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Regulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase mRNA Levels by L-Triiodothyronine†

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ABSTRACT: In hypophysectomized rats, hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, immunoreactive 97-kilodalton (97-kDa) protein, and mRNA were all reduced to undetectable levels. Administration of triiodothyronine (T_3) resulted in large increases in all three after a 36-h lag period. HMG-CoA reductase activity, immunoreactive 97-kDa protein levels, and reductase mRNA levels were tightly correlated. Feeding hypophysectomized rats diets containing the bile acid sequesterant colestipol, together with the potent reductase inhibitor mevinolin, resulted in an increase in HMG-CoA reductase activity similar to that seen with T_3 but a lesser stimulation of reductase mRNA levels. These results suggest that agents which cause depletion of mevalonate-derived products may share in part with T_3 a common mechanism for increasing levels of HMG-CoA reductase activity in order to satisfy cellular needs for these products. Dexamethasone treatment, which is known to prevent the T_3-mediated stimulation of reductase activity, caused a marked decrease in 97-kDa immunoreactive material but had little effect on reductase mRNA levels.

Hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] reductase, the enzyme which catalyzes the rate-limiting reaction of cholesterol biosynthesis, plays a key role in maintaining cholesterol homeostasis (Spady et al., 1985). Both rapid and large changes in reductase activity occur in response to body needs for cholesterol, its products, and other mevalonate-derived metabolites. These changes are effected by both dietary and hormonal signals. Perhaps the best studied regulatory process is the LDL-mediated feedback system. It has been shown that the decrease in reductase activity caused by LDL is due to suppressed transcription of the reductase gene and accelerated degradation of the reductase protein (Faust et al., 1982; Edwards et al., 1983; Luskey et al., 1983; Gil et al., 1985; Chin et al., 1985).

In contrast with regulation by LDL, relatively little is known concerning the mechanisms by which various hormones regulate HMG-CoA reductase activity. With respect to pituitary hormones, it has been shown that hepatic reductase activity is reduced to very low levels in hypophysectomized rats (Ness et al., 1973). Administration of T_3 to such animals increases reductase activity to levels about 3-fold above normal. Administration of hydrocortisone inhibited the T_3-mediated stimulation. Actinomyocin D and cycloheximide also blocked the T_3-mediated increase in reductase activity. This suggested

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; T_3, triiodothyronine; kDa, kilodalton(s); LDL, low-density lipoprotein; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; kb, kilobase(s); EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

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that the T_3 effect might be due to increased RNA and protein synthesis.

In this study, the effects of T_3 and glucocorticoids on reductase mRNA and protein were determined by using a labeled cDNA probe (Chin et al., 1982) and antisera to the homogeneous enzyme (Singer et al., 1984).

Materials and Methods

Animals. Hypophysectomized male Sprague-Dawley rats weighing 150–175 g at the time of surgery were obtained from Altech Laboratories, Madison, WI. They were housed in a reverse-cycle light-controlled room with a 14-h light period followed by a 10-h dark period. The animals were fed Purina Rodent Laboratory Chow 5001 ad libitum. Where indicated, rats were fed ground chow containing 2% colestipol and 0.04% mevinolin for 3 days. Animals were used in experiments 17–24 days after surgery and typically weighed 125 g at this time. The animals ate normally, based on stomach contents, and were alert and active. L-Triiodothyronine was given as a single intraperitoneal injection of 100 μg/100 g of body weight. This dosage is sufficient to saturate nearly 90% of the nuclear T_3 receptors (Jump et al., 1984). Dexamethasone in corn oil was injected subcutaneously at doses of 50 μg/100 g of body weight 24 and 48 h after T_3.

Determination of HMG-CoA Reductase Activity and Immunoreactive Protein. Lysosome-free microsomes were prepared as described previously (Ness et al., 1986). HMG-CoA reductase activity was measured as previously described (Ness et al., 1986) using 10 mM dithiothreitol and 1 mM NADPH in phosphate buffer at pH 7.1. The labeled mevalonolactone was isolated by thin-layer chromatography. Microsomal protein concentrations were determined by a biuret method using bovine serum albumin as the standard (Lee & Lardy, 1965). Immunoreactive reductase protein was estimated by immunoblotting as described elsewhere (Ness et al., 1986).

Isolation of Rat Liver Cytoplasmic Poly(A+) RNA. A 2-g portion of rat liver was homogenized in a Dounce homogenizer with 7.5 mL of 0.25 M sucrose and then centrifuged at 16000*g* for 15 min. The supernatant fraction was combined with an equal volume of 7 M urea, 0.35 M sodium chloride, 50 mM Tris-HCl, pH 7.4, and 1% SDS. Then an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the mixture was shaken for 10 min (the phenol was redistilled, and 8-hydroxyquinoline was added to 0.1% before use). This extraction was repeated until there was no visible protein interface, usually 3 times. The aqueous phase was then extracted with chloroform/isoamyl alcohol (24:1). Total cytoplasmic RNA was precipitated from the aqueous phase by the addition of 2 volumes of ethanol and storage overnight at −20 °C. This method (Berk et al., 1979) is rapid, convenient for processing multiple samples, and quite applicable to liver tissue. The ethanol-precipitated RNA was collected by centrifugation at 12000*g* for 20 min, and poly(A+) RNA was isolated by chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972). The intactness and biological activity of the isolated poly(A+) RNA were examined by in vitro translation after hybrid selection and by Northern blot hybridization. These analyses showed that reductase mRNA directed the synthesis of a polypeptide of 100 kDa and exhibited a size of 4.5–5.0 kb.

Determination of HMG-CoA Reductase mRNA Levels. Poly(A+) RNA was denatured by adding 3 volumes of 6.15 M formaldehyde, 1.5 M sodium chloride, and 0.15 M trisodium citrate and heating at 65 °C for 15 min. Aliquots ranging from 0.1 to 4 μg of RNA were applied to nitrocellulose by using a dot blot apparatus. Alternatively, 1 μg of each RNA sample was applied to nitrocellulose by using a slot blot apparatus. The nitrocellulose was baked at 80 °C for 2 h in a vacuum oven. Prehybridization was carried out overnight at 42 °C in 50% formamide, 0.5% SDS, 200 μg/mL salmon sperm DNA, 0.9 M sodium chloride, 5 mM EDTA, 50 mM sodium phosphate, pH 7.7, 0.1% Ficoll, 0.1% poly(vinylpyrrolidone), and 0.1% BSA. The plasmid pRED-10 was labeled with ^{32}P to specific activities ranging from 3×10^8 to 6×10^8 cpm/μg of DNA by nick translation using [α-^{32}P]-dCTP purchased from New England Nuclear. Hybridization was carried out overnight at 42 °C with the same solution used for prehybridization except that the salmon sperm DNA concentration was decreased to 100 μg/mL and approximately 1×10^7 cpm/mL of the ^{32}P-labeled probe was added. The nitrocellulose was then washed in 0.3 M sodium chloride, 0.03 M trisodium citrate, and 0.1% SDS for 5 min at room temperature and then in fresh solution for 15 min. Washing was continued in 0.015 M sodium chloride, 0.0015 M trisodium citrate, and 0.5% SDS for 2 h at 60 °C. This was followed by two additional washes in this media at 60 °C for 30 min each. These relatively stringent conditions were found to be necessary for reduction of background. Autoradiography was performed at −70 °C using two intensifying screens and Kodak X-Omat AR film. For comparative purposes the slot blots were photographed. For quantitation, dot blots were cut out and counted. Typically, the amount of ^{32}P-labeled pRED-10 hybridized was linear up to 2 μg of RNA. As a control, ^{32}P-labeled pBR322 was hybridized to a parallel series of RNA dots. Typically, less than 10 cpm were hybridized per microgram of RNA.

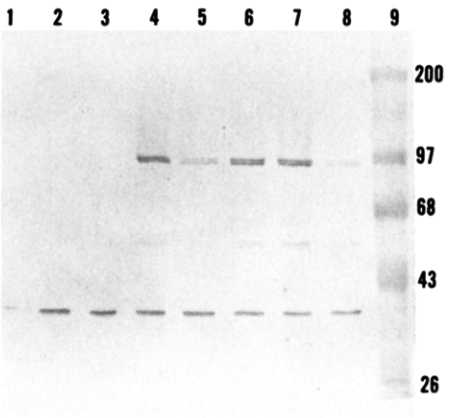

FIGURE 1: Immunoblotting of HMG-CoA reductase protein from T_3-treated hypophysectomized rats. Each lane contained 100 μg of microsomal protein. Prestained protein standards are shown in lane 9. Hours after T_3 administration and corresponding HMG-CoA reductase activities in nanomoles per minute per milligram, respectively, are the following: lane 1, 0 h, 0; lane 2, 24 h, 0; lane 3, 36 h, 0; lane 4, 48 h, 2.0; lane 5, 60 h, 0.6; lane 6, 72 h, 1.0; lane 7, 96 h, 1.3; and lane 8, 120 h, 0.3. Molecular weights ($\times 10^{-3}$) are indicated.

Results

Triiodothyronine. The effect of T_3 treatment on levels of immunoreactive microsomal HMG-CoA reductase in livers of hypophysectomized rats was examined by immunoblotting analysis (Figure 1). After a lag of about 36 h, a band of immunoreactive material appeared corresponding to a molecular mass of about 97 kDa. The relative intensity of this band correlated quite well with HMG-CoA reductase activity. Invariably, an intense band of 35 kDa was seen in all liver microsomes from hypophysectomized rats.[2] This band was not seen in microsomes from normal animals and was essen-

[2] The lack of an intense band in lane 1 of Figure 1 is atypical and may be due to an edge effect.

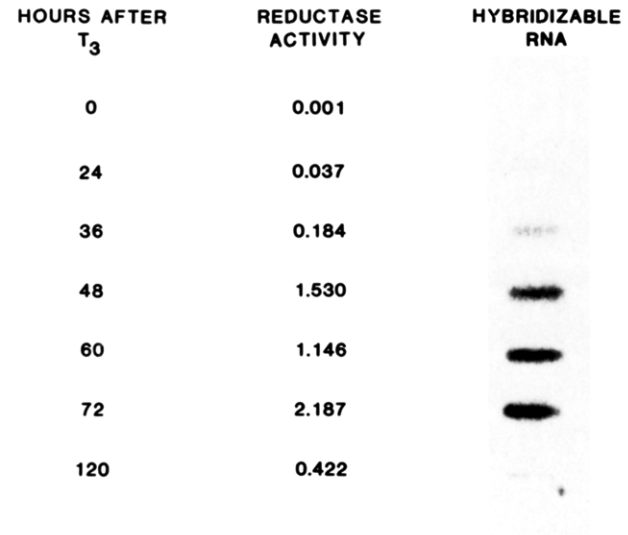

FIGURE 2: Effect of T_3 on HMG-CoA reductase activity and hybridizable mRNA in hypophysectomized rats. Livers were divided for isolation of microsomes and poly(A+) RNA. HMG-CoA reductase activity is expressed as nanomoles per minute per milligram.

Table I: HMG-CoA Reductase Activity and mRNA Levels in Hypophysectomized Rats Treated with T_3 or Fed Mevinolin and Colestipol

animal condn[a]	reductase mRNA (cpm hybridized/μg)[b]	reductase act. (nmol min^{-1} mg^{-1})
H	<10	<0.01
H + T_3	340 ● 40	3.3 ● 1.2
H + M + C	70 ± 20	3.0 ± 1.2
N	220 ± 60	0.6 ± 0.3
N + M + C	1500 ± 400	18 ± 6

[a] Animal conditions: H, hypophysectomized; T_3, 1 μg/g of body weight was given 72 h prior to being killed; M + C, fed diets containing 40 mg of mevinolin and 2 g of colestipol per 100 g of diet for 3 days prior to being killed; N, normal. All rats were killed at the fourth hour of the dark period (diurnal high). [b] The probe used for all RNA samples was pRED-10 labeled with ^{32}P to a specific activity of 4.3×10^8 cpm/μg of DNA. Values are means ± SD with three rats in each group.

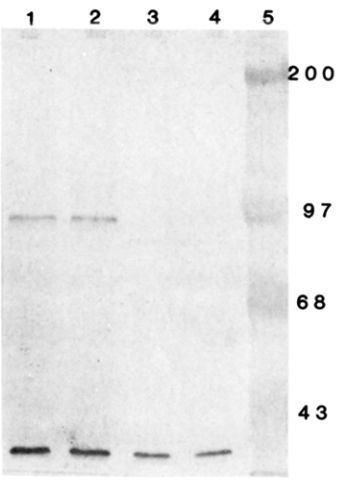

FIGURE 3: Immunoblotting of HMG-CoA reductase protein from hypophysectomized rats treated with T_3 with or without dexamethasone. Each lane contained 50 μg of microsomal protein from rats injected with T_3 with (lanes 3 and 4) or without (lanes 1 and 2) dexamethasone. Administration of T_3 was 72 h before death; dexamethasone was given at 24 and 48 h after T_3. Molecular weights ($\times 10^{-3}$) are indicated.

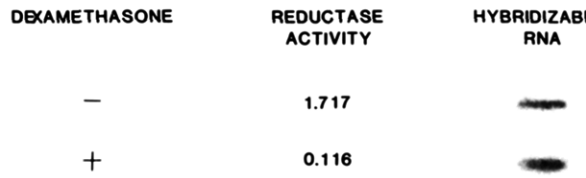

FIGURE 4: Effect of dexamethasone on HMG-CoA reductase activity and hybridizable mRNA in T_3-treated hypophysectomized rats. Rats were injected with T_3 only (−) or with T_3 and dexamethasone (+). Livers were divided for isolation of microsomes and poly(A+) RNA. HMG-CoA reductase activity is expressed as nanomoles per minute per milligram.

tially unaffected by T_3 treatment. The presence of this band provides an explanation for our prior observation that total immunoreactive HMG-CoA reductase protein increased less than enzyme activity in hypophysectomized rats treated with T_3. This band might represent an intermediate in the degradation of HMG-CoA reductase which accumulates in hypophysectomized rats due to an impairment in protein degradation (Pain, 1980).

To determine whether the large T_3-promoted increase in immunoreactive 97-kDa protein is associated with a corresponding rise in mRNA, reductase mRNA levels were determined by hybridization analysis. As shown in Figure 2, the amount of hybridizable RNA follows HMG-CoA reductase activity quite closely. A more quantitative analysis is presented in Table I.

Mevinolin and Colestipol. It was of interest to examine the specificity of the T_3 response in hypophysectomized rats. It has been shown that feeding normal rats diets containing mevinolin and the bile acid sequesterant cholestyramine markedly increases HMG-CoA reductase activity and mRNA levels (Clark et al., 1984). Feeding only the bile acid sequesterant colestipol or only the reductase inhibitor mevinolin to hypophysectomized rats resulted in increases of reductase activity which ranged from 10% to 40% of those seen with T_3. Increasing the concentrations of these drugs as much as 5-fold resulted in further small increases. Mevinolin alone was more effective than colestipol. Feeding the combination of colestipol and mevinolin resulted in reductase activity comparable to that seen in T_3-treated hypophysectomized rats. Hybridizable RNA was not increased to the same extent (Table I). This difference in the fold increase of mRNA compared to reductase activity is also seen in normal animals fed mevinolin and colestipol (Table I). Reductase activity was increased 30-fold while reductase mRNA levels increased 7-fold. This could well be due to a longer half-life for HMG-CoA reductase protein from animals fed mevinolin (Sinensky & Logel, 1983).

Glucocorticoids. Previously, we (Ness et al., 1973) demonstrated that administration of glucocorticoids could prevent the T_3-promoted increase in HMG-CoA reductase in hypophysectomized animals. Thus, it was of interest to examine their effect on reductase protein and mRNA levels. As shown in Figure 3, immunoreactive 97-kDa protein was markedly decreased by administering dexamethasone to T_3-treated hypophysectomized rats. In contrast, HMG-CoA reductase mRNA levels were not significantly affected (Figure 4). A more quantitative analysis of reductase mRNA levels was performed by dot blotting. Dexamethasone treatment typically decreased reductase mRNA levels about 2-fold. In a representative experiment, reductase mRNA was decreased from 114 to 58 cpm hybridized/μg of RNA while reductase activity

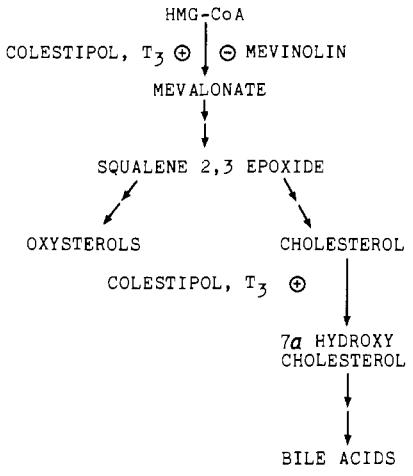

FIGURE 5: Effects of colestipol, mevinolin, and T_3 on cholesterol and bile acid synthesis.

decreased from 0.79 to 0.02 nmol min^{-1} mg^{-1}. These findings suggest that either rates of HMG-CoA reductase synthesis or rates of degradation, or both, are altered by glucocorticoids. To measure these parameters, true solubilization followed by immunoprecipitation of the native form of HMG-CoA reductase is required.

Discussion

The dramatic increase in HMG-CoA reductase activity seen after T_3 treatment of hypophysectomized rats has been shown to be the result of increased reductase mRNA levels leading to increased levels of 97-kDa reductase protein. An altered rate of reductase degradation also contributing to the accumulation of reductase protein cannot be excluded. The effect of T_3 was mimicked at least in part by feeding mevinolin and colestipol, indicating that T_3 is not an absolute requirement. This finding, taken together with the 36-h lag period (Figures 1 and 2), suggests that T_3 may exert its effects in an indirect manner and could share with agents that lower levels of mevalonate-derived products a common pathway for stimulating HMG-CoA reductase activity.

Perhaps thyroid hormones exert their indirect effects on HMG-CoA reductase by altering the levels of oxygenated sterols. These compounds are believed to be the mediators of the cholesterol feedback repression of HMG-CoA reductase (Kandutsch et al., 1978). This view is further supported by the characterization of a high-affinity cytosolic oxysterol binding protein which may function similarly to steroid hormone receptors (Kandutsch et al., 1984). Recently, it was reported (Panini et al., 1984) that these regulatory oxysterols could be derived from squalene 2,3:22,23-dioxide. This metabolite is in turn derived from squalene 2,3-epoxide, a normal intermediate in cholesterol synthesis.

The known effects of T_3, colestipol, and mevinolin on cholesterol and bile acid synthesis are depicted in Figure 5. T_3 acts to increase the conversion of cholesterol to bile acids (Kritchevsky, 1960), thereby lowering the levels of intermediates in the cholesterol biosynthetic pathway. This would result in decreased levels of precursor for oxysterol production and hence initiation of HMG-CoA reductase activity. Feeding colestipol would be expected to exert a similar effect. Adding mevinolin to a colestipol-containing diet would act synergistically to decrease oxysterol levels by decreasing mevalonate production.

Although feeding mevinolin and colestipol to hypophysectomized rats results in substantial increases in reductase mRNA and enzyme activity, the levels attained are not nearly as high as those in normal animals fed this diet (Table I). This coupled with the finding that feeding colestipol alone did not entirely mimic the T_3 effect would argue for a unique role for thyroid hormones in promoting the accumulation of HMG-CoA reductase mRNA. Determinations of rates of HMG-CoA reductase mRNA synthesis and degradation in these various states may provide information on whether these dietary agents and thyroid hormones share a common mechanism for promoting HMG-CoA reductase synthesis. In preliminary studies, we[3] have determined that the half-live of both HMG-CoA reductase mRNA and immunoreactive protein is 3 h in normal rats. The decline in mRNA levels preceded that in enzyme levels. In T_3-treated hypophysectomized rats, both half-lives were increased to about 12 h. A similar increase was seen in rats fed mevinolin and colestipol.

It is of interest that nine different rat liver mRNA-encoded products can be regulated both by dietary manipulation (high carbohydrate diet) and by thyroid hormones (Liaw et al., 1983). These include 6-phosphogluconate dehydrogenase (Miksicek & Towle, 1983) and malic enzyme (Siddiqui et al., 1981). Thus, the present observations concerning dietary factors replacing T_3 for promoting accumulation of HMG-CoA reductase mRNA are not unique.

From the data presented in Figures 3 and 4, it appears that glucocorticoids prevent the T_3-mediated induction of HMG-CoA reductase protein by acting either to decrease translation or to increase degradation of reductase protein. Usually glucocorticoids exert their effects by altering rates of transcription. In the present situation, glucocorticoids could, for example, promote the synthesis of an mRNA which codes for a protein essential for the degradation of HMG-CoA reductase.

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Registry No. T_3, 6893-02-3; HMG-CoA reductase, 9028-35-7; dexamethasone, 50-02-2.

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Rapid and Efficient Purification of Plasma Membrane from Cultured Cells: Characterization of Epidermal Growth Factor Binding†

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ABSTRACT: We have devised a rapid and simple protocol for the purification of the plasma membrane from several lines of transformed cultured cells. A431 or KB plasmalemma was purified in 90 min with a two-step centrifugation cycle after selectively inducing microsomal aggregation by the addition of calcium to homogenized cells. Relative specific activity analysis using membrane marker enzymes on the various fractions indicated that the isolated plasmalemma was purified 8–12-fold over the starting homogenate and contained a high density of epidermal growth factor (EGF) receptors. Transmission electron microscopy showed the final membrane suspension consisted of unilamellar vesicles with an average diameter of approximately 100 Å. The purified membrane vesicles avidly bound to ^{125}I-EGF and reached equilibrium within 30 min. Microfiltration assays indicated more than 90% of the total binding can be displaced by excess unlabeled ligand. Equilibrium binding analysis showed a single class of high-affinity ^{125}I-EGF binding site, with K_d = 0.14 nM and B_{max} = 0.1 pmol/mg of protein for purified KB membrane and K_d = 1.2 nM and B_{max} = 5.26 pmol/mg of protein for purified A431 membrane. Gel electrophoresis of ^{125}I-EGF cross-linked to membrane EGF receptors showed a distinct autoradiographic band at 170 kilodaltons, which could be displaced with excessive amounts of unlabeled EGF. Finally, EGF-dependent autophosphorylation of the EGF receptor was clearly demonstrated with the purified membrane preparation. Membrane vesicles purified in this manner can be stored in liquid nitrogen for several months without losing their biological activity.

Epidermal growth factor (EGF), a single-chain polypeptide with a molecular weight of 6,000, is a potent mitogen for several cell types (Das, 1983; Carpenter & Cohen, 1981; Adamson & Rees, 1981). The transduction of the mitogenic signal across the cell membrane is believed to be mediated by the EGF receptor, a 170-kilodalton (kDa) integral plasmalemma protein (Buhrow et al., 1982; Cohen et al., 1980). Although it is well-known that the EGF receptor is autophosphorylated primarily at the tyrosine-1173 residue upon stimulation with EGF (Downward et al., 1984), neither the role of autophosphorylation nor potential secondary substrates have been clearly identified. The existence of an EGF receptor has been documented in various tissues and in cultured cells (Makku & Stancel, 1985; O&Keefe et al., 1974; Rubin & Earp, 1983; Fernandez-Pol, 1981; Toyota et al., 1986). Due to the unusually high number of EGF receptors found to be present on the A431- and KB-transformed human carcinoma (Cohen, 1983; King & Cuatrecasas, 1982), many previous studies have utilized these two cell lines. We have recently developed a new membrane extraction procedure that allows rapid purification of the cell membrane from these transformed cell lines.

The extraction of plasmalemma from cultured cell lines is an intrinsically difficult process since it is usually limited by the small amount of starting material available and the long lag time needed to grow a sufficient number of cells. Previous methods for cell membrane purification from cultured cells typically required multistep gradient ultracentrifugation ranging from 1 to 16 h (Cohen, 1983; Decker, 1984; Butters & Hughes, 1974; Thom et al., 1977). By selectively inducing

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