was repeated. The detergent-treated microsomal membranes were filtered through a 0.22-μm filter and chromatographed on a Superose 6 column from which the DGAT activity was recovered in a high molecular mass fraction. A monoclonal antibody that reacted with this fraction was raised and used in immunoaffinity experiments. This antibody removed 93 ± 6% (mean ± SD, n = 4) of the DGAT activity present in solution and 44 ± 6% (mean ± SD, n = 5) of the applied activity could be recovered after desorption. The antibody recognized a 60 kDa protein upon Western blot of rat liver microsomal proteins as well as of the DGAT-containing fraction from the Superose 6 column. A 60 kDa protein was highly enriched in the DGAT-containing retained fraction from the immunoaffinity chromatography. This 60 kDa protein reacted with the monoclonal antibody on Western blot. In addition to the 60 kDa protein, the retained fraction from the immunoadsorber contained a 77 kDa protein. This protein did not react with the monoclonal antibody on Western blots. Neither the 60 nor the 77 kDa protein reacted with antibodies to mouse immunoglobulins or showed any unspecific reaction with immunoglobulins.—**Andersson, M., M. Wettesten, J. Borén, A. Magnusson, A. Sjöberg, S. Rustaeus, and S-O. Olofsson.** Purification of diacylglycerol: acyltransferase from rat liver to near homogeneity. *J. Lipid Res.* 1994. **35:** 535–545.

Supplementary key words diacylglycerol:acyltransferase (EC 2.3.1.20) • rat liver microsomes • detergent solubilization • monoclonal antibody • triglyceride biosynthesis

The synthesis of triacylglycerol has been suggested to occur in the endoplasmic reticulum (ER) and most of the enzyme activities involved appears to be bound to or associated with the membrane of this organelle (1). The majority of the steps involved in the biosynthesis of the triacylglycerol molecule are also used for the formation of the phosphoglycerides and the only unique reaction is the last step in the process (2), i.e., the acylation of the diacylglycerol molecule to form triacylglycerol. This step is catalyzed by the enzyme diacylglycerol:acyltransferase (DGAT, EC 2.3.1.20). Results by Mayorek, Grinstein, and Bar-Tana (3) indicate that this unique last step is rate-limiting in the biosynthesis of the triacylglycerol. On the other hand Brindley (4) and his co-workers have presented evidence that the dephosphorylation of phosphatidic acid could serve as a step in which hormones and fatty acids may influence the rate of triacylglycerol (and phosphoglyceride) formation.

The triacylglycerol biosynthesis that occurs in the liver is of great importance for the secretion of the apoB-100-containing lipoproteins. Thus it has been shown that an increased biosynthesis of triacylglycerol in the cell is a major stimuli for apoB-100 secretion (5, 6).

The assembly of these lipoproteins appears to take place in regions of the ER membrane that are rich in DGAT activity (7) and the importance of the enzyme for the assembly and secretion of lipoproteins is further underlined by the observation (8) that the inhibitory effect of n-3 fatty acids on the secretion of triacylglycerol-rich lipoproteins has been suggested to be caused by an inhibition of the DGAT activity.

Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micellar concentration; DGAT, diacylglycerol:acyltransferase (EC 2.3.1.20); DOC, sodium deoxycholate; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.05% Tween 20; PMSF, phenylmethylsulfonyl fluoride; SD, standard deviation; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline with 0.1% Tween 20.

[1]To whom correspondence should be addressed.

Some attempts have been made to isolate the enzyme DGAT. A 9-fold increase in the specific activity has been achieved by gradient ultracentrifugation after detergent treatment of rat liver microsomes (9), and a 145-fold purification was obtained from the intestine after solubilization in taurocholate followed by phenyl Sepharose chromatography (10). In both cases only partial purifications were obtained.

Purification of the enzyme from soybean cotyledons has also been reported (11). Analyses of these preparations by electrophoresis in polyacrylamide gels containing SDS suggested that the enzyme had a subunit structure.

In this report we present a method to isolate DGAT from rat liver microsomes to near homogeneity.

MATERIALS

Superose 6 prep grade, FPLC columns, Sephadex G 25, Q Sepharose fast flow, Sephacryl S-200 HR, CNBr-activated Sepharose 4B, Protein G Sepharose fast flow and pre-packed disposable PD-10 columns were purchased from Pharmacia (Uppsala, Sweden); Chemibond® was from Chemicon International Inc. (Temecula, CA); palmitoyl CoA, L-phosphatidylcholine (from egg yolk), 1,2-dioleoyl-*sn*-glycerol, L-phosphatidyl-L-serine, and pepstatin A were from Sigma (St. Louis, MO). Sucrose was purchased from International Biotechnology Inc. (New Haven, CT); [1-^{14}C]palmitoyl CoA, rainbow markers (14,300–200,000) and blotting detection kit for mouse antibodies were from Amersham Int. (Amersham, United Kingdom). Ready Safe® was from Beckman (Fullerton, California); pre-coated TLC plates with Silica gel 60 were from Merck (Darmstadt, Germany); mini-PROTEAN II Ready Gels and Silver Stain Plus Kit were from Bio-Rad (Richmond, CA); BCA protein Assay Reagent was from Pierce (Rockford, IL). Trioleate, calpain inhibitor I and calpain inhibitor II were purchased from Boehringer Mannheim (Mannheim, Germany); microtiter plates PVC M 24 were from Dynatech Labor Inc. (Chantilly, VA); urease-conjugated sheep anti-mouse Ig fraction was from Sera-lab Limited (Crawley Down, England).

Millex AA filter 0.8 μm, Millex GV filter 0.22 μm, and the Milli-Q UF plus equipment (used for the purification of all water that was used in the experiments) were from Millipore Intertech (Bedford, MA).

METHODS

Isolation of rat liver microsomes

The method described earlier (5) for cells was scaled up and modified to make it possible to handle livers from 50 Sprague-Dawley rats.

The rats were killed by decapitation and the livers were removed and washed with 3 mM imidazole, pH 7.4, with 125 mM sucrose. The livers were cut into small pieces and homogenized in 3 mM imidazole, pH 7.4, with 125 mM sucrose (20 ml buffer/liver) by five strokes with a rotating Teflon pestle in a glass homogenizer. The homogenate was centrifuged for 10 min in a Beckman J-21 centrifuge at 1700 rpm and +4°C using a JA-14 rotor. The pellet was washed with the imidazole buffer (4 ml buffer/liver) under the same conditions and the combined supernatants were centrifuged for 20 min in a Beckman J-21 at 13,000 rpm and +4°C using a JA-14 rotor, and the obtained supernatant was ultracentrifuged for 63 min at +4°C and 35,000 rpm in a Beckman Ti 50 rotor. The pellet was recovered and suspended (at a concentration of approximately 30 mg microsomal protein/ml) in 3 mM imidazole, pH 7.4, with 125 mM sucrose and homogenized with 17 strokes in a Dounce homogenizer. The obtained microsomes were frozen in aliquots of 120 mg and kept at −80°C until used.

Detergent treatment of the microsomes

Microsomes (120 mg) (measured as protein) were diluted to 30 ml with 50 mM Tris-HCl, pH 7.5, with 300 mM sucrose and 1.25 mM EDTA. The following protease inhibitors were used: calpain inhibitor I (17 μg/ml), calpain inhibitor II (7 μg/ml), and 1 μM pepstatin A. Sodium deoxycholate (DOC) was added to a final concentration of 1 mM (i.e., 0.1 mg/mg microsomal protein) (12). The mixture was left on ice for 60 min and was then centrifuged in a Beckman Ti 90 rotor at 40,000 rpm and +4°C for 60 min. The supernatant was removed and the tube and the pellet were carefully washed twice with 2 ml ice-cold 50 mM Tris-HCl, pH 7.5, with 300 mM sucrose and 1.25 mM EDTA. To each pellet (recovered from 50 mg microsomes) was added 2 ml of 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1.25 mM EDTA, 1.6 mg DOC/ml, calpain inhibitor I and II (17 and 7 μg/ml), and 1 μM pepstatin A.

The pellet was first dispersed by forcing it through a 1-ml tip of an Eppendorf pipette approximately 10 times and then subjected to short (1–2 sec long) sonications in a MSE soniprep 150 at the following setting; 6, 10, 14, 18, 22, 26, and 30 microns. Four sonications were carried out at each setting.

CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; 5 mg/ml) was added to the solution and it was again subjected to sonication as described above at the following settings; 14, 22, 26, and 30 microns. The obtained suspension/solution was filtered through a 0.22-μm filter.

Diacylglycerol:acyltransferase assay (DGAT)

1,2-Dioleoyl-*sn*-glycerol was dispersed into portions enough for five assays and stored under nitrogen in sealed

glass ampules at −80°C. The storage time was usually below 30 days.

1,2-Dioleoyl-*sn*-glycerol (1 mg/assay) was dissolved in chloroform and mixed with 0.8 mg phosphatidylcholine and 0.8 mg phosphatidylserine and dried under nitrogen; 0.4 ml of 10 mM Tris-HCl, pH 8.0, was added and the mixture was sonicated for 60 sec on ice (using an MSE 8 ultrasonic at maximal setting).

Palmitoyl CoA (final concentration in assay 0.2 mM) and 0.188 μCi [1-^{14}C]palmitoyl CoA were added to the substrate, and the mixture was vortexed until all palmitoyl CoA had been solubilized.

The assay system contained 0.4 ml of the substrate solution, 0.150 ml of a 1.4 M solution of magnesium chloride, 0.6 ml 10 mM Tris-HCl, pH 8.0, and fatty acid-free bovine serum albumin (BSA) to a final concentration of 1 mg/ml. Fifty μl of the enzyme source was added to the assay simultaneously with the substrate solution, and the mixture was vortexed.

After incubation at 37°C, the assay system was transferred to 6 ml of chloroform–methanol 1:1 containing 0.1 mg trioleate, and the formed triacylglycerols were extracted in a two-phase system (13). In short, 4 ml of chloroform was added and the tubes were shaken and stored at −20°C for 10–12 h. Two ml of an acidified solution of sodium chloride (17 mM NaCl and 1 mM of H_2SO_4) was added, the tubes were again shaken and then centrifuged at 2500 rpm for 10 min at room temperature in a Beckman CPR centrifuge.

The upper phase was removed and the lower phase was recovered and evaporated to dryness under nitrogen.

The sample was dissolved in chloroform and subjected to thin-layer chromatography in chloroform–acetic acid 96:4. This chromatography gave a good separation be-

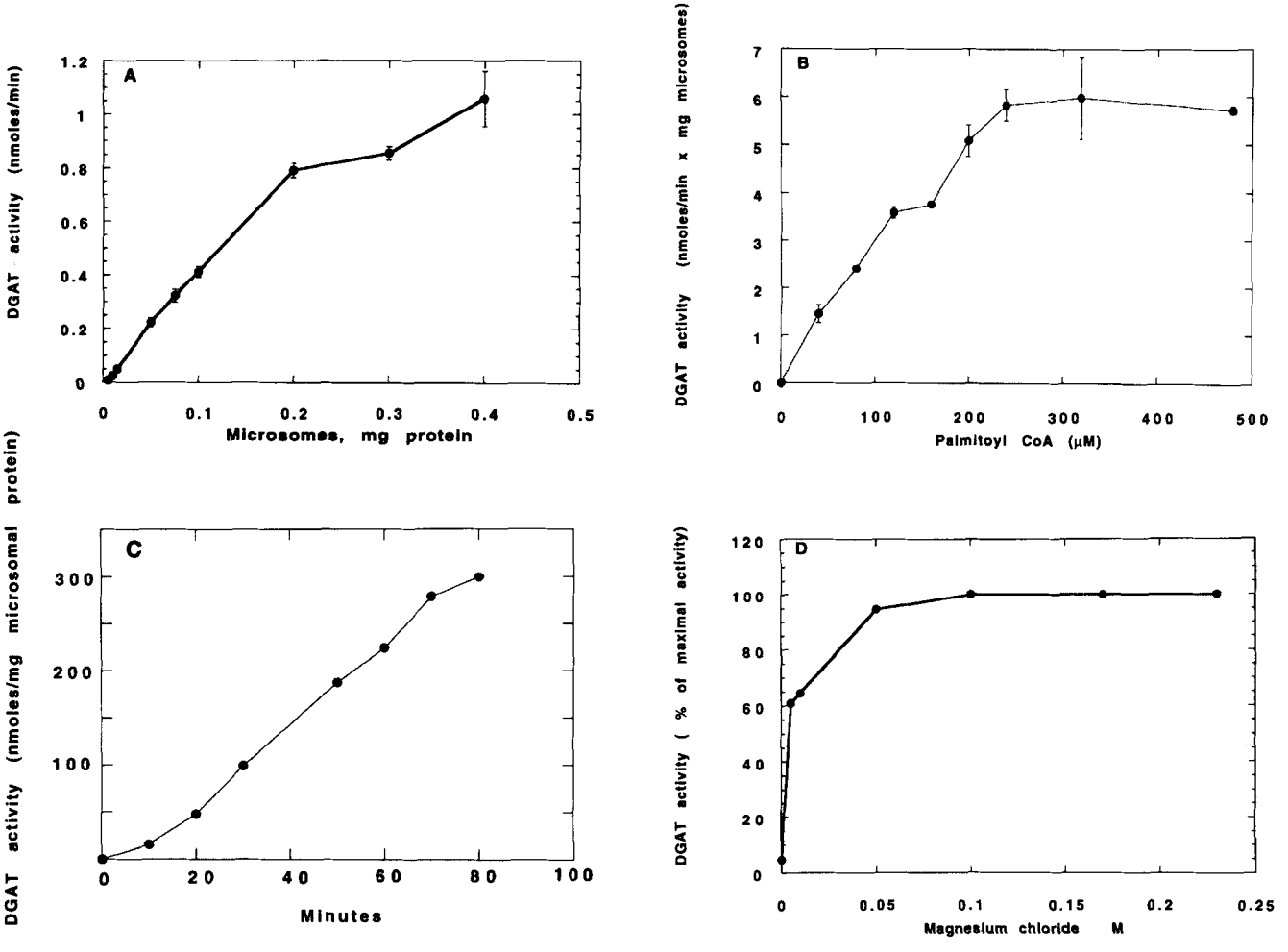

Fig. 1. A: The relation between the amount of microsomal protein and the DGAT activity (nmole formed triacylglycerol/min). Results are given as mean ± SD, n = 5. B: The relation between the DGAT activity (nmole formed triacylglycerol/min and mg protein) and the concentration of palmitoyl CoA in the assay. Results are given as mean ± SD, n = 5. C: The relation between the DGAT activity (nmole formed triacylglycerol/mg microsomal protein) and the length of the incubation. Results are given as mean of three experiments. D: The relation between the DGAT activity (given as % of maximal activity) and the concentration of magnesium chloride in the assay. Results are given as mean of three experiments.

tween triacylglycerol (R_f value: 0.78) and diacylglycerol (R_f value: 0.43), free fatty acids (R_f value: 0.47) and acyl-CoA (which remained on the application point). The spot corresponding to triacylglycerol was identified after staining in iodine vapor, scraped off, extracted in 1 ml chloroform, and counted in Ready Safe in a Beckman LS 6000 TA Liquid Scintillator. Correction for quenching was made.

The assay was totally dependent on microsomes or solubilized DGAT and there was a linear relation between the amount of triacylglycerol formed and the mass of microsomal protein between 0.05 mg and 0.2 mg (**Fig. 1A**).

The assay was dependent on both acyl-CoA (Fig. 1B) and diacylglycerol. When microsomes were used as the enzyme source, we could estimate that less than 2% of the activity was not dependent on exogenous diacylglycerol. The activity determined in isolated microsomes varied between 2 and 6 nmol/min and mg microsomal protein which was within the range reported by other authors (9).

Time course showed a linearity between 20 and 70 min (Fig. 1C) whereas the curve showed a tendency to level off after 70 min incubation. We routinely used a 60-min incubation.

The reaction was totally dependent on $MgCl_2$ and a reaction maximum was reached at a concentration of 50 mM (Fig. 1D). We have chosen to use a higher concentration, i.e., 150 mM.

The within assay variation was 6% (n = 28).

To determine the hydrolysis of acyl-CoA, the samples were incubated with 0.2 mM palmitoyl CoA together with 0.04 μCi [1-^{14}C]palmitoyl CoA, in 10 mM Tris-HCl, pH 8.0, with 150 mM $MgCl_2$ and 1 mg/ml BSA for 60 min. Palmitic acid was added as carrier and the lipids were extracted and analyzed by TLC as described above. The spot corresponding to palmitic acid was scraped off and the radioactivity was counted as described above. Correction for quenching was made.

Chromatography on Superose 6

The Superose 6 column (Pharmacia HR 16/50) was equilibrated with 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1.25 mM EDTA, 1.6 mg/ml DOC, and 1 μM pepstatin A. One ml of the filtered detergent-treated microsomes was applied to the column and the chromatography was carried out at a flow rate of 1 ml/min using an LKB 2150 HPLC pump (Pharmacia Uppsala, Sweden). The absorbance at 280 nm was measured in an LKB 2158 UVICORD SD (Pharmacia Uppsala, Sweden) and fractions of 1 ml were collected. The chromatography was carried out at room temperature but the fractions containing the DGAT activity were rapidly combined and placed on ice.

Q Sepharose and immunoaffinity chromatography

The DGAT-containing fractions from the Superose 6 chromatography were combined and dithiothreitol (DTT) was added to a final concentration of 2 mM. This was essential for the stabilization of the activity during the following steps in the isolation procedure. The combined fractions were chromatographed on a 2-ml column of Q Sepharose equilibrated with 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1.25 mM EDTA, 1.6 mg/ml DOC, 5 mg/ml CHAPS, 2 mM DTT, and 1 μM pepstatin A (this buffer is referred to as buffer A in the following). The unretained material (eluted with the same buffer) was collected on ice.

The DGAT-containing fraction from the Q Sepharose was chromatographed on an immunoadsorber based on a monoclonal antibody (see below). The antibody was purified from the hybridoma culture medium by chromatography on Chemibond® and immobilized on Sepharose (see below). The adsorber (volume 2–3 ml) was poured on top of a Sephadex G 25 column (volume 3 ml) and equilibrated with buffer A. The unretained material was eluted with 10 column volumes of this buffer. To desorp the retained proteins we applied 0.5 ml of 3 M sodium thiocyanate in buffer A to the column. The column was then eluted with buffer A. Fractions of 0.15–0.3 ml were collected on ice and analyzed for the presence of DGAT activity. The analysis was carried out within an hour.

Chromatography on Protein G Sepharose and Chemibond®

The DGAT-containing fractions were chromatographed on a 0.6-ml column of Protein G Sepharose or Chemibond® equilibrated with buffer A. The unretained material was collected in 0.3-ml fractions on ice.

Immunoglobulins from the hybridoma supernatant were isolated by chromatography on Chemibond® using the protocol recommended by the manufacturer, i.e., 50 ml of hybridoma supernatant was applied to a 1-ml column of Chemibond® equilibrated with phosphate-buffered saline (PBS) pH 7.4. The column was washed with PBS until all the phenol red dye was removed from the column (50–100 ml). The bound antibodies were eluted with 0.05 M sodium acetate, pH 2.8, precipitated with ammonium sulfate and resolubilized in 0.1 M $NaHCO_3$, pH 8.3, and 0.5 M NaCl.

Isolation of monoclonal antibodies

The base for the strategy to isolate a monoclonal antibody to DGAT was the observation from radiation inactivation studies done by other investigators (14) that DGAT had a molecular mass of about 70 kDa. The aim was, therefore, to isolate a fraction enriched in microsomal proteins about 70 kDa and to use that fraction for immunization and primary screening of the obtained hybridomas. Several methods have been tried but the following turned out to be useful.

The rat liver microsomes were diluted with 3 mM imidazole, pH 7.4, containing 125 mM sucrose to a final

concentration of 0.6 mg/ml (measured as protein). In general, we started each isolation with 1200 mg microsomes. Sodium carbonate (1 M) was added to a final concentration of 0.1 M and a pH of 10.3, and the solution was incubated on ice for 30 min with intermittent stirring. This was followed by centrifugation for 60 min in a Beckman J-21 centrifuge at 10,000 rpm in a JA-14 rotor at +4°C.

The supernatant (2400 ml) was directly loaded onto a 170 ml Q Sepharose column (5 × 8 cm) equilibrated with 50 mM Tris, pH 10.3, with 300 mM sucrose, 5 mM $MgCl_2$, 1.25 mM EDTA, 1 mM PMSF, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin (this buffer will be referred to as buffer B in the following) and chromatographed at a flow rate of 60 ml/h. The unretained fraction was collected and the column was washed with two volumes of buffer B. The chromatography was carried out at +4°C.

The unretained fraction was lyophilized and resolubilized in approximately 500 ml water containing 1 mM PMSF, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin. The sucrose concentration of the resolubilized sample was kept between 40 and 60% w/v.

The solubilized material was homogenized by 15 strokes in a Dounce homogenizer with a tight-fitting glass pestle, and filtered through a series of glass filters with decreasing pore size (Jena Glas G2, G3, and G4), followed by two passages through a 3-μm Millipore filter and finally one passage through a 0.8-μm filter.

The filtered solution was chromatographed on a 5 × 110 cm column of Sephacryl S-200 HR, equilibrated with buffer B. The chromatography was carried out at +4°C at a flow rate of 40–60 ml per h and the absorbance at 280 nm was constantly monitored by a UVICORD S (Pharmacia LKB Uppsala, Sweden). Based on the obtained chromatogram (**Fig. 2A**), fractions of 50–100 ml were collected, dialyzed against water, and lyophilized. The fractions were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS. In this way we obtained a fraction that was enriched in proteins with a molecular mass between 50 and 80 kDa (Fig. 2B). This fraction was used for immunization of Balb C mice.

A primary injection was followed by a booster injection after 5, 7, and 9 weeks. Spleen cells were hybridized with Sp/2/O-Ag-14 cells and cultured in the presence of mouse peritoneal macrophages as feeder cells.

The clones were tested with ELISA (see below) against the fractions used for immunization. Positive clones were recloned by the method of single-cell cloning (6). The primary screening of the clones, obtained after recloning, was carried out with ELISA against the fraction used for immunization as well as against the DGAT-containing fraction from the Superose 6 column (see Results). Positive clones were analyzed with Western blots to identify clones that reacted with a protein with a molecular mass of 60–80 kDa, i.e., similar to that determined for DGAT by radiation inactivation studies (14). Such clones were

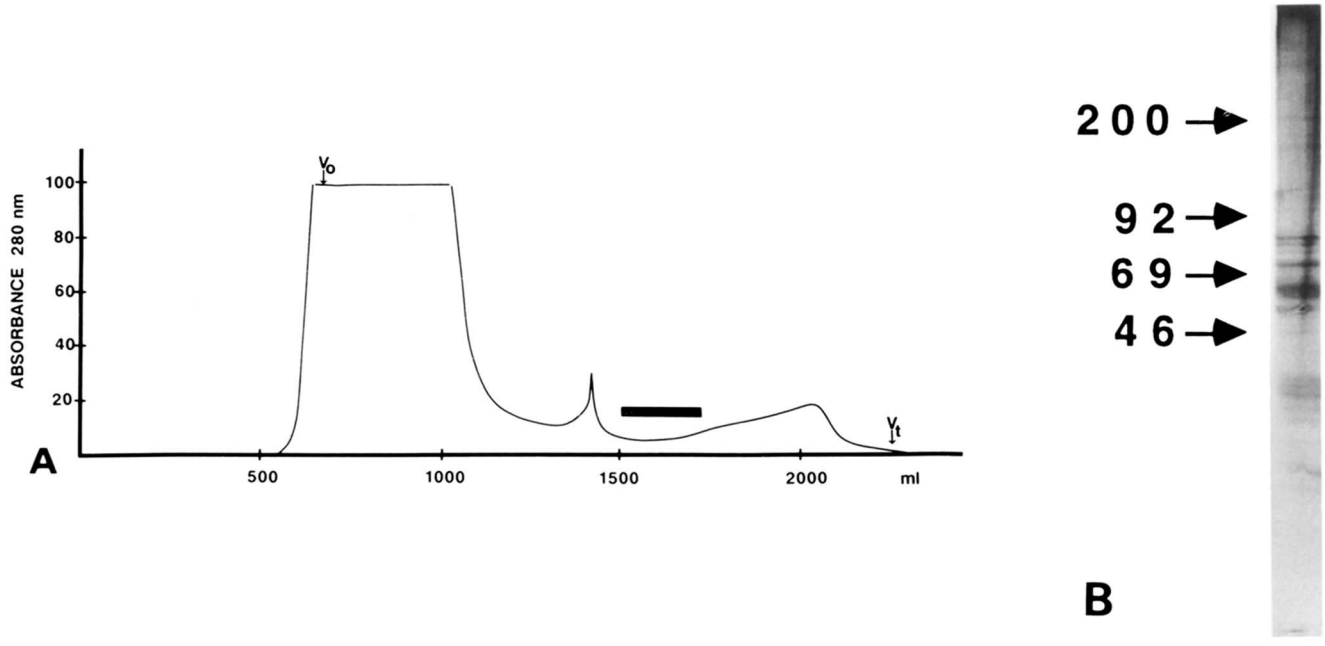

Fig. 2. A: Chromatography of the unretained fraction from the ion exchange (Q Sepharose) chromatography on Sephacryl S-200 HR equilibrated with 50 mM Tris, pH 10.3, with 300 mM sucrose, 5 mM $MgCl_2$, 1.25 mM EDTA, 1 mM PMSF, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin. The chromatography was carried out at +4°C with a flow rate of 60 ml/h. The absorbance at 280 nm was measured continuously; V_0, void volume; V_t, total volume. The fraction that was used for immunization is indicated with a bar in the chromatogram. B: Electrophoresis in 4–20% polyacrylamide gradient gels containing SDS of the fraction from the Sephacryl S-200 HR chromatography that was used for immunization of Balb C mice. (The fraction is indicated with a bar in Fig. 2A.) Arrows indicate the migration of the 200, 92, 69, and 46 kDa standards. The gel is silver stained.

tested for their ability to inactivate DGAT and, after immobilization, for their ability to retain the DGAT activity.

In order to prepare immunoadsorbers, the immunoglobulin (Ig) fraction was recovered from the culture medium by chromatography on Chemibond® followed by precipitation with ammonium sulfate (see above). The Ig fraction was solubilized in 0.1 M $NaHCO_3$, pH 8.3, and 0.5 M NaCl and coupled to CNBr-activated Sepharose 4B as recommended by the manufacturer (Pharmacia Uppsala, Sweden).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was carried out on microtiter plates (PVC M 24). The wells were coated with either the fraction from the Sephacryl S-200 HR chromatography or the DGAT-containing fraction from the Superose 6 chromatography.

The fraction from the Sephacryl S-200 HR column was dialyzed against a 50 mM sodium carbonate buffer, pH 9.6, with 3 mM sodium azide before it was used to coat the wells, while the DGAT-containing fraction from the Superose 6 chromatography was desalted on a PD 10 column, equilibrated with the same buffer, before the fraction was used for the coating. The coating was carried out overnight at +4°C, and was followed by three washes with PBS containing 0.05% Tween 20 (PBS-T). Residual binding sites in the wells were blocked by incubation with PBS containing 3% BSA for 30 min at room temperature.

Incubation with the hybridoma supernatant was carried out for 1 h at 37°C followed by three washes with PBS-T. To detect the bound antibodies, we used a urease-conjugated sheep anti-mouse Ig fraction in PBS-T containing 0.25% BSA. The incubation was carried out for 1 h at 37°C and was followed by six washes with PBS-T and three washes with water. After development, the microtiter plates were read at 570 nm in a microplate reader model 450 (Bio-Rad).

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out either in 4–20% gradient gels (using Mini-PROTEAN II Ready Gels, Bio-Rad), that had been pre-run for 30 min or in 3–15% gradient gels. To avoid degradation during dialysis, lyophilization, or concentration, we analyzed the material as it was eluted from the immunoadsorber. SDS (final concentration of 2.3%) and DTT (final concentration 75 mM) were added to the sample and it was boiled for 5 min. The electrophoresis was carried out at 20 mA/gel with the equipment placed in an ice-bath. The gels were stained with silver, using the Silver Stain Plus Kit, as recommended by the manufacturer (Bio-Rad). Rainbow molecular weight markers were used to standardize the gels. This kit contains prestained myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa).

Immunoblots were carried out on a Multiphore II (Pharmacia LKB Uppsala, Sweden) as recommended by the manufacturer. The blots were blocked with 5% non-fat dry milk in 20 mM Tris-HCl, pH 7.6, containing 137 mM NaCl and 0.1% Tween 20 (TBS-T) overnight. This was followed by a 1-h incubation with the antibody in 20 mM Tris-HCl, pH 7.6, containing 137 mM NaCl (TBS) with 5% non-fat dry milk. The blots were washed with TBS-T for 2 × 1 and 3 × 5 min. To detect the bound antibody we used a biotinylated antibody to mice Ig followed by streptavidin-conjugated alkaline phosphatase, using the Amersham blotting detection kit for mice (i.e., monoclonal) antibodies. After the incubation with the second antibody, the filter was washed with TBS-T for 2 × 1, 2 × 15, and 1 × 5 min. The incubation with the alkaline phosphatase was followed by washes with TBS-T for 2 × 1 and 3 × 5 min. To control for unspecific binding, we used an irrelevant monoclonal antibody, i.e., a monoclonal antibody obtained from Balb C mice that had not been immunized with rat proteins.

Protein determination

The protein content of microsomes, solubilized microsomes, and fractions from the Superose 6 column was determined by the BCA method, using BSA as standard. Both DOC and CHAPS influence this method, but this influence could be overcome by diluting the sample 200-fold with 0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl, and 1% Triton X-100. To estimate the protein content in the immunoaffinity-purified enzyme, we scanned the silver-stained SDS-polyacrylamide gels. The electrophoresis and silver staining were carried out as described above. As standards we used a dilution series of BSA run in duplicate at the same time as the samples. The gels were scanned at 500 nm with a dual-wavelength flying-spot Shimadzu CS-9000 scanner equipped with a Shimadzu DR-13 computer unit. A linear relation between the absorbance and the amount of protein was obtained between 0.025 and 0.1 μg BSA. All samples were within this linear part of the curve.

RESULTS

Solubilization and gel chromatography of diacylglycerol:acyltransferase (DGAT)

The solubilization procedure started with an extraction of luminal and loosely bound proteins from the microsomes by sodium deoxycholate (DOC) below the critical micellar concentration (CMC). During this extraction 3% of the DGAT activity and all of the acyl-CoA hydrolase activity were recovered in the supernatant after

TABLE 1. Recovery of protein and DGAT activity during purification of the enzyme

	Recovery DGAT Total Activity In Step	Recovery Protein	DGAT Activity	Purification
	%	%	*nmol/min × mg protein*	*fold*
Microsomes	100	100	2	1
Solubilized microsomes	46	46	21	5	2.5
Solubilized microsomes after filtration	37	83	20	5	2.5
Superose 6 chromatography	17	45	5	7	3.5
Q Sepharose and immunoaffinity chromatography	4	22[a]	0.0045	830	415

Results are mean of three different experiments.
[a]The recovery of the applied activity in the unretained fraction from the Q Sepharose was 45% and in the retained fraction from the immunoaffinity column was 49%.

ultracentrifugation. Thus no hydrolase activity could be detected in the membrane pellet or in any of the fractions recovered from gel chromatography (described below).

The membrane pellet was sonicated in DOC and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) at concentrations above the CMC, and filtered through a 0.22-μm filter. Thirty seven percent (**Table 1**) of the initial activity present in the total microsome was recovered in the filtrate. The filtrate was chromatographed on a Superose 6 column, from which the DGAT activity was eluted in a high molecular weight peak (**Fig. 3**). Forty five percent (Table 1) of the applied activity was recovered during this chromatography; this corresponded to a recovery of 17% of the total activity present in the microsomes.

The DGAT-containing fraction from the Superose 6 column chromatography was heterogeneous as judged from its appearance on polyacrylamide gel electrophoresis (Fig. 4, lane 2).

Isolation of a monoclonal antibody that could be used for immunoadsorbtion of DGAT

As a result of three different hybridizations, we found one clone that fulfilled the criteria that were set up. Thus this clone reacted on ELISA with the fraction used for immunization as well as with the DGAT-containing fraction from the Superose 6 chromatography (**Fig. 5**). Moreover, it reacted with a 60 kDa protein on Western blot of solubilized microsomal proteins (**Fig. 6A**) as well as with the DGAT-containing fraction recovered from the Superose 6 column chromatography (Fig. 6B).

The monoclonal antibody did not react with rat serum or rat albumin (not shown).

The obtained clone produced an antibody of the IgG3 class. As the antibody did not inhibit the DGAT reaction (not shown) we used immunoaffinity chromatography in order to investigate its relation to the DGAT activity. The DGAT-containing fraction from the Superose 6 column was chromatographed on a 2-ml immunoaffinity column

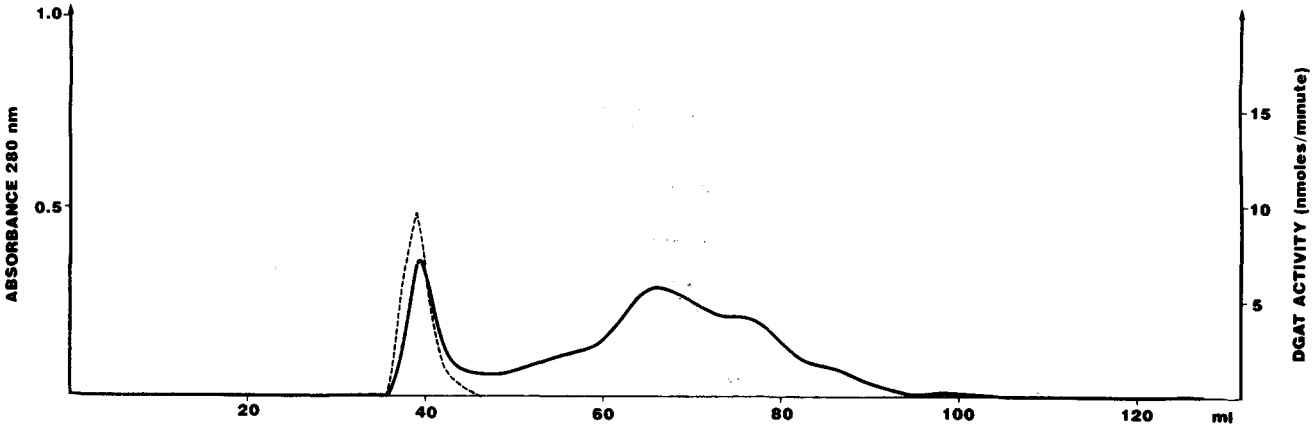

Fig. 3. Gel chromatography of detergent-treated microsomes on Superose 6. One ml of the filtered detergent-treated microsomes was applied to a 1.6 × 45 cm column in 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1.25 mM EDTA, 1.6 mg/ml sodium deoxycholate, and 1 μM pepstatin A. The column was eluted at a rate of 1 ml/min. The effluent was constantly monitored for the absorbancy at 280 nm (smooth line) and fractions of 1 ml were collected and analyzed for DGAT activity (nmole formed triacylglycerol/min, dashed line).

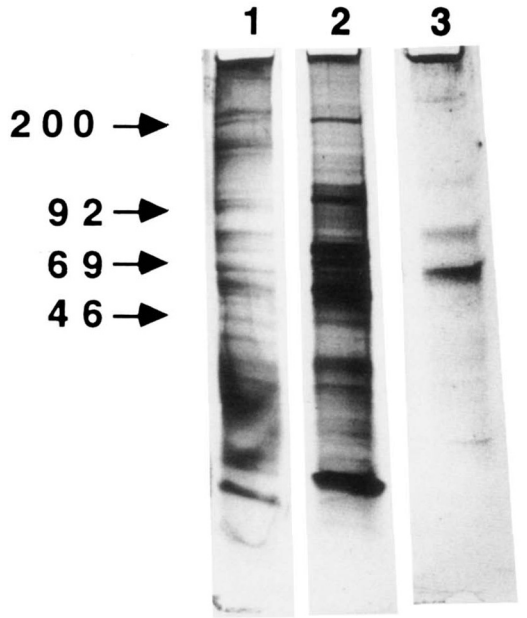

Fig. 4. Electrophoresis in 4–20% polyacrylamide gradient gels containing SDS of total rat liver microsomes (lane 1); the DGAT-containing fraction from the Superose 6 column (lane 2); and the retained (DGAT-containing) fraction from the immunoaffinity chromatography (lane 3). The arrows mark the migration of the 200, 92, 69, and 46 kDa standards.

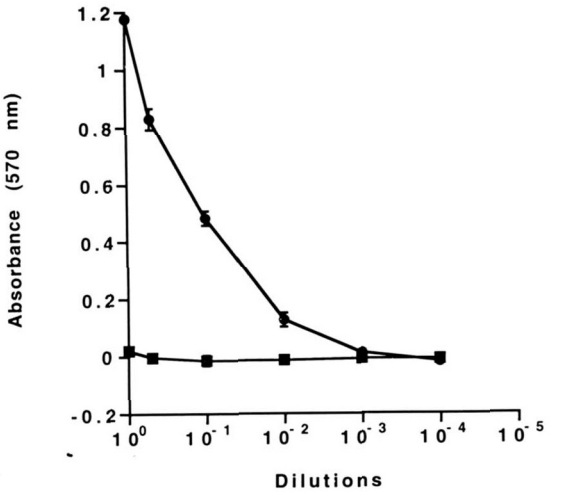

Fig. 5. The reaction between the DGAT-containing fraction from the Superose 6 chromatography and different dilutions of the hybridoma supernatant (●) as well as an irrelevant antibody (■). The reaction was investigated with an ELISA system in which the wells were coated with the DGAT fraction from the Superose 6 chromatography. The bound antibody was detected with a goat anti-mouse antibody coupled to urease. Results are given as mean ± SD, n = 5.

(a total activity of 1 nmol formed triacylglycerol/min was applied to each column). 93 ± 6% (mean ± SD, n = 4) of the applied activity was retained by the immunoadsorber.

The desorption of the enzyme was carried out with sodium thiocyanate which, however, inhibited the activity (not shown). In order to limit this inhibition, we poured the immunoadsorber on top of a small column of Sephadex G 25; thus the desorbed enzyme was immediately separated from the bulk of the sodium thiocyanate. Using this method we could recover 44 ± 6% (mean ± SD, n = 5) of the applied DGAT activity from the immunoaffinity column after the desorption.

The results indicated that the monoclonal antibody could be used to isolate the enzyme by immunoaffinity chromatography.

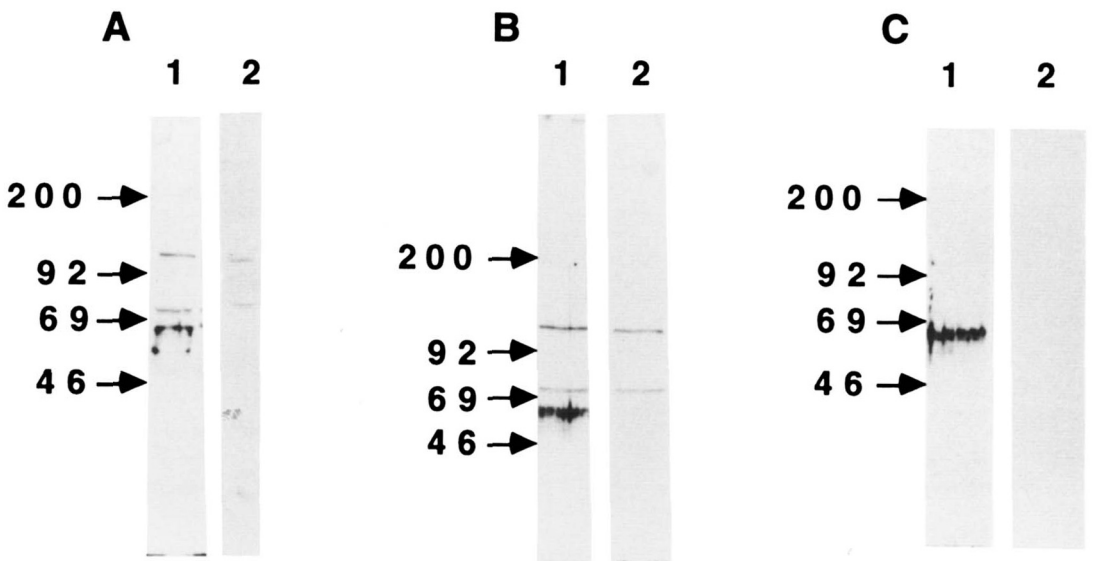

Fig. 6. Immunoblot of rat liver microsomes (0.23 mg/slot) (A), the DGAT-containing fraction from the Superose 6 chromatography (0.05 mg/slot) (B), and immunopurified DGAT (C). The blots were probed with the monoclonal antibody to DGAT (lane 1) or an irrelevant monoclonal antibody (lane 2). The electrophoresis was carried out in 3–15% polyacrylamide gradient gels containing SDS. The bound antibodies were detected by a biotinylated sheep anti-mouse antibody followed by incubation with streptavidin-conjugated alkaline phosphatase. The arrows mark the migration of the 200, 97, 69, and 46 kDa standards.

Q Sepharose and immunoaffinity chromatography of the DGAT-containing fraction from the gel chromatography

The DGAT-containing fraction from the gel chromatography was first chromatographed on a Q Sepharose from which 74 ± 14% (mean ± SD, n = 5) of the recovered activity was eluted in an unretained fraction. Forty five percent of the applied activity was recovered in this unretained fraction.

The unretained fraction from the Q Sepharose chromatography was subjected to immunoaffinity chromatography on the obtained monoclonal antibody and the retained fraction containing the DGAT activity was analyzed by electrophoresis in polyacrylamide gradient gels (PAG) containing SDS. The gels showed, after silver staining, a marked enrichment in a protein with a molecular mass of 60 kDa as well as a 77 kDa protein (Fig. 4, lane 3). These proteins were only present in the fractions that contained the DGAT activity. In addition to these proteins we could detect trace amounts of proteins with molecular masses of 200 and 120 kDa. A pre-run of the fraction on an adsorber, containing an irrelevant antibody before the immunoaffinity chromatography, did not change the electrophoresis pattern (not shown). We also chromatographed the retained fraction from the immunoaffinity chromatography on a Protein G Sepharose column and on a Chemibond® column to remove immunoglobulins that might have leaked from the adsorber. This did not influence the electrophoresis pattern (not shown).

The 60 and 77 kDa bands were also present after rechromatography on the immunoaffinity column (not shown).

Immunoblot showed that the monoclonal antibody reacted with the 60 kDa proteins but not with the 77 kDa protein (Fig. 6C). As discussed above, the antibody also reacted with a 60 kDa protein on Western blot of both solubilized microsomes and of the DGAT-containing fraction recovered from the Superose 6 column (Fig. 6, A and B). In addition to this reaction, there were proteins present in the solubilized microsomes as well as in the DGAT-containing fraction from the Superose 6 chromatography that reacted unspecifically with immunoglobulins or were lit up by the second antibody (i.e., the sheep anti-mice Ig). These proteins were not present after the immunoaffinity purification (Fig. 6C) and their nature has not been investigated further.

DISCUSSION

We have developed a method to purify diacylglycerol: acyltransferase (DGAT) from rat liver microsomes to near homogeneity. The method was based on the solubilization of the DGAT activity from the microsomes by sonication in sodium deoxycholate (DOC) and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) followed by gel chromatography and immunoaffinity chromatography, utilizing a monoclonal antibody.

Radiation inactivation studies (14) indicate that DGAT has a molecular mass of 72 ± 4 kDa; we therefore developed a method to isolate a fraction enriched in microsomal proteins with molecular masses between 50 and 80 kDa and raised monoclonal antibodies to this fraction. One antibody was obtained that reacted with a 60 kDa protein within the microsomal proteins as well as in DGAT-containing fractions from gel chromatography. The antibody did not inhibit DGAT but could, when used in immunoaffinity experiments, quantitatively remove the DGAT activity from a solution. Moreover, 44% of the applied activity could be recovered after desorption of the enzyme from the immunoaffinity column. The discrepancy between the adsorbed and recovered DGAT activity is not unexpected and could most likely be explained by: *i)* a denaturation of the enzyme during the adsorption–desorption procedure; *ii)* an inhibition of the enzyme activity by the sodium thiocyanate used for desorption (although attempts were made to rapidly separate the enzyme from the sodium thiocyanate, it is most likely that this separation is incomplete and that the thiocyanate contaminates the last portion of the enzyme peak) or *iii)* perhaps a failure to desorb all enzyme.

The purification method gave rise to a 415-fold increase in the specific activity. It should be pointed out that it is possible that this is an underestimation because, as discussed above, the enzyme may to some degree be inactivated during the immunoaffinity chromatography.

The antibody recognized a protein with an estimated molecular mass of 60 kDa on Western blot of microsomal proteins. A protein of the same size was also highly enriched in the retained fraction from the immunoaffinity chromatography that contained the enzyme activity. In addition, this fraction contained a 77 kDa protein. The relation between these two proteins is not clear. The observation that the epitope for the antibody resides on the 60 kDa protein and that the two proteins are desorbed together from the immunoaffinity column, even after washes of the column with detergent-containing buffers prior to the desorption, indicates a strong interaction between the two components.

The possibility that the two proteins are generated by proteolysis should be considered. The observation that the monoclonal epitope is confined to the 60 kDa protein argues against the possibility that this protein is generated from the 77 kDa protein by proteolysis. It is, however, possible that the 60 and 77 kDa proteins are generated by proteolysis of a larger protein. Thus, for example, a hinge region between two domains may provide a site for such a proteolysis. However, one would have anticipated that at least a portion of the tentative full-length protein could be detected by the antibody during the Western blot analysis.

It is possible that the 77 kDa protein represents a unrelated contaminant. If this is the case our results suggest that the 77 kDa protein is either derived from the immunoadsorber or is a protein that has a high unspecific affinity for immunoglobulins. Against these possibilities argue the results from the immunoblotting experiment (Fig. 6C) which indicated that the protein neither reacted with the sheep anti-mice Ig (used as the second antibody) nor with unspecific immunoglobulins; moreover, neither chromatography on an adsorber with an irrelevant antibody nor chromatography on protein G and/or Chemibond® removed the 77 kDa protein.

A final possibility that has to be considered is that DGAT has a subunit structure. In fact, the failure of the monoclonal antibody to inhibit the enzyme activity prevents us from concluding that the active site is present on the 60 kDa protein. However, a protein with a molecular mass close to 140 kDa would not be supported by the results from the radiation inactivation studies (14), while both molecular masses of 60 kDa and 77 kDa would be compatible with these observations.

A subunit structure has been suggested for the enzyme that was isolated from soybean cotyledons (10), however none of the identified subunits of this enzyme appear to correspond to the proteins reported in this paper. Moreover, the enzyme isolated from the liver microsomes appeared to have much (near 1000-fold) higher specific activity than the enzyme isolated from the cotyledons.

The DGAT activity is eluted in a high molecular weight fraction from the Superose 6 column. Even if the enzyme consists of two subunits with a combined molecular mass of near 140 kDa, this could not explain its appearance on gel chromatography, suggesting that the enzyme occurs in a high molecular weight complex when extracted from the microsomes. Indeed, results from studies in rat intestine indicate that the whole triacylglycerol synthetase complex could be solubilized in the presence of taurocholate (10).

It has recently been demonstrated that the microsomes of rat intestine contain an enzyme that forms triacylglycerol by transacylation (15). This transacylase was not dependent on acyl-CoA, and it differed in molecular weight from the enzyme isolated in this study.

Rat liver contains an acyl-CoA hydrolase activity that generates fatty acids from acyl-CoA. The thin-layer chromatography system used in this study does separate fatty acids from triacylglycerol. Moreover, the acyl-CoA hydrolase appeared to be completely removed during the first extraction of the microsomes as we could not detect the activity in the membrane pellet or in the fractions from the gel column.

In conclusion, the results presented in this paper suggest that DGAT consists of a 60 kDa protein and perhaps also a 77 kDa protein. Further studies including cloning and expression of the enzyme are needed to unequivocally clarify the relation between these proteins.

The excellent technical assistance of Margareta Evaldsson is greatly acknowledged. We would like to thank Dr. Eva Sjögren-Jansson for expert advice and help during the preparation of the monoclonal antibody. This work was supported by grants 7142 and 8862 from the Swedish Medical Research Council, the Swedish National Association against Heart and Lung Disease, The Oleo-Margarine Foundation of Nutritional Research, King Gustav V's Foundation, The Swedish National Board for Technical Development, The Insulin Foundation, the Göteborg Medical Society, Torsten and Ragnar Söderbergs Foundation, Ulf Widenberg Foundation, and British Biotechnology Limited.

Manuscript received 31 July 1992 and in revised form 20 August 1993.

REFERENCES

1. Bell, R. M., L. M. Ballas, and R. A. Coleman. 1981. Lipid topogenesis. *J. Lipid Res.* **22:** 391–403.
2. Bell, R. M. 1980. Enzymes of glycerolipid synthesis in eukaryotes. *Annu. Rev. Biochem.* **49:** 459–487.
3. Mayorek, N., I. Grinstein, and J. Bar-Tana. 1989. Triacylglycerol synthesis in cultured rat hepatocytes. The rate-limiting role of diacylglycerol acyltransferase. *Eur. J. Biochem.* **182:** 395–400.
4. Brindley, D. N. 1984. Intracellular translocation of phosphatidate phosphohydrolase and its possible role in the control of glycerolipid synthesis. *Prog. Lipid Res.* **23:** 115–133.
5. Boström, K., J. Borén, M. Wettesten, A. Sjöberg, G. Bondjers, O. Wiklund, P. Carlsson, and S-O. Olofsson. 1988. Studies on the assembly of apoB-100-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* **263:** 4434–4442.
6. Pullinger, C. R., J. D. North, B. B. Teng, V. A. Rifici, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* **30:** 1065–1077.
7. Borén, J., M. Wettesten, A. Sjöberg, T. Thorlin, G. Bondjers, O. Wiklund, and S-O. Olofsson. 1990. The assembly and secretion of apoB-100-containing lipoproteins in HepG2 cells. Evidence for different sites for protein synthesis and lipoprotein assembly. *J. Biol. Chem.* **265:** 10556–10564.
8. Rustan, A. C., J. O. Nossen, E. N. Christiansen, and C. A. Drevon. 1988. Eicosapentaenoic acid reduces hepatic synthesis and secretion of triacylglycerol by decreasing the activity of acyl-coenzyme A:1,2-diacylglycerol acyltransferase. *J. Lipid Res.* **29:** 1417–1426.
9. Polokoff, M. A., and R. M. Bell. 1980. Solubilization, partial purification and characterization of rat liver microsomal diacylglycerol acyltransferase. *Biochim. Biophys. Acta.* **618:** 129–142.
10. Manganaro, F., and A. Kuksis. 1985. Rapid isolation of triacylglycerol synthetase complex from rat intestinal mucosa. *Can. J. Biochem. Cell Biol.* **63:** 107–114.
11. Kwanyuen, P., and R. F. Wilson. 1986. Isolation and purification of diacylglycerol acyltransferase from germinating soybean cotyledons. *Biochim. Biophys. Acta.* **877:** 238–245.
12. Morimoto, T., M. Arpin, and S. Gaetani. 1983. Use of proteases for the study of membrane insertion. *Methods Enzymol.* **96:** 121–150.

13. Olegård, R., and L. Svennerholm. 1970. Fatty acid composition of plasma and red cell phosphoglycerides in full term infants and their mothers. *Acta Paediatr. Scand.* **59:** 637–647.
14. Ozasa, S., E. S. Kempner, and S. K. Erickson. 1989. Functional size of acyl-coenzyme A:diacylglycerol acyltransferase by radiation inactivation. *J. Lipid Res.* **30:** 1759–1762.
15. Lehner, R., and A. Kuksis. 1993. Triacylglycerol synthesis by an *sn*-1,2(2,3)-diacylglycerol transacylase from rat intestinal microsomes. *J. Biol. Chem.* **268:** 8781–8786.