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Thyrotropin-Releasing Hormone-Induced Subcellular Redistribution and Down-regulation of $G_{11\alpha}$: Analysis of Agonist Regulation of Coexpressed $G_{11\alpha}$ Species Variants

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SUMMARY

Human embryonic kidney 293 cells that had been transfected to express the long isoform of the rat thyrotropin-releasing hormone (TRH) receptor (clone E2) were further transfected with a cDNA encoding the murine version of $G_{11\alpha}$. A clone was isolated (clone E2M11) that stably expressed murine as well as the endogenous human $G_{11\alpha}$. Subcellular fractionation demonstrated identical cellular distribution of the two species variants of this G protein. Sustained exposure of clone E2M11 cells to TRH resulted in substantial cellular redistribution and reduction in total cellular levels of $G_{11\alpha}$ immunoreactivity. Fractions of both the exogenously introduced murine and endogenously expressed human isoforms of $G_{11\alpha}$ were transferred from plasma membranes to low density membranes (detected as a shift from middle to low density regions on sucrose density gradients) and cytosol fractions. The plasma membrane redistribution to low density membrane was accompanied by a parallel redistribution of G protein β subunits; however, there was no increase in β subunits in the cytosol. The total cellular amount of $G_{11\alpha}$ subunits was decreased to 21% and 59% for human and murine isoforms, respectively, and β subunits were decreased to 68% after sustained treatment with TRH compared with controls (100%). Such data are consistent with the notion that the agonist-occupied long isoform of the rat TRH receptor may be able to partially differentiate between the endogenous (human) and exogenous (murine) $G_{11\alpha}$. This was not a reflection that the murine G protein was expressed but incorrectly folded as both species variants of $G_{11\alpha}$ were solubilized equally from E2M11 membranes by sodium cholate. Using this system, we demonstrate both agonist-induced subcellular redistribution and down-regulation of $G_{11\alpha}$ and β subunit proteins in response to activation of a phopholipase C coupled receptor.

Stimulations of S49 lymphoma cells by isoprenaline (1) or of mastocytoma cells by iloprost (2) have been reported to result in a transfer of a significant portion of $G_{s\alpha}$ from membranes to cytosol. In contrast, stimulation of neuroblastoma X glioma (NG108–15) cells by prostaglandin E_1 or iloprost (3–5), of white adipocytes by phenylisopropyladenosine (6), of CHO cells transfected to express the A_3 adenosine receptor with 5′-*N*-ethylcarboxamidoadenosine (7), or direct activation of $G_{s\alpha}$ (8) results in a specific decrease in membrane content of either G_s or G_i α subunits without producing a corresponding increase in the cytosol. A similar decrease in cellular levels of $G_q/G_{11\alpha}$ (two G proteins demonstrated to allow receptor coupling to phospholipase Cs of the β subclass in a pertussis toxin-insensitive manner) has been observed in CHO cells transfected with either the muscarinic m1 (9) or m3 (10) acetylcholine receptor on carbachol stimulation and for a variety of other receptors that couple to these two G proteins (11–13). Thus, two complementary regulatory features seem to exist to allow agonist-induced changes in plasma membrane G protein content on agonist activation of relevant receptors (for a review, see Ref. 14). We recently extended the above observations to show that carbachol can cause a subcellular redistribution within the particulate membrane fractions of muscarinic m1 acetylcholine receptor expressing CHO cell line. On challenge with carbachol, a fraction of the G protein population was translocated to a low density vesicle-containing population (15). Because the absolute levels of translocated $G_q/G_{11\alpha}$ were relatively small, the prospect of quantitative analysis was limited; therefore, for the current study, we generated a cell line from HEK 293 cells by two sequential transfections. We first established a clonal cell line (clone E2) that expresses high levels of the

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ABBREVIATIONS: TRH, thyrotropin-releasing hormone; HEK, human embryonic kidney; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

long isoform of the rat TRH receptor (13) and then established a clonal cell line (clone E2M11) by transfecting clone E2 with a cDNA encoding murine $G_{11\alpha}$ to substantially increase levels of cellular $G_{11\alpha}$. Cells of clone E2M11 thus provide a combination of robust stimulus produced via the high number of activated TRH receptors with high detection capacity for potentially redistributed $G_{11\alpha}$. We recently developed methods that allow concurrent detection and unambiguous assignment of identity to species isoforms of $G_{11\alpha}$ and showed that we can express either murine or bovine $G_{11\alpha}$ in monkey COS cells and subsequently separate the exogenously introduced $G_{11\alpha}$ from the endogenously expressed simian version of the polypeptide (16).

We demonstrate in unstimulated E2M11 cells that the distribution of human and murine $G_{11\alpha}$ is identical and that the introduced murine $G_{11\alpha}$ displays the same ability as the endogenous human $G_{11\alpha}$ to be solubilized by treatment with sodium cholate. We then demonstrate and analyze a major subcellular redistribution of the $G_{11\alpha}$ proteins in these cells in response to treatment with TRH.

Experimental Procedures

Materials. All materials for tissue culture were obtained from Life Technologies (Paisley, Strathclyde, UK). [^{3}H]TRH (56 Ci/mmol) and *myo*-[^{3}H]inositol were obtained from Amersham International. Sucrose (Aristar grade) was purchased from BDH. All other chemicals were obtained from either Sigma or BDH and were of the highest quality available.

Generation and isolation of clones E2 and E2M11. A full-length rat (long isoform) TRH receptor cDNA (2.2 kilobases) was subcloned into the eukaryotic expression vector pcDNA1 (Invitrogen) that is driven by the CMV promoter. Monolayer cultures of HEK 293 cells (50% confluent) were cotransfected overnight with linearized pcDNA1/TRH receptor (800 ng) and pSP neo (200 ng, Invitrogen) with the use of lipofectin reagent (30 mg, Life Technologies) in serum-free DMEM. After 24 hr, medium was replaced with DMEM containing 800 μg/ml geneticin sulfate (Life Technologies). Resultant geneticin sulfate- (800 μg/ml) resistant clones were picked, grown, and assayed for the presence of the TRH receptor. Cells were trypsinized, transferred to 24-well plates, and labeled with *myo*-[^{3}H]inositol (2 μCi/ml for 48 hr) in inositol-free serum containing [1% (v/v) dialyzed] DMEM medium. Total inositol phosphate production was then measured in vehicle- and TRH-treated cells (1 μM for 30 min) (17). TRH receptor-containing clones were identified as those in which TRH produced a rise in total inositol phosphate production. Expression of the TRH receptor in membranes from these clones was assessed by the specific binding of [^{3}H]TRH. Clone E2, which expresses ~14 pmol of the receptor/mg membrane protein (13), was selected for further transfection in these studies. Cells of clone E2 were cotransfected with plasmid pCMV, into which a cDNA encoding murine $G_{11\alpha}$ was inserted, and the plasmid pBABE hygro, which allows expression of resistance to the antibiotic hygromycin B in a 10:1 ratio. Clones were selected on the basis of resistance to hygromycin B (200 μg/ml), and the continued expression of the TRH receptor and the novel expression of murine $G_{11\alpha}$ were examined (see Results). Clone E2M11 was selected for detailed analysis.

Cell growth. Clones E2 and E2M11 of HEK 293 cells were grown in tissue culture in DMEM containing 5% (v/v) newborn calf serum and maintained in the presence of geneticin sulfate (800 μg/ml) (both cell lines) and hygromycin B (200 μg/ml) (only clone E2M11). Before confluency, they were either split 1:10 into fresh tissue culture flasks or harvested. Membrane fractions were prepared from cell pastes that had been stored at −80° after harvest essentially as described previously (18). Frozen cell pellets were suspended in 5 ml of 10 mM Tris·HCl, 0.1 mM EDTA, pH 7.5 (buffer A), and rupture of the cells was achieved with 25 strokes of a hand-held Teflon-on-glass homogenizer. The resulting homogenate was centrifuged at 500 × *g* for 10 min in a Beckman L5–50B centrifuge with a Ti50 rotor to remove unbroken cells and nuclei. The supernatant fraction from this treatment was then centrifuged at 48,000 × *g* for 10 min, and the pellet from this treatment was washed and resuspended in 10 ml of buffer A. After a second centrifugation at 48,000 × *g* for 10 min, the membrane pellet was resuspended in buffer A to a final protein concentration of 1–3 mg/ml and stored at −80° until required.

Production of antisera and immunoblotting. Antiserum CQ (19) was produced by a New Zealand White rabbit after immunization with a glutaraldehyde conjugate of keyhole limpet hemocyanin (Calbiochem) and a synthetic peptide (QLNLKEYNLV) that represents the carboxyl-terminal decapeptide that is conserved between $G_{q\alpha}$ and $G_{11\alpha}$ (20). As such, this antiserum cannot distinguish between these two polypeptides unless they are resolved by electrophoretic techniques. This antiserum has been shown directly, however, to identify both polypeptides equally (21). Antiserum BN3 was generated against a synthetic peptide corresponding to the amino-terminal 10 amino acids (MSELDQLRQE) of the β1 subunit of G proteins. It is able to identify the polypeptides derived from both the β1 and β2 genes. Characterization of this antiserum has previously been defined (7). The $G_{11\alpha}$-specific antiserum E976 (22) was a kind gift from Dr. J. H. Exton (Vanderbilt University, Nashville, TN).

Molecular mass determinations were based on prestained molecular mass markers (Bethesda Research Laboratories). SDS-PAGE [12.5% (w/v) acrylamide/0.0625% (w/v) bisacrylamide containing a linear gradient of 4–8 M urea] was carried out overnight at 100 V.

Quantification of immunoblots. After SDS-PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 hr in 5% (w/v) gelatin in PBS, pH 7.5. Primary antisera were added in 1% (w/v) gelatin in PBS containing 0.2% (v/v) Nonidet P40 and incubated for ≥2 hr. The primary antiserum was then removed, and the blot was washed extensively with PBS containing 0.2% (v/v) Nonidet 40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase; Scottish Antibody Production Unit, Wishaw, Scotland) was added [1:200 dilution in 1% (w/v) gelatin in PBS containing 0.2% (v/v) Nonidet 40] and incubated with the nitrocellulose for 2 hr. The antiserum was then removed, and after extensive washing of the blot with PBS containing 0.2% (v/v) Nonidet 40 and finally with PBS alone, the blot was developed with o-dianisidine hydrochloride (Sigma) as the substrate for horseradish peroxidase as previously described (23). The developed blots were scanned with either a Shimadzu CS-9000 dual-wavelength flying-spot laser densitometer on reflectance mode at 500 nm or a Bio-Rad GS 670 imaging densitometer to enable quantification of the immunoblots. Preliminary experiments were performed to assess the range of linearity of the assay for the antiserum. Amounts of membranes used to assess the effects of TRH treatment on levels of the variants of $G_{11\alpha}$ were, in all cases, within the observed linear region.

Subcellular fractionations: Preparation of crude membrane and cytosol fractions. Suspensions of E2 or E2M11 HEK 293 cells were centrifuged for 10 min at 900 × *g*; washed twice in 140 mM NaCl, 20 mM Tris·HCl, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4; and homogenized in 20 mM Tris, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4 (TME buffer) with the use of an Elvehjm-Potter Teflon-on-glass homogenizer. The cell homogenate was centrifuged for 2 hr at 50,000 rpm in a Beckman Ti50 rotor, resulting in sediment (crude membranes) and supernatant (cytosol). The membrane sediment was suspended with rehomogenization in TME buffer (3–5 mg protein/ml) and stored at −80° until use.

Subcellular fractionation of E2M11 cells on sucrose density gradients: Purification of plasma membranes. E2M11 HEK 293 cells (6 × 75-cm^2 flasks/sample) were harvested by centrifugation for 10 min at 900 × *g*; washed twice in 140 mM NaCl, 20 mM Tris·HCl, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4; and homogenized in 2.5 ml of TME buffer with the use of an Elvehjm-Potter Teflon-on-glass homogenizer. Two milliliters of homogenate (after being frozen at −80°

for ≥1 hr) were layered on the top of a discontinuous sucrose density gradient consisting of (from top to bottom) 19% (5 ml), 23% (5 ml), 27% (5 ml), 31% (5 ml), 35% (5 ml), and 43% (10 ml) (all w/w) sucrose, 20 mM Tris·HCl, 3 mM $MgCl_2$, and 1 mM EDTA, pH 8.0. The gradient was centrifuged for 30 min at 27,000 rpm in a Beckman SW 28 rotor and fractionated manually from the meniscus (fractions 1–7, 5 ml each). The first 5 ml (fraction 1) represented an interphase between the overlayed homogenate and 19% (w/w) sucrose. To separate the low density (light vesicles) membranes from cytosol (15), fraction 1 was diluted 1:1 with redistilled water and centrifuged for 120 min at 50,000 rpm in Beckman Ti50 rotor, and the resulting pellet (fraction 1P) was suspended by rehomogenization in 0.3 ml of TME buffer. The supernatant represented cytosol fraction 1S. The TME buffer was used also to resuspend the gradient pellet (fraction 8). The gradient fractions were frozen at −80° until use.

Measurement of plasma membrane marker enzyme activities. For measurement of plasma membrane marker enzyme activities, sucrose fractions 1–8 (5 ml) were diluted 1:1 with redistilled water and centrifuged for 120 min at 50,000 rpm in a Beckman Ti50 rotor. The resulting membrane pellets were rehomogenized in 20 mM Tris·HCl, and 1 mM EDTA, pH 7.4 (TE buffer) and stored at −80° until use.

Basal and forskolin (10 μM)-amplified adenylyl cyclase activities were measured as described previously (24). Each assay contained 100 mM Tris·HCl, pH 7.5, 20 mM creatine phosphate, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM cAMP, 1 μM GTP, 10 units of creatine phosphokinase, and 0.2 mM ATP containing 1 μCi of [α-^{32}P]ATP. Separation of radiolabeled cAMP and ATP was achieved with use of the double-column method described by Johnson and Salomon (25).

Na^+/K^+-ATPase activity (E.C. 3.6.1.3) was determined as inorganic phosphate released/min/mg of membrane protein in $Na^+/K^+/Mg^{2+}$ medium A (100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 100 mM Tris·HCl, pH 7.6, 2.5 mM ATP) minus that in Na^+/Mg^{2+}/ouabain medium B (120 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris·HCl, pH 7.6, 2.5 mM ATP plus 0.2 mM ouabain) as described by Svoboda *et al.* (26).

Ouabain binding was measured as described previously (27) with 50 nM [^{3}H]ouabain in Pi/Mg^{2+} medium (50 mM Tris·HCl, 5 mM $MgCl_2$, 5 mM NaH_2PO_4). Whatman GF/C filters were used for separation of bound and free radioactivity. Nonspecific binding was determined in the presence of 100 μM unlabeled ouabain. Specific binding was defined as the difference between the total and nonspecific binding.

Results

The generation and analysis of clone E2 HEK 293 cells, which express high levels of the long isoform of the rat TRH receptor, and the interaction of this receptor with the cell signaling machinery to cause stimulation of phosphoinositidase C activity have been described in detail (13). These cells were further cotransfected with the plasmid pCMV, harboring a cDNA encoding murine $G_{11\alpha}$ (20), and the plasmid pBABEhygro, which allows expression of resistance of cells to hygromycin B in a 10:1 ratio. Clones were selected by maintenance in the presence of hygromycin B (200 μg/ml). Membranes derived from a number of individual clones were examined for their ability to specifically bind [^{3}H]TRH. A clone designated E2M11 bound [^{3}H]TRH with both high capacity and high affinity as assessed by analysis of [^{3}H]TRH versus TRH competition curves performed in the presence of guanosine-5′-(β,γ-imido)triphosphate (100 μM) (13). Binding parameters were estimated to be B_{max} of 26–31 pmol/mg membrane protein with K_d for TRH of 120–140 nM when analysis was performed by either Scatchard analysis or application of the formalisms of DeBlasi *et al.* (28). Furthermore, after labeling of these cells with *myo*-[^{3}H]inositol, a large stimulation of accumulation of [^{3}H]inositol phosphates was produced by the addition of TRH to Li^+-treated cells (data not shown).

Sustained incubation of clone E2 cells with TRH (16 hr, 10 μM) resulted in a large decrease in the level of human $G_{11\alpha}$ in a membrane fraction (Fig. 1, *top*), as we have previously described (13). Equivalent incubation of clone E2M11 cells with TRH resulted in a reduction in membrane levels of both the human and murine isoforms of $G_{11\alpha}$ as detected immunologically after resolution in a urea-containing SDS-PAGE system that we have previously demonstrated to be able to resolve primate and rodent variants of $G_{11\alpha}$ (16) (Fig. 1, *bottom*). No effect of TRH was observed in untransfected HEK 293 cells (data not shown). Surprisingly, the observed degree of reduction of murine $G_{11\alpha}$ caused by TRH treatment was much less than of the human $G_{11\alpha}$ subunit. The human isoform was diminished to very low levels (20%) compared with controls, whereas the murine $G_{11\alpha}$ was decreased to only 60% compared with untreated cells (see Table 1).

To obtain information about the total cellular content and distribution of $G_{11\alpha}$ proteins in E2M11 cells, homogenates prepared from untreated and TRH-treated (10 μM, 16 hr) cells were centrifuged for 2 hr at 200,000 × *g*, and crude membrane and cytosol fractions were prepared and examined (Fig. 1, *bottom*). As in Fig. 1 (*top*), the decrease in both human and murine $G_{11\alpha}$ in the crude membrane fractions was clear. However, a substantial amount of immunodetectable murine $G_{11\alpha}$ and a clearly detectable amount of human $G_{11\alpha}$ were also noted in the cytosol fractions of both control and TRH-treated cells, and this was greater in cells that had been treated with TRH. The cytosolic content of $G_{11\alpha}$ of control samples was calculated to represent 7.5 ± 3.2% (mean ± standard error, four experiments) of the total cellular pool of a combination of human and murine $G_{11\alpha}$. In

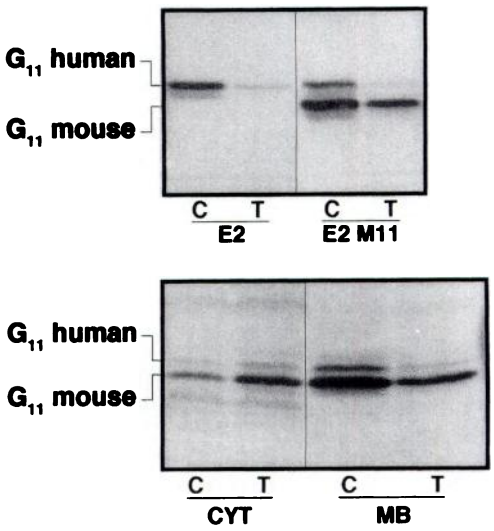

Fig. 1. The effect of TRH on the content of $G_{11\alpha}$ in crude membrane and cytosol fractions of E2 and E2M11 cells. Tissue culture flasks (75 cm^2) of either E2 (*top left*) or E2M11 cells (*top right and bottom*), grown to 70% confluency, were incubated for 16 hr with (*T*) or without (*C*) 10 μM TRH. Crude membrane (*MB*) and cytosol (*CYT*) fractions were prepared as described in Experimental Procedures. The $G_{11\alpha}$ proteins in crude membranes (*top*) or crude membranes and cytosol (*bottom*) were resolved by urea-containing SDS-PAGE with 60 μg (*top*) or 150 μg (*bottom*) of protein/lane and identified by imunoblotting with antiserum CQ. Results are from a representative experiment.

TABLE 1

TRH-induced alterations in membrane to cytosol distribution and downregulation of human and murine $G_{11\alpha}$ in E2M11 cells

The immunological signal (arbitrary units) corresponding to species variants of $G_{11\alpha}$ was analyzed with densitometric scanning and related to the amount of protein present in membrane and cytosol fractions. Down-regulation by TRH (10 μM for 16 hr) is expressed as a percentage of total signal (membranes plus cytosol) in untreated cells. Data represent an average ± standard error of four experiments.

	Membranes	Cytosol	Total	Down-regulation
				%
Human $G_{11\alpha}$				
Control	51.2 ± 12.7	2.3 ± 0.9	53.5 ± 14.6	0
16 hr	2.2 ± 0.8	9.1 ± 2.7	11.3 ± 3.2	78.9
Mouse $G_{11\alpha}$				
Control	236.8 ± 46.1	25.4 ± 5.6	262.2 ± 51.7	0
16 hr	113.7 ± 18.5	40.4 ± 12.9	154.1 ± 35.4	41.2

contrast, cytosol from the TRH-treated cells contained 29.5 ± 2.3% (mean ± standard error, four experiments) of the remaining total cellular content of these proteins (Table 2). However, the net increase in cytosol located $G_{11\alpha}$ proteins in E2M11 cells, i.e., from 95.2 to 209.7 arbitrary units as shown in Table 2 (expressed per the same number of cells), was much less than the amount of $G_{11\alpha}$ removed from the total cellular membrane fraction by treatment with TRH, i.e., from 1314.7 to 505.4 arbitrary units as shown in Table 2. Therefore, the amount of $G_{11\alpha}$ no longer associated with the total membrane fraction after TRH treatment of E2M11 cells could not be quantitatively recovered in the cytosolic fraction. Calculation of the total cellular amount of $G_{11\alpha}$ proteins (membranes plus cytosol) indicated a significant down-regulation in response to TRH, i.e., a decrease of 50% when compared with controls (Table 2).

To examine the time course of TRH-induced redistribution of $G_{11\alpha}$ proteins, E2M11 cells were incubated with 10 μM TRH for times between 10 min and 16 hr. Murine and human variants of $G_{11\alpha}$ were resolved by urea-containing SDS-PAGE as above and densitometric scanning of the developed immunoblots. As demonstrated in Fig. 2, the decrease in both $G_{11\alpha}$ species variants in the crude membrane fraction was accompanied by an increase in these polypeptides in the cytosol, but this effect could be reproducibly detected only at time points of >1 hr. The membrane-to-cytosol redistribution of $G_{11\alpha}$, as well as down-regulation, i.e., decrease in total cellular amount (Table 1), was considerably more pronounced for the human variant than for murine $G_{11\alpha}$. The cytosolic human $G_{11\alpha}$ of untreated cells, which represented 4.3% of the total cellular pool, increased to 80.5% of the total immunodetectable pool after 16 hr of TRH treatment, whereas the corresponding change in murine $G_{11\alpha}$ was from 9.7% in controls to 26.2% in TRH-treated samples (Table 1). The total

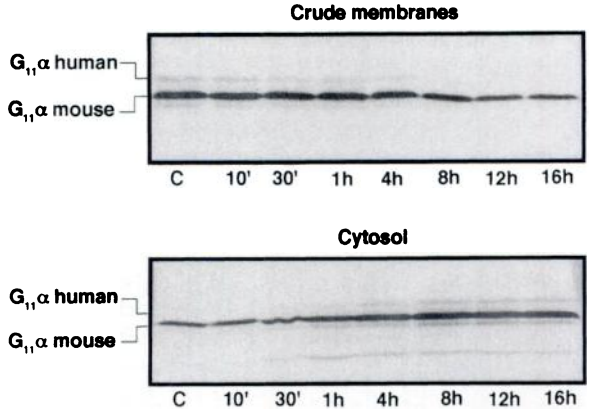

Fig. 2. Time course of membrane removal and cytosolic accumulation of variants of $G_{11\alpha}$. E2M11 cells were untreated (C) or treated with TRH (10 μM) for varying times and crude membrane and cytosolic fractions subsequently prepared and immunoblotting with antiserum CQ was performed after resolution of the fractions (crude membranes, 60 μg; cytosol, 150 μg) in urea-containing SDS-PAGE, as described in legend to Fig. 1.

cellular amount (sum of membrane plus cytosol pools) of $G_{11\alpha}$ proteins was reduced by TRH to 21% and 59% for human and murine $G_{11\alpha}$, respectively, compared with controls (100%) (Table 1).

The TRH-induced changes in subcellular localization of the coexpressed $G_{11\alpha}$ species variants were further analyzed with sucrose density gradient centrifugation to allow partial resolution of plasma membranes from other membrane structures. Application of 19%, 23%, 27%, 31%, 35%, and 43% (w/w) sucrose density gradients, which have been used previously (see Ref. 15 and references therein) for subcellular fractionation of S49 lymphoma and CHO cell lines and for brown adipose tissue homogenates, indicated that the cellu-

TABLE 2

Distribution of $G_{11\alpha}$ in clone E2M11 cells and effect of TRH

The immunological signals (arbitrary units) corresponding to both human and murine variants of $G_{11\alpha}$ were analyzed with densitometric scanning and related to the amount of protein present in membrane and cytosol fractions prepared from the same number of cells (1 × 75-cm² flask of cells). Data represent the mean ± standard error from four independent experiments.

	$G_{11\alpha}$	Down-regulation
		%
Control		
Membranes	1314.7 ± 261.8 (92.5 ± 3.2%)	
Cytosol	95.2 ± 41.5 (7.5 ± 3.2%)	
Total	1409.8 ± 256.5 (100%)	0
TRH treated (10 μM for 16 hr)		
Membranes	505.4 ± 28.7 (70.5 ± 2.3%)	
Cytosol	209.7 ± 55.1 (29.5 ± 2.3%)	
Total	715.1 ± 181.7 (100%)	49.8 ± 9.7

lar distribution profiles of human and murine versions of $G_{11\alpha}$ were identical (Fig. 3A) and demonstrated a high enrichment of the $G_{11\alpha}$ proteins in untreated E2M11 cells in fractions (centered on fraction 4) previously characterized in other systems to contain plasma membranes (15) (Fig. 3). The specific content (density of the immunoblot signal divided by the total protein content) of $G_{11\alpha}$ proteins in fraction 4 was nearly 100-fold higher than in fraction 1S (cytosol) and ~20-fold higher than in fraction 8 (nucleus plus cell debris), the fractions that contained the majority (~85%) of the total cellular proteins (Fig. 4).

TRH treatment of clone E2M11 cells (Figs. 3B and 4) resulted in a dramatic shift of the particulate $G_{11\alpha}$ polypeptides from high and medium density (fractions 4–7) to low density regions of the gradient (fractions 1S, 1P, 2, and 3). The greatest effect of TRH treatment on $G_{11\alpha}$ distribution was observed in the cytosol fraction 1S, where the specific content of $G_{11\alpha}$ increased ~30-fold, i.e., from 11.7 to 309.4 arbitrary units (defined as density of immunoblot signal divided by the amount of protein applied per lane; Fig. 4A) or from 1.2% to 29.2% of total cellular $G_{11\alpha}$ (Fig. 4B). These values agree well with those obtained above with the simple crude membranes versus cytosol distribution studied with differential centrifugation (Fig. 1 and Tables 1 and 2) and represented an unparalleled, original finding. This was achieved without an overall gross alteration in the protein

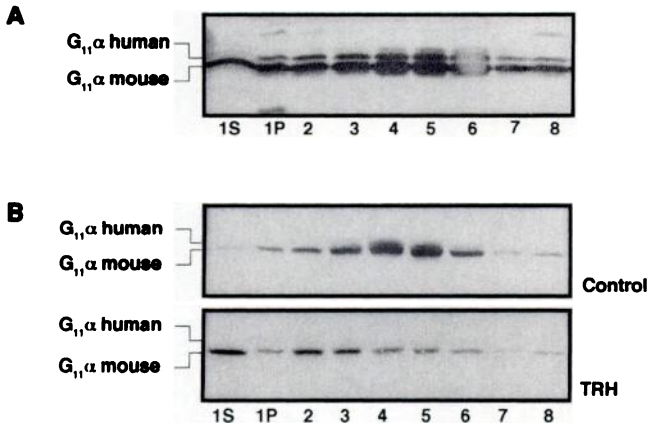

Fig. 3. TRH treatment of E2M11 cells resulted in a transfer of $G_{11\alpha}$ variants from the plasma membrane to low density membrane fractions. In immunoblot studies, homogenates prepared from the same amount of untreated (control) (A and B, top) or TRH-treated cells (10 μM, 16 hr) (B, bottom) (each derived from 6 × 75-cm^2 tissue culture flasks) were fractionated on discontinuous sucrose density gradients as described in Experimental Procedures, and the plasma membrane-containing fractions (centered on *fraction 4*) were separated from cytosol (*1S*), light vesicles (*1P*), and low density (microsomes, endoplasmic reticulum, *fractions 2 and 3*) and high density (mitochondria, *fractions 6 and 7*) membranes as well as the gradient pellet (nucleus, cell debris, fraction 8). A, Endogenous human $G_{11\alpha}$ and exogenously introduced murine $G_{11\alpha}$ display identical cellular distributions. Fractions (0.75-ml) from a sucrose density gradient of the homogenate from untreated E2M11 cells were concentrated by trichloroacetic acid precipitation [6% (w/v) for 30 min on ice] and resolved on urea-containing SDS-PAGE as described in legend to Fig. 1 and immunoblotted to detect the presence of the human and murine variants of $G_{11\alpha}$. B, TRH caused a redistribution of cellular $G_{11\alpha}$ from the plasma membrane to low density membrane fractions. Homogenates from control and TRH-treated E2M11 cells were subjected to centrifugation on discontinuous sucrose density gradients as described above; proteins were resolved by SDS-PAGE and immunoblotted with antiserum CQ. Data are from a representative experiment.

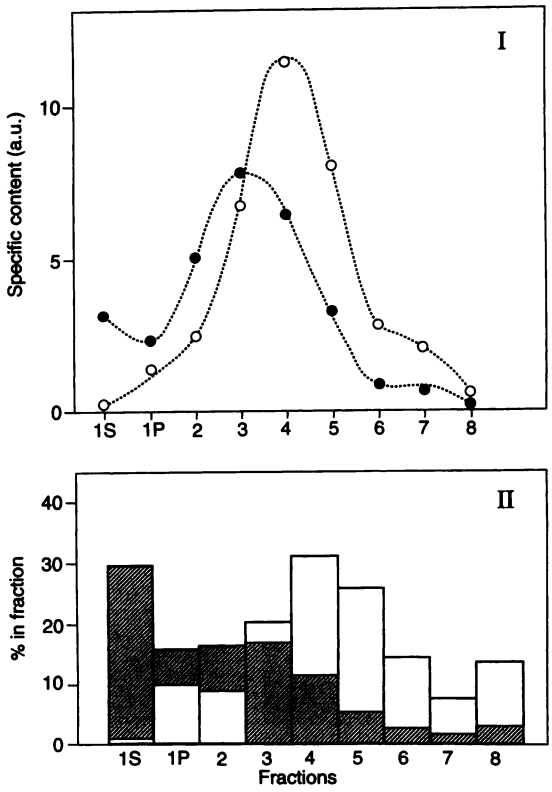

Fig. 4. Specific content and redistribution of $G_{11\alpha}$ proteins in control and TRH-treated E2M11 cells. I, Specific content of $G_{11\alpha}$ (both variants) present in gradient fractions 1S, 1P, and 2–8 of control (○) and TRH-treated (10 μM, 16 hr) (●) E2M11 cells was calculated from immunoblots such as those displayed in Fig. 3 by dividing the immunological signals by the amount of protein applied to the gel. The amount of protein applied was determined in parallel samples trichloroacetic acid-precipitated from 0.75-ml aliquots of the gradient fractions. II, The relative amount of $G_{11\alpha}$ present in a given fraction was expressed as a percentage over the entire gradient. *Open bars*, controls; *filled bars*, TRH treated.

profile of the gradient (data not shown) or a comparable change in distribution of the plasma membrane markers, basal- and forskolin-stimulated adenylyl cyclase, Na^+/K^+-ATPase/ and specific [^{3}H]ouabain binding (Fig. 5). The results shown in Fig. 5 did, however, demonstrate a small redistribution of these plasma membrane markers to regions of lower densities in the gradient. This applied both to the peak levels detected in the middle of the gradient (fractions 4 and 5) and to a minor plasma membrane pool recovered in the low density end of the gradient (fractions 1P and 2). The lack of presence of plasma membrane markers in the cytosol fraction 1S in untreated E2M11 cells was unchanged by TRH treatment. Therefore, it cannot be excluded that the marked redistribution of the $G_{11\alpha}$ proteins is related to a redistribution of some very minor plasma membrane fraction; such a plasma membrane pool, however, would have to be highly enriched in G protein content to account for the results (see Ref. 27).

A direct comparison of the redistribution of human and murine $G_{11\alpha}$ in key fractions of the sucrose density gradients is shown in Fig. 6. The plasma membrane-containing fractions (fractions 3–5) displayed a substantial reduction in immunodetectable levels of both human and murine $G_{11\alpha}$ in response to TRH with virtually complete removal of the hu-

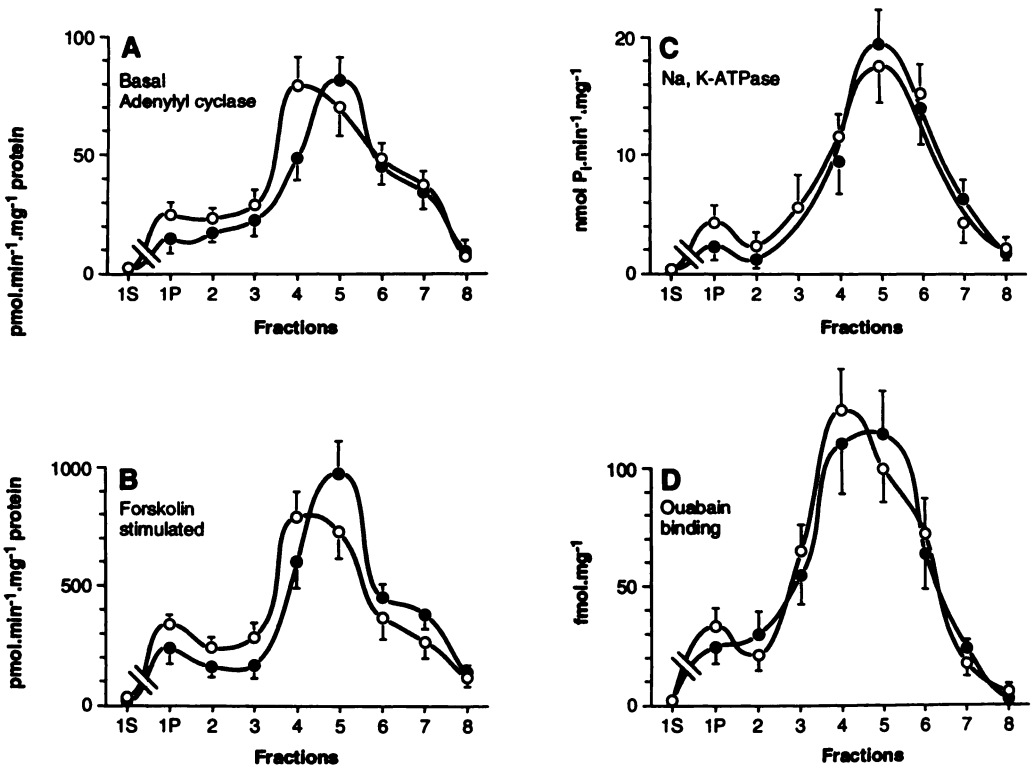

Fig. 5. Subcellular distribution of plasma membrane markers in control ([ ]) and TRH-treated ([ ], 10 μM for 16 hr) E2M11 cells. Basal (A) or forskolin-stimulated (B) adenylyl cyclase, Na^+/K^+-ATPase (C) and specific [^{3}H]ouabain binding (D) were measured in membrane sediments of gradient *fractions 1–7* prepared by high-speed centrifugation and in *fraction 8* representing the gradient pellet as described in Experimental Procedures. *Fractions 1S* (cytosol) and *1P* represent the supernatant and membrane sediment prepared from fraction 1. The results shown represent the average ± standard error of three experiments (Na^+/K^+-ATPase and [^{3}H]ouabain binding) or representative examples measured in triplicate (adenylyl cyclase). Adenylyl cyclase activity is expressed as pmol of cAMP produced/min/mg of membrane protein; Na^+/K^+-ATPase activity is expressed as nmol of inorganic phosphate produced/min/mg of membrane protein; and specific [^{3}H]ouabain binding is expressed as fmol/mg of protein ●, control; ○, TRH-treated.

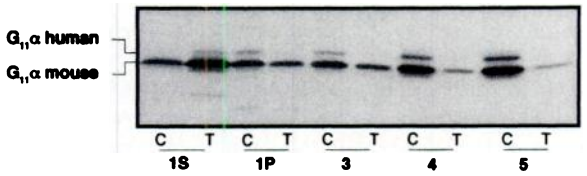

Fig. 6. TRH treatment of E2M11 cells results in a greater cellular redistribution and down-regulation of human $G_{11\alpha}$ than murine $G_{11\alpha}$. *Fractions 1S* (320 μg), *1P* (170 μg), *3* (40 μg), *4* (35 μg), and *5* (40 μg) (the protein content of 0.3 ml of each fraction after trichloroacetic acid precipitation) from centrifugation on a discontinuous sucrose density gradient of control (*C*) and TRH-treated (*T*) (10 μM for 16 hr) E2M11 cells were resolved in urea-containing SDS-PAGE and immunoblotted with antiserum CQ.

Fig. 7. Both human and murine $G_{11\alpha}$ are efficiently solubilized from membranes of E2M11 cells by sodium cholate. Crude membranes of E2M11 cells were either untreated (*1*) or treated with sodium cholate (1% w/v, 1 hr at 4°) (*2 and 3*). The sodium cholate-treated samples were then resolved into remaining particulate (*2*) and solubilized (*3*) fractions by centrifugation (200,000 × g, 30 min). Samples were resolved with urea-containing SDS-PAGE and immunoblotted with antiserum CQ.

man variant and somewhat lower reductions in levels of the murine form. In contrast, elevated levels of both species variants of $G_{11\alpha}$ were noted in the cytosol after treatment with TRH.

A potential explanation for quantitative differences in TRH receptor regulation of the human and murine variants of $G_{11\alpha}$ in these cells might have reflected a difference in the physical state of endogenously expressed human $G_{11\alpha}$ compared with the exogenously introduced murine $G_{11\alpha}$, perhaps representing a nonphysiological aggregation of the introduced proteins as we have observed for murine $G_{11\alpha}$ after high level transient expression in COS cells (29). However, treatment of membranes of E2M11 cells with the detergent sodium cholate [1% (w/v) for 1 hr at 4°] resulted in equivalent and essentially complete solubilization of both human and murine versions of $G_{11\alpha}$ (Fig. 7).

To examine potential changes in the distribution and levels of G protein β subunits in response to TRH, parallel samples of gradient fractions analyzed in Fig. 3 were tested immunologically for their content of β subunit (Fig. 8). There was little if any detectable β subunit in the cytosolic fraction 1S before treatment with TRH, and this was unchanged by TRH treatment. However, similar to the results presented for the $G_{11\alpha}$ subunits, a major plasma membrane-to-low density membrane (light vesicle) redistribution occurred with TRH treatment (Fig. 8). Resolution of crude membrane and cytosol fractions from control and TRH-treated E2M11 cells also indicated a down-regulation of the total cellular amount of β subunits in TRH-treated cells to 67% of control levels (Table 3). To examine whether the observed β subunit redistribution in response to TRH reflected the high levels of expression of the introduced murine $G_{11\alpha}$, the E2 cell line was also examined for β subunit redistribution in response to TRH. Al-

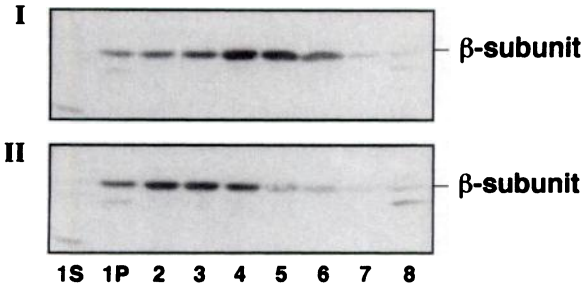

Fig. 8. The cellular distribution of G protein β subunit is altered by treatment of E2M11 cells with TRH. Homogenates of control (*I*) and TRH-treated (10 μM for 16 hr) (*II*) E2M11 cells were centrifuged on discontinuous sucrose density gradients as described in the legend to Fig. 3. Trichloroacetic acid precipitates (0.75-ml aliquots) of gradient fractions used in Fig. 3 were then resolved in SDS-PAGE (without urea). β Subunit was subsequently detected with immunoblotting with antiserum BN3. Data represent results from a typical fractionation procedure.

TABLE 3

G protein β subunits in E2M11 cells: regulation by treatment with TRH

E2M11 cells (1 × 75-cm² flask), which were either control or treated with TRH (10 μM for 16 hr), were homogenized in 0.5 ml of TME buffer and centrifuged for 2 hr at 50,000 rpm. The sediment was suspended with rehomogenization in 0.5 ml of TME buffer (crude membranes), and the supernatant represented the cytosol fraction. The immunodetectable signal corresponding to G protein β subunits signal (arbitrary units) was normalized to the amount of protein in these fractions. Down-regulation by TRH was expressed as percentage of total signal (membranes plus cytosol) in untreated cells (100%) minus 100. Data represent an average ± standard error of three experiments.

	β Subunit	Down-regulation
		%
Control		
Membranes	437.0 ± 27.1 (99%)	
Cytosol	6.6 ± 1.9 (1%)	
Total	443.6 (100%)	0
TRH treated (10 μM for 16 hr)		
Membranes	294.2 ± 22.8 (99%)	
Cytosol	2.4 ± 0.5 (1%)	
Total	296.6 (100%)	32.2

though evident, the redistribution of β subunits in E2 cells was much lower than in E2M11 cells (data not shown). Therefore, the high level of expression of $G_{11\alpha}$ subunits seems to be a key factor in TRH receptor-induced intermembrane redistribution of β subunits.

Discussion

Cellular redistribution of the α subunit of the stimulatory G protein G_s from membrane to cytosol has been recorded in a number of studies after agonist occupation of a receptor that links to this G protein (1, 2). However, the quantitative details relating to the fraction of the G protein redistributed and the time course of this effect have either been somewhat variable or not been examined in detail. Furthermore, except in the study of Haraguchi and Rodbell (30), there has been little previous attempt to analyze whether the activated G protein might redistribute within membrane fractions on agonist activation of a receptor. We have recently reported that sustained agonist occupation of a human muscarinic m1 acetylcholine receptor stably transfected into CHO cells results in a cellular redistribution of the α subunits of the phosphoinositidase C-linked G_q and G_{11} (15). We also provided strong evidence that maintained exposure of a cell to an agonist can result in a reduction in cellular levels of the G protein or proteins activated by the receptor for that agonist (4–14), an effect that seems to reflect accelerated degradation of the activated G proteins (9–12). There are, thus, clearly a variety of dynamic processes in which cellular G proteins are participants after receptor activation of a cell. In the current study, we established a cellular system that has allowed us to examine concurrently both the qualitative and quantitative details of many of these processes and to examine whether the quantitative details of TRH receptor interactions with the same G protein derived from different species are identical. This latter point is of considerable importance given the widespread use of transfection studies with G protein-coupled receptors that often pay little attention to the genetic background of the host cells versus the transfected receptor species.

The system we used is a clonal cell line derived from the HEK 293 cell line by two consecutive stable transfections. In the first stage, the cells were transfected to express high levels of the long isoform of the rat TRH receptor, and subsequently a clone from this transfection (E2) (13) was transfected to express the murine form of $G_{11\alpha}$ (clone E2M11). The requirement for high levels of a receptor was based on our previous observations that to record agonist-mediated down-regulation of a G protein, high levels of receptors are required (see Ref. 5 for an example) as a significant fraction of the cellular population of the relevant G protein must be activated by the receptor, and we reasoned that higher levels of expression of a relevant G protein would provide sensitivity in detection of agonist-induced cellular redistribution of the G protein if there were sufficient receptor present to activate this excess G protein.

We have previously noted that rodent and primate variants of $G_{11\alpha}$ migrate in markedly different positions in urea-containing SDS-PAGE (16). As such, in the current study, we transfected the HEK 293 cells with murine $G_{11\alpha}$ so it would be possible to study concurrently the expression of both variants of this G protein. Indeed, we were able to demonstrate conclusively that clone E2M11 but not clone E2 expressed murine $G_{11\alpha}$, whereas both of these clones express human $G_{11\alpha}$ endogenously due to the genetic background of the HEK 293 cells. As shown in Fig. 1, murine $G_{11\alpha}$ is expressed in substantially higher levels than the human variant in E2M11 cells (this ratio varied somewhat in individual passages of the cells, a feature previously well appreciated for a variety of polypeptides in many transfected cell lines). Importantly, before these studies could progress, we were able to demonstrate that the cellular distributions of the endogenous human $G_{11\alpha}$ and the introduced murine $G_{11\alpha}$ were identical (Fig. 3A), as based on their codistribution in various membrane fractions prepared through centrifugation of cellular homogenates on sucrose density gradients. Furthermore, both human and murine $G_{11\alpha}$ were effectively solubilized from a crude membrane fraction by exposure to sodium cholate (Fig. 7). We have previously observed that transient transfection of murine $G_{11\alpha}$ into monkey COS cells can result in a substantial fraction of the particulate immunodetectable murine G_{11} being resistant to solubilization (29), a situation that we have interpreted to reflect either incorrect folding or aggregation of the expressed protein (29). It was thus vital to demonstrate that in the current study, this would not have an adverse effect on conclusions.

As we have previously shown for clone E2 (13), sustained exposure of E2M11 cells to a maximally effective concentration of TRH resulted in a marked reduction in $G_{11\alpha}$ levels in a crude particulate (total membrane) fraction of these cells, confirming that TRH treatment of these cells can result in down-regulation of a substantial fraction of the expressed $G_{11\alpha}$. We did note, however, that down-regulation of human $G_{11\alpha}$ seemed to be quantitatively more effective than down-regulation of murine $G_{11\alpha}$. One interpretation of these observations would be to suggest that the rat TRH receptor may not interact identically with the same G protein from two separate species when presented with a concurrent choice between them. However, because the level of expression of the murine $G_{11\alpha}$ was greater than its human counterpart and because we do not have a more direct way to examine this point, this must remain speculative and open to a number of other interpretations. In many ways, it would be surprising if there were selectivity of interaction, as the two species variants of $G_{11\alpha}$ are ~97% identical in primary sequence and are very highly conserved in regions believed to play important roles in interactions with receptors. Furthermore, in a range of studies, we have observed little to suggest that receptors can functionally select between the α subunits of endogenously expressed G_{11} and G_q (11–13, 21), the prevalent and widely expressed phosphoinositidase C-linked G proteins that are highly homologous (20), although derived from distinct genes. It should be noted, however, that differences in the interactions of the mammalian neuromedin B receptor with *Xenopus* forms of $G_{q\alpha}$ and $G_{11\alpha}$ have been recorded with an antisense-based approach (31) and between murine $G_{q\alpha}$ and $G_{11\alpha}$ with the murine TRH receptor after their expression in *Xenopus* oocytes (32).

By simple fractionation of E2M11 cell homogenates into total particulate and cytosolic fractions, we noted that in the unchallenged cells only ~7% of the total cellular content of the $G_{11\alpha}$ species variants could be found in the soluble fraction of the cell. However, after challenge of the cells with TRH, an increase in the fraction of $G_{11\alpha}$ present in the cytosol was observed (Fig. 1, *bottom*; and Fig. 2). This could be observed for both the human and murine variants of $G_{11\alpha}$ and was observed to increase over an 8-hr period. When presented as a fraction of the total cellular content of $G_{11\alpha}$ at any time period, the cytosolic fraction increased to ~30% of the total (Table 2), which is clearly a substantial fraction, but it must be remembered that this value is made apparently more impressive as the total cellular content of $G_{11\alpha}$ decreases (it is down-regulated). The increase in cytosolic content of $G_{11\alpha}$ was insufficient to account for the bulk loss of $G_{11\alpha}$ from the particulate fraction, confirming a true cellular reduction in $G_{11\alpha}$ levels. A number of other studies have reported an accumulation of cytosolic $G_{s\alpha}$ after agonist challenge of cells (for example, see Refs. 1 and 2), but in general the time course of appearance of the G protein in the cytosolic fraction has been more rapid than observed in the current study.

Recently, there has been considerable emphasis on examining the interaction of G protein α subunits with membranes, as all of the widely expressed α subunits have been shown to be palmitoylated close to their amino terminus (for reviews, see Refs. 33 and 34). Studies indicate that the palmitoylation status (of at least $G_{s\alpha}$) is dynamic and regulated by the activation status of the G protein (33, 34). Furthermore, cytosolic G protein α subunits have been reported to be devoid of palmitate. It will thus be of interest to examine, in time, the relative palmitoylation status of $G_{11\alpha}$ in the various cellular compartments during and after TRH treatment of E2M11 cells.

Separation of the total particulate fraction of control and TRH-treated E2M11 cells into a series of membrane fractions by centrifugation of cellular homogenates on discontinuous sucrose density gradients (15) demonstrated a further clear and novel cellular redistribution of $G_{11\alpha}$ in response to TRH. Although the distribution of $G_{11\alpha}$ in the untreated cells indicated that a large preponderance of the G protein was in the plasma membrane, as might be expected given its role, TRH treatment produced a profound membrane redistribution, so that the bulk of the remaining immunodetectable $G_{11\alpha}$ was now located in lighter density membrane fractions (see Fig. 3B for an example, where the main peak of $G_{11\alpha}$ immunoreactivity is in fractions 2 and 3 after TRH treatment compared with fractions 4 and 5 in the untreated samples). This redistribution of $G_{11\alpha}$ was achieved without a bulk redistribution of protein as the gross pattern of protein expression in the various sucrose density gradient fractions was not altered by TRH treatment of the cells (data not shown). Somewhat unexpectedly, the membrane redistribution of G protein subunits on TRH treatment was not restricted to $G_{11\alpha}$. Equivalent analysis of the particulate distribution of G protein β subunit indicated that there was also a substantial movement of immunodetectable β subunit from the plasma membrane containing fractions to light membrane fractions (Fig. 8). In contrast to $G_{11\alpha}$, however, there was no increase in detection of cytosolic β subunit on TRH treatment, and this remained an insignificant fraction. Thus, the hormone-induced movement of $G_{11\alpha}$ subunits to the cytosol induced by TRH stimulation of E2M11 cells must be associated with physical dissociation from β subunits that remain in the particulate fraction. The high level of mouse $G_{11\alpha}$ expressed in E2M11 clone may be an important factor in TRH-induced redistribution of β subunits as equivalent treatment of parental E2 cells (which express the same high levels of the TRH receptor but only the endogenous, human isoform of $G_{11\alpha}$) induced only a minor alteration in β subunit distribution (data not shown).

The physical processes involved in alteration in the membrane fraction distribution profile of $G_{11\alpha}$ and β subunits on sustained TRH stimulation remain undefined, but these may contribute to the processes of desensitization. We have previously provided data to support the concept that down-regulation of G proteins can play an important role in the maintenance of long term desensitization phenomena (6, 14), and the observed intermembrane redistribution in these studies may relate to the types of ligand-induced internalization and recycling of a variety of receptors, including the TRH receptor, previously observed by others (for examples, see Refs. 35–39). This is particularly relevant as the low density membranes represent a heterogeneous mixture of membrane structures (endosomes, endoplasmic reticulum, golgi) containing the type of structures previously reported to contribute to internalization of hormone receptors after agonist challenge again as part of mechanisms designed to regulate cellular sensitivity to agonists (35–37).

As demonstrated by plasma membrane marker measurements (Fig. 5), we were able to detect a small degree of

redistribution of plasma membrane markers after TRH treatment. It is possible that the low density vesicles enriched in G protein subunits may represent a continuous sequence of vesicles, including endosomes, which at the beginning bear plasma membrane markers but that later (on the way to lysosomes) loose them. Caveolae may represent candidates for such structures (40, 41).

The redistributed plasma or nonplasma membrane form of G protein subunits may be regarded as intermediate pools between internalization, recycling, and the down-regulation/degradation pathways. The amount of receptors and G proteins detected in the light-vesicular pool must represent a steady state, dynamic equilibrium between these pathways. Depending on relative capacities of these pathways, the steady state level of receptors and/or G proteins inside the cell may differ among cell types.

The results presented in this study demonstrate a complex pattern of cellular G protein redistribution after agonist-induced activation of receptors that may be as complex as that of the G protein-linked receptors themselves. The detailed mechanisms responsible for agonist-induced cellular G protein redistribution will form the basis of future studies.

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