Epidermal Growth Factor-induced Vacuolar (H⁺)-ATPase Assembly
A ROLE IN SIGNALING VIA mTORC1 ACTIVATION

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Using proteomics and immunofluorescence, we demonstrated epidermal growth factor (EGF) induced recruitment of extrinsic V1 subunits of the vacuolar (H⁺)-ATPase (V-ATPase) to rat liver endosomes. This was accompanied by reduced vacuolar pH. Bafilomycin, an inhibitor of V-ATPase, inhibited EGF-stimulated DNA synthesis and mammalian target of rapamycin complex 1 (mTORC1) activation as indicated by a decrease in eukaryotic initiation factor 4E-binding 1 (4E-BP1) phosphorylation and p70 ribosomal S6 protein kinase (p70S6K) phosphorylation and kinase activity. There was no corresponding inhibition of EGF-induced Akt and extracellular signal-regulated kinase (Erk) activation. Chloroquine, a neutralizer of vacuolar pH, mimicked bafilomycin effects. Bafilomycin did not inhibit the association of mTORC1 with Raptor nor did it affect AMP-activated protein kinase activity. Rather, the intracellular concentrations of essential but not non-essential amino acids were decreased by bafilomycin in EGF-treated primary rat hepatocytes. Cycloheximide, a translation elongation inhibitor known to augment intracellular amino acid levels, prevented the effect of bafilomycin on amino acid levels and completely reversed its inhibition of EGF-induced mTORC1 activation. In vivo administration of EGF stimulated the recruitment of Ras homologue enriched in brain (Rheb) but not mammalian target of rapamycin (mTOR) to endosomes and lysosomes. This was inhibited by chloroquine treatment. Our results suggest a role for vacuolar acidification in EGF signaling to mTORC1.

After ligand binding, epidermal growth factor receptors (EGFRs) are rapidly activated by tyrosine autophosphorylation and internalized into endosomes (ENs) (1) from where they undergo dephosphorylation and recycling or are escorted to late endosomes and multivesicular bodies for degradation (2, 3). It has been clearly demonstrated that insulin and EGF signaling, initiated at the cell surface, is sustained and amplified in endosomes (4–6). There is also evidence showing that signals can be uniquely generated within the endosomal compartment (7, 8). Therefore, endosomes are a key site for mediating and modulating peptide hormone and growth factor signaling (9, 10).

Characterization of detergent-resistant membranes (DRMs) prepared from subcellular fractions has been used to infer biochemical characteristics of lipid rafts that are the subcompartments of a given organelle, such as endosomes (11). DRMs have been identified in a number of organelles including plasma membrane (12), endoplasmic reticulum, Golgi (13, 14), and endosomes (15, 16). We have previously observed that DRMs in endosomes and plasma membrane are greatly enriched in tyrosine-phosphorylated EGFR and downstream signaling molecules, consistent with a role for lipid-rich membrane domains in EGF signaling (15, 17).

EGFR-dependent signaling contributes to liver cell proliferation and is an important regulator of hepatic regeneration (18). Thus, mice lacking hepatic EGFR display reduced hepatocyte proliferation with decreased and delayed expression of cyclin D1 (19). In previous studies it was found that the inhibi-

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tion of mTORC1 by rapamycin reduced EGF-stimulated DNA replication (20, 21), protein synthesis, and cyclin D1 levels (21).

An important feature of the endosomal system is the progressively decreasing pH (from ~6.0 in early endosomes to 5.0–5.5 in lysosomes (22)) which ligand-receptor complexes experience while traversing the system (23). This may reflect in part differences in the vacuolar (H\(^+\))-ATPase (V-ATPase) content/function between early endosomes and lysosomes (24). The V-ATPase is a multisubunit enzyme composed of a peripheral V\(_1\) complex, mediating the hydrolysis of ATP, and a membrane-bound V\(_0\) complex, translocating protons across the membrane (25). Reversible assembly/disassembly of V\(_1\) and V\(_0\) sectors is an important mechanism of physiological regulation of V-ATPase (24) and is apparently widely conserved from yeast to mammalian cells (26–28).

In this study we found that EGF-induced recruitment of V\(_1\) subunits of the vacuolar ATPase to rat liver endosomes/lysosomes was accompanied by a reduction in vacuolar pH. This appears to be important for EGF-induced mTORC1 activation.

EXPERIMENTAL PROCEDURES

Materials

Porcine insulin was a gift from Lilly. EGF was from BD Biosciences. Antibodies against EGFR (#sc-03, used for immunoblotting) and phosphorytrosine proteins (PY99, #sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against REDD1 was from Abanova (Walnut, CA). Antibodies against phospho-p70S6K(Thr-389), p70S6K, 4E-BP1, phospho-Erk1/2(Thr-202/Tyr-204), Erk1/2, caspase 3, poly(ADP-ribose) polymerase, phospho-PRAS40(Thr-246), PRAS40, phosphor-AMPK(Thr-172), AMPK, phosphor-TSC2(Thr-1462), TSC2, mTOR, regulatory-associated protein of mTOR (Raptor), RagA, and RagC were from Cell Signaling Technologies (Beverly, MA), 3-(2, 4-Dinitroanilino)-3(Raptor), RagA, and RagC were from Cell Signaling Technologies (Beverly, MA), 3-(2, 4-Dinitroanilino)-3'(Raptor), RagA, and RagC were from Cell Signaling Technologies (Beverly, MA), 3-(2, 4-Dinitroanilino)-3'-amino-N-methyl-dipropylamine (DAMP) and polyclonal anti-DNP were from Oxford Biomedical Research (Oxford, MI). l-Amino acid quantitation kit was from Biovision (Mountain View, CA). Antibodies for immunofluorescence are described below. Bafilomycin A1, chloroquine, cycloheximide, and DMSO were from Sigma.

Animals

Sprague-Dawley rats were purchased from Charles River Canada Ltd. (St. Constant, Quebec), housed in an animal facility with 12-h light cycles at 25 °C, and fed ad libitum on Purina normal chow. Animals were fasted overnight (16–18 h) before preparation of liver subcellular fractions. All animal protocols have been approved by McGill University.

Proteomic Sample Preparation and Analysis

All methods were described previously (15). Briefly, equal quantities of subcellular fractions from three independent preparations of endosomes and endosomal DRMs were loaded on a 7–12% gradient gel and stained with Coomassie G. Each gel was sliced by hand so each lane was ~66 equal bands. Each band was minced into 1-mm\(^2\) pieces and subjected to alkalyation followed by in gel tryptic digestion and peptide extraction using a MassPrep Work station (Micromass, Manchester, UK). Peptides were then subjected to LC-MS in a Micro Q-TOF (Micromass, UK). Equivalent bands from each sample were placed on the same 96-well tray and, therefore, subjected to all manipulations and MS as identically as possible. Peptides were identified using Mascot and subjected to clustering using an in-house data base (CellMapBase) (15) to produce a non-redundant list of proteins. More detailed information about the “peptide counting” has been previously described (15).

Preparation of Microsomes and Endosomes from Rat Liver

Rats (160–180 g of female Sprague-Dawley) were anesthetized and sacrificed by decapitation after intraperitoneal injections at the indicated times as described in the appropriate figures and legends. Livers were exsanguinated, rapidly excised, and minced at scissor point in ice-cold buffer (5 mM Tris-HCl buffer, pH 7.4, containing 0.25 m sucrose, 1 mM benzamidine, 1 mM PMSF, 1 mM MgCl\(_2\), 2 mM NaF, and 2 mM Na\(_2\)VO\(_4\)). Endosomes and microsomes were prepared as previously described (15).

Preparation of Lysosomes from Rat Liver

Lysosomes were prepared as described in detail by Wattiaux et al. (29).

Isolation of Endosomal Detergent-resistant Membranes (DRMs) from Rat Liver

DRMs were isolated as described in detail by Balbis et al. (15).

In Vivo Chloroquine Treatment

Chloroquine was dissolved in 0.9% NaCl (normal saline). The pH of the solution was adjusted to 7.4 with NaOH, and the volume was then adjusted to a final concentration of 10 mg chloroquine/ml. Animals received 10 mg/200 g of body weight of chloroquine by intraperitoneal injection, 2 and 1 h before EGF stimulation. Control animals received a comparable volume of normal saline.

Primary Hepatocyte Cultures

Primary rat hepatocytes were prepared from male Sprague-Dawley rats (~130 g) by collagenase perfusion and maintained as described previously (30). All studies were performed in a humidified 37 °C incubator with 5% CO\(_2\). For the preparation of samples for immunoblotting, cells were rinsed twice with ice-cold PBS (pH 7.4) and solubilized in lysis buffer as described previously (30).

Immunofluorescence Studies

Preparation of Cells—Primary hepatocytes harvested by collagenase in situ perfusion (as described above), were grown on glass coverslips coated with collagen and starved for 2 days. Incubations with EGF (100 nM) were for 5 min at 37 °C after which cells were washed rapidly with cold PBS and fixed with pre-chilled methanol at ~20 °C for 15 min. Cells were washed 3 times with cold PBS and kept at 4 °C until the labeling procedure.

Labeling of the Cells—Fixed cells were first blocked in 5% inactivated goat serum (Invitrogen) in PBS for 30 min at room
temperature. Primary antibody incubation (in 2% inactivated goat serum) was for 45 min at 37 °C. Cells were then washed 3 times in PBS before a 30-min incubation with secondary antibody (diluted in 2% inactivated goat serum) at room temperature in the dark. For double labeling, the two primary or two secondary antibodies were prepared in the same solution. After secondary antibody incubation, cells were washed three times with PBS then three times with water and mounted on slides using Prolong anti-fade mounting medium (Molecular Probes, Burlington, ON, Canada).

Antibodies—The following summarizes the antibodies used and the dilutions at which they were used. Anti-V, E chicken antibody, A22284F (GenWay Biotechnologies, San Diego, CA) was used at 1:2000. Anti-early endosome antigen 1 (EEA1) rabbit polyclonal, ab2900 (Abcam, Cambridge, UK), was used at 1:500. Anti-Rab5 rabbit polyclonal, sc-309 (Santa Cruz) was used at 1:50. Anti-lyssosomal-associated membrane protein-1 (LAMP1) rabbit polyclonal, ab24170 (Abcam), was used at 1:25. Goat anti-chicken IgY-Alexa Fluor 488 (A11039) was used at 1:1000. Goat anti-rabbit IgG-Alexa Fluor 594 (A11037) was used at 1:1000. Goat anti-rabbit IgG-Alexa Fluor 750 (A21039) was used at 1:250. All the goat antibodies were obtained from Molecular Probes.

Immunofluorescence Microscopy—The cells were examined under an epifluorescence Zeiss Axiovert 200M inverted microscope using a 100× objective. Digital pictures were taken with a Roper Scientific CoolSNAP ES digital camera and processed with a PerkinElmer Metamorph Imaging system (Santa Clara, CA) for adding pseudo-colors and merging the double labeling. Images were finally saved in Tiff format. All immunofluorescence data shown are representative of at least three independent experiments.

To assess the V, E distribution pattern, each coverslip was divided into 5 regions, and at least 6 cells per region were analyzed (~30 cells per coverslip, from at least 10 different fields of view across the coverslip). The criteria for selecting the cells were as follows; in each of the five regions the first six cells seen that did not display excessive amounts of secreted material masking the cell cytoplasm were evaluated. Cells were double-blindly assigned “vesicular,” “diffuse,” or “unclear” labeling pattern.

The co-localization of V, E with LAMP1, EEA1, and Rab5, respectively, was examined in at least 10 cells per EGF-treated coverslip. The percentage overlap between V, E and LAMP1, EEA1, and Rab5 was estimated in three independent experiments with each antibody. The cell selection criteria were the same as described above. In the course of these studies we encountered cells in which there was notable autofluorescence as previously observed by others (31). Autofluorescence is visible in all channels but is much weaker in the infrared channel. Therefore, we could falsely interpret autofluorescence as co-localization, as it would appear yellow in a merged image. We employed an antibody coupled to a fluorophore that emits in the infrared range to limit the amount of autofluorescent material appearing in the co-localization studies. Comparison of the specific infrared fluorescence to channels in the visible light range allows identification of interfering autofluorescent material. Thus, use of the infrared channel allowed us to verify that our co-localization was true V, E-LAMP1 co-localization and not autofluorescent material.

DAMP Labeling of Vesicles in Rat Primary Hepatocytes

The DAMP labeling of vesicles was performed using an Acidic Granule Kit (D09, Oxford Biomedical Research). For DAMP labeling quantification, 10 cells were selected randomly in each condition. For each selected cell a digital picture was taken with a Roper Scientific CoolSNAP ES digital camera. Each picture was analyzed with the PerkinElmer Metamorph Imaging system. The fluorescence intensity of all the vesicles labeled in the cell was measured. Total fluorescence intensity for each cell was calculated using the Metamorph software. The mean ± S.E. of 10 intensities was calculated for each condition.

Protein Quantification

Protein content of all samples was performed using a kit (Bio-Rad) with bovine serum albumin as an internal standard as described in the company manual.

Immunoblotting

Reagents for electrophoresis and for measuring the protein content of the liver fractions were from Bio-Rad. PVDF Immobilon-P transfer membranes were from Millipore Ltd. (Mississauga, ON, Canada). Anti-mouse IgG developed in goat (GAM, #M-8642), anti-rabbit IgG developed in goat (GAR, #R-2004), and most of the chemicals were purchased from Sigma. GAR and GAM were labeled with Na125I as described before (32) and were used as secondary antibodies for immunoblotting.

[3H]Thymidine Incorporation Assay

[3H]Thymidine incorporation was measured as previously described (30).

In Vitro S6 Kinase Assay

The in vitro S6 kinase assay was performed using the S6 protein substrate as previously described (33).

7-Methyl-GTP-Sepharose 4B Pulldown Assay

7-Methyl-GTP-Sepharose 4B pulldown assay was performed as previously described (34).

Leucine Uptake Assay

Serum-starved primary hepatocytes were incubated with either serum-free medium or warm PBS in the presence of DMSO or bafilomycin for 30 min. Cells were then incubated with 10 nM EGF or insulin and 1 μCi [3H]leucine (PerkinElmer) (humidified 37 °C, 5% CO2) for 15 min, then washed 3 times with ice-cold PBS, and solubilized in 1 ml of 1 M NaOH. Subsequently 1 ml of 1 M HCl was added to neutralize the solubilized total cell extract, which was analyzed by scintillation counting for 3H.

Amino Acid Analysis by HPLC

Cells were washed 3 times with cold PBS, scraped into 1 ml cold saline, and spun down by centrifugation. Pellets were resuspended in 200 μl of saline, sonicated on ice, and then centrifuged to obtain the supernatant. From cell lysate samples,
proteins were precipitated with perchloric acid, and supernatants were neutralized with potassium carbonate. Diluted samples (with water) were then derivatized with o-phthalaldehyde reagent before injection into the HPLC system (Beckman Coulter System Gold® 508 Autosampler, 126 Solvent Module, 32 Karat 5.0 software). Amino acids were separated in reversed phase on a C18 silica column (4.6 mm × 15 cm, 3-μm particles, from Supelco) using a sodium acetate buffer and increasing gradient of methanol in about 52 min. Detection was accomplished by fluorescence at excitation 340 nm and emission at 450 nm (Jasco FP 2020 Plus). Amino acid concentrations were quantified using standard curves run in duplicate at the beginning, middle, and end of an overnight sequence of injections. Using this approach, 21 individual amino acids were well separated and quantified. Intra-assay coefficient of variation was <5.0% for all amino acids except ornithine and lysine (±20%). Concentrations of amino acids (nmol/ml) in primary hepatocytes were calculated using an estimated cell volume of 1/15 ml per 5 × 10⁶ hepatocytes.

**Statistical Analysis**

All statistical analyses were carried out by unpaired Student’s t test. The number of replicates is indicated in the figure legends.

**RESULTS**

**Proteomic Analysis of Rat Liver Endosomes**—We previously observed that an endosomal DRM subfraction is greatly enriched in tyrosine-phosphorylated EGF receptors and downstream signaling molecules (15). A more detailed and complete proteomic analysis of hepatic endosomal DRMs derived from control versus EGF-treated rats identified 1472 proteins of which 442 reproducibly increased or decreased in abundance after EGF. These are listed in functional categories in supplemental Table 1. Of interest is the large number of signaling and trafficking proteins that change in response to EGF stimulation, consistent with the dual signaling and trafficking roles of the endosomal system (17, 35, 36). Also noteworthy are eight ubiquitination-related proteins whose presence emphasizes the important role of ubiquitin-related processes in trafficking events (37).

The V-ATPase is a multimeric structure (Fig. 1A) responsible for the acidification of intracellular organelles such as endosomes and lysosomes (23). It is structurally similar to the F-type ATP synthase, the H⁺-dependent ATP synthase of mitochondria, and chloroplast (for review, see Ref. 23) and is composed of a V₁ (extrinsic) domain that consists of eight different subunits involved in the binding and hydrolysis of ATP as well as a Vₒ domain consisting of multiple intrinsic membrane components responsible for H⁺ transport across the membrane. A striking observation was that, after EGF treatment, almost all the extrinsic V₁ subunits of the V-ATPase were observed to increase 1.5–4.3-fold in endosomal DRMs, whereas the intrinsic Vₒ subunits did not change except for subunit d. The variation in -fold increase in the V₁ subunits may reflect a stepwise assembly of V₁ in association with Vₒ as suggested by Kane et al. (38). The change in subunit d, which is not an integral membrane protein, may reflect stepwise assembly of the full Vₒ domain as suggested by Forgac (39) (Fig. 1B). The proteomic studies suggest that EGF stimulates the rapid assembly of the V-ATPase at the endosomal membrane.

**FIGURE 1.** Proteomic analysis of EN-DRMs/rafts reveals an EGF-dependent change in abundance of V-ATPase V₁ but not Vₒ intrinsic subunits. A, a schematic of the V-ATPase holoenzyme is shown (based on the model from Nishi and Forgac (23)). V₁ subunits are shown in light gray, and Vₒ subunits are in dark gray. B, proteomic results for the identification of V-ATPase subunits in hepatic endosomal rafts are shown. Endosomal rafts were prepared from rat livers at 5 min after EGF (1.0 μg/100 g body weight) or vehicle administration and subjected to proteomic analyses as described under “Experimental Procedures.” The Mascot data base identifiers for each subunit are noted. The mean peptide “count” for each subunit was calculated by summing the results of all the subunit isoforms and is listed for three replicate studies of controls (C ± S.D.) and EGF-treated (E ± S.D.) rats. The -fold change in peptide count for EGF-treated versus control endosomal rafts demonstrates an EGF-dependent augmentation of V₁ but not Vₒ subunits as shown in the upper and lower panels, respectively.

**EGF-induced Recruitment of V₁ Subunits to the Vacuolar System**—The proteomic results, indicating V₁ subunit recruitment, were confirmed by immunological methods. Immunoblotting of rat liver-combined endosomal fractions and endosomal DRMs for subunit V₁E and Vₒa demonstrated recruitment of the former but not the latter after EGF administration (Fig. 2, A and B). It should be noted that this endo-
somal fraction is depleted of lysosomes (40), which are known to contain a high concentration of V-ATPase. In addition, we employed immunofluorescence microscopy to localize V1E in primary rat hepatocytes (Fig. 2, C and D). After EGF stimulation for 5 min, V1E labeling can be seen to shift from a primarily diffuse basal pattern to a more vesicular pattern consistent with labeling of the vacuolar system. We did observe V1E-labeled vesicles in the basal state, but this population of structures, which are most likely lysosomes and late endosomes, increased markedly after EGF treatment. A negative control antibody (non-immune chicken IgY) was used to demonstrate the specificity of the V1E labeling (Fig. 2C). Double-blind quantification of multiple independent experiments confirmed differential localization of V1E after EGF treatment (Fig. 2D). Comparable results were obtained using an antibody against V1B (data not shown); however, detailed studies were pursued with antibody to V1E as this proved of superior quality in our hands.

The rapid effect of EGF on V1E localization to vacuolar structures prompted us to identify them more fully. Fig. 2E shows that after EGF stimulation, V1E co-localizes with LAMP1, which is a late endosomal and lysosomal marker but not EEA1 or Rab5. These data taken together with the recruitment of V1E to lysosome-free endosomal fractions leads us to conclude that EGF stimulates the rapid recruitment of V1E to late endosomes as well as lysosomal structures.
Because the V-ATPase is responsible for the acidification of intracellular organelles, we sought to determine whether EGF-induced recruitment of V1 V-ATPase subunits to the vacuolar system could cause a decrease in the pH of these structures. We performed DAMP labeling in primary hepatocytes and found that EGF induced acidification of vacuolar structures which was sustained for as long as 20 min (Fig. 2F).

**Effect of Bafilomycin on EGF Action**—Bafilomycin, a pleco-macrolide antibiotic that specifically and potently inhibits the vacuolar ATPase (41), has been shown to bind to both the V0c (42) and V0a (43) subunits. Bafilomycin was previously observed to inhibit insulin-stimulated mitogenesis in primary hepatocytes (44), Swiss 3T3 cells (45), BNL (murine embryonic liver) cells (46), and eight different human cancer cell lines (47). In this study we showed that EGF-stimulated DNA synthesis in primary rat hepatocytes was also inhibited by bafilomycin using both 10 and 100 nM EGF (Fig. 3A). Notably bafilomycin maintained the cellular content of the EGFR for 6 h, during which time EGFR remained tyrosine-phosphorylated (Fig. 3, B and C). Thus, bafilomycin treatment decoupled the relationship between EGFR tyrosine phosphorylation status and its mitogenic potential.

To determine the basis by which bafilomycin inhibited EGF-induced DNA synthesis, we extended our studies on EGF signaling. Activation of both the PI3K/Akt (30) and Erk pathways is important for EGF-dependent mitogenesis (30) and Erk pathways is important for EGF-dependent mitogenesis in primary rat hepatocytes. An assessment of the time course of EGF-induced phosphorylation of Akt and Erk clearly demonstrated no inhibitory effect of bafilomycin on these processes (supplemental Fig. S1).
Bafilomycin Inhibits EGF-induced mTORC1 Activation—
However, bafilomycin markedly inhibited EGF-stimulated activation of p70S6K as measured by Thr(P)-389 (Fig. 4, A and B) and p70S6 kinase activity (Fig. 4C) as well as EGF-stimulated phosphorylation of 4E-BP1 (Fig. 4D). Both p70S6K and 4E-BP1 are downstream of the mammalian target of rapamycin (mTOR) and generally reflect mTORC1 activity (48). The inhibitory effect of bafilomycin on p70S6K and 4E-BP1 phosphorylation is compatible with previous data showing that rapamycin inhibited EGF-dependent DNA synthesis in hepatocytes (20).

Chloroquine Mimics the Effect of Bafilomycin—To confirm that the bafilomycin effect was due to its inhibition of endosomal acidification, we investigated the effect of chloroquine, an acidotropic inhibitor of vacuolar acidification (49). As shown in supplemental Fig. S2, A and B, chloroquine also inhibited activation of p70S6K and 4E-BP1 but had no inhibitory effect on Akt and Erk signaling. Under our experimental conditions bafilomycin and chloroquine did not induce cell apoptosis as judged by the absence of cleavage of caspase 3 and no change in that of poly(ADP-ribose) polymerase (supplemental Fig. S2C).

We also assessed the effect of bafilomycin on insulin signaling and found that here as well there was no effect on Akt and Erk activation by insulin but a noticeable reduction in TORC1 activation as inferred from the inhibition of p70S6K and 4E-BP1 phosphorylation (supplemental Fig. S3). Therefore, it appears that the acidic pH of the endosomal milieu is required for hormone and growth factor activation of mTORC1-dependent signaling.

Bafilomycin and Upstream Regulators of TORC1 Activation—
Mammalian TORC1 is composed of mTOR, Raptor, mammalian lethal with Sec13 protein 8 (mLST8), and proline-rich Akt substrate 40 kDa (PRAS40) and is sensitive to the inhibition of macrolide antibiotic rapamycin. Its activation and downstream events can be influenced by many factors, including amino acids, glucose (for review, see Ref. 48) and growth factors. We demonstrated that mTOR and Raptor association status was
unchanged by bafilomycin (supplemental Fig. S4A), so the inhibitory effect of bafilomycin on mTORC1 is not a “rapamy-
cin-like” effect.

Mammalian TORC1 activity is linked to energy status in a
manner independent from nutrient status (50). AMP-activated
protein kinase (AMPK) is the sensor of ATP levels in cells (51),
and AMPK has been shown to inhibit mTORC1 by phosphor-
ylation of tuberous sclerosis complex 2 (TSC2), an upstream
GTPase activating protein (GAP) that regulates Rheb activity
(52). We, therefore, assessed AMPK status by immunoblotting
for pT172 of AMPK, the target of upstream liver kinase B1 (53)
and a readout of AMPK activity status (54). Supplemental Fig.
S4B shows that EGF and insulin gradually deactivated AMPK
in primary hepatocytes, and bafilomycin did not interfere with
this process.

The interaction of PRAS40 with mTORC1 inhibits the activ-
ity of mTORC1 (55). Phosphorylation of PRAS40 reduces its
binding to mTOR, thus, relieving its inhibitory constraint on
mTORC1 activity (56). Growth factor-dependent phosphory-
lization of PRAS40 is effected by Akt activation and modulated
by intracellular amino acid levels (57). We found that EGF-in-
duced phosphorylation of PRAS40 at 15 min was decreased by
both bafilomycin (by ~20%) and chloroquine (by ~30%) (Fig.
5A). This was not due to an inhibition of PIM1 kinase (data not
shown), which was recently reported to regulate mTOR activity
by phosphorylation of PRAS40 at Thr-246 (58). Because bafi-
loymycin had no effect on EGF-induced Akt activation (sup-
plemental Fig. S1A) as further confirmed by lack of change in
Akt-dependent phosphorylation of TSC2 at Thr-1462 site (sup-
plemental Fig. S4C), we inferred that bafilomycin might inhibit
EGF-induced mTORC1 activity through influencing amino
acid sufficiency.

**Bafilomycin and the Levels of Intracellular Amino Acids**—
Using a chromogenic technique to measure amino acids (L-
amino acid quantitation kit from Biovision), we found that bafi-
loymycin had no effect on total free amino acid levels in primary hepatocytes (supplemental Fig. S4D). This
prompted us to do further individual intracellular amino acid
analysis by HPLC. Consistent with our data using the chromo-
genic method, we found total intracellular amino acid levels to
be unchanged after bafilomycin. However, interestingly, the
total concentration of essential amino acids decreased dramat-
ically with bafilomycin treatment (Fig. 5B, top), whereas that of
non-essential amino acids remained unchanged in EGF-treated
primary hepatocytes. Notably, the intracellular concentration
of the amino acids leucine, isoleucine, and arginine, whose suf-
ciency is critical for mTORC1 activation (59), decreased dra-
matically with bafilomycin in EGF-treated primary hepatocytes
(Fig. 5B, bottom, and supplemental Table S2). It is also noteworthy
that the concentrations of essential and non-essential amino acids
in the medium were 2764 and 4259 nmol/ml respectively, which were ~10 times higher than the corre-
sponding intracellular amino acid levels (supplemental Table
S2). Thus intracellular amino acid levels in cultured hepatocytes
appear to reflect primarily the balance between the intracellu-
lar processes of protein synthesis and proteolysis.

To complement these observations, we sought to investigate
whether neutralizing endosomal pH could affect amino acid
transport into cultured hepatocytes. This is relevant in view of
the report that in skeletal muscle chloroquine can inhibit the
movement of amino acid transporters to the plasma mem-
brane, thus impairing amino acid uptake (60). The branched
chain amino acid leucine is sensed by cells as a reporter of exter-
nal nutrient status. We thus determined leucine uptake in the
absence and presence of bafilomycin. Neither EGF nor insulin
stimulated an increase in leucine uptake (supplemental Fig.
S4E) nor did bafilomycin inhibit uptake. To further eliminate

**FIGURE 5.** Effect of bafilomycin on PRAS40 phosphorylation and intracellu-
lar amino acid levels. A, hepatocytes were pretreated with DMSO, 100 nM
bafilomycin, or 100 μM chloroquine for 30 min and then incubated with 10 nM
EGF for the times shown. Cell lysates were subjected to SDS-PAGE followed by
immunoblotting with anti-phospho-PRAS40 (Thr-246), PRAS40 antibody, and
Hsp90 antibody (loading control) (top). The results are from a single exposed
gel that was sliced to allow side-by-side comparison of the data. Densitomet-
ric quantification was performed on immunoblotting data of at least three
independent experiments. *, p < 0.05; bars indicate S.E. (bottom). B, hepa-
tocytes were pretreated with DMSO ± 100 nM bafilomycin for 30 min and then
incubated with 10 nM EGF for 30 min. Individual intracellular amino acids were
determined by HPLC analysis as described under “Experimental Procedures.”
Total concentration of essential amino acids and non-essential amino acids
were calculated by adding the respective individual amino acids concentra-
tion together. Quantification was performed on three independent experi-
ments. *, p < 0.001 (top); *, p < 0.05; **, p < 0.005 (bottom). Bars indicate S.E.
FIGURE 6. Effect of cycloheximide on mTORC1 activation and intracellular amino acid levels. A, hepatocytes were pretreated with either DMSO, 100 nM bafilomycin (Baf), 25 μg/ml cycloheximide (CHX), or Baf and CHX together (Baf+CHX) for 30 min and then incubated with 10 nM EGF for another 30 min. Individual intracellular amino acids were determined by HPLC analyses as described under “Experimental Procedures.” Total concentrations of essential and non-essential amino acids were calculated by adding the respective individual amino acids concentration together. Quantification was performed from three independent experiments. *, p < 0.005; bars indicate S.E. B, cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-phospho-p70S6K (Thr-389) and p70S6K antibody (top). Quantification was performed from three independent experiments. *, p < 0.0005; bars indicate S.E. (bottom). C, cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-phospho-p70S6K (Thr-389) and p70S6K antibody (top). Quantification was performed from three independent experiments. *, p < 0.0005; bars indicate S.E. (bottom). D, hepatocytes were treated as indicated, samples of cell lysates were subjected to affinity chromatography on 7-methyl-GTP-Sepharose, and bound materials were subjected to SDS-PAGE followed by immunoblotting with anti-eIF-4E, eIF-4G, and 4E-BP1 antibody. The signals of 4E-BP1 and eIF-4E were quantified and presented as a ratio. Quantification was performed from three independent experiments. *, p < 0.005; bars indicate S.E. E, hepatocytes were pretreated with 50 μM MG132 for 5 h or/and 25 μg/ml cycloheximide for 30 min and then incubated with 10 nM EGF for the times shown. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-p70S6K (Thr-389) and p70S6K antibody (top). Quantification was performed from three independent experiments. *, p < 0.05; **, p < 0.005; bars indicate S.E. (bottom). The right top figure shows induction of REDD1 by CoCl2, as a positive control. Quantification was performed on six independent replicates. Data are normalized to control values. *, p < 0.05; bars indicate S.E. (bottom). F, hepatocytes were pretreated with 50 μM MG132 for 5 h or/and 25 μg/ml cycloheximide for 30 min and then incubated with 10 nM EGF for the times shown. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-RED1 p70S6K antibody (bottom).
markedly inhibited EGF-stimulated activation of p70S6K as measured by Thr(P)-389. At the same time, cycloheximide dramatically reversed the inhibition of MG132 by restoring the phosphorylation of p70S6K in EGF-treated hepatocytes (Fig. 6F).

**Effect of in Vivo Chloroquine on mTOR Signaling**—We sought to evaluate the effect of inhibiting vacuolar acidification on mTOR signaling in vivo. Fasted rats were treated with chloroquine or normal saline, and EGF was then administered. At 15 min after EGF administration, rat liver endosomes and cytosols were prepared and subjected to Western blot analysis. As shown in Fig. 7A, chloroquine administration markedly inhibited EGF-stimulated activation of p70S6K as measured by Thr(P)-389, during which time there was augmented accumulation of EGFR and tyrosine-phosphorylated EGFR in endosomes after chloroquine (Fig. 7B). Ras homologue enriched in brain (Rheb) activates mTOR after binding directly to its kinase domain (63). Recent reports have suggested a movement of mTOR to subcellular structures where Rheb resides in response to amino acids (64, 65). Intriguingly, in our study EGF induced a marked recruitment of Rheb into endosomes, whereas chloroquine significantly inhibited this recruitment. The endosomal content of mTOR was unaffected by either EGF or chloroquine treatment. EGF also induced an increase of Rheb in rat liver lysosomal fractions, whereas the content of mTOR was unchanged (Fig. 7C).

**DISCUSSION**

In this study we extended earlier observations on endosomal events after EGF treatment (6, 10, 15, 66). Of particular note was the finding that EGF induced the accumulation of V1 components of the V-ATPase in endosomal DRMs/rafts, whereas the V0 components showed little or no change in this fraction. The augmentation of V1 components in DRMs/rafts did not solely reflect translocation of V1 components from non-hydrophobic to hydrophobic membrane domains as we could dem-
Demonstrate that there was net recruitment of V₁ subunits to intact endosomes.

Disassociation of the V₁ and V₀ components of the holoenzyme was first observed in insect tissue (67), and their reversible assembly/disassembly was documented in Saccharomyces cerevisiae (26) and renal epithelial cells (28). This is now recognized as an important regulatory mechanism of V-ATPase function (68, 69). It is possible that EGF-induced V₁/V₀ assembly follows activation of PI3K in analogy with the demonstrated mechanism by which glucose promotes the assembly of V₁ and V₀ complexes in renal epithelial cells (28).

Using DAMP fluorescence to measure vacuolar pH, we found that the recruitment of V₁ components was accompanied by augmented vacuolar acidification. This represents the first demonstration of a growth factor inducing the rapid assembly of the V-ATPase as a functional holoenzyme.

The mTORC1 molecular complex is a highly conserved regulator of cell growth and proliferation, responding to a range of signals relating to the energy and nutrient status of the cell (48). In previous work we showed that mTOR is the critical regulator of EGF-induced cell growth and DNA synthesis in primary hepatocytes (20). In this study we examined the role of EGF-induced vacuolar acidification on EGF action by neutralizing vacuolar pH during EGF treatment. Bafilomycin, a specific inhibitor of V-ATPase, inhibited EGF-induced mTORC1 activation. Notably bafilomycin did not block EGF-induced Erk or Akt activation nor did it alter AMPK activity. That this effect derived from the inhibition of vacuolar acidification was supported by the use of the acidotropic agent chloroquine (70), which like bafilomycin, selectively inhibited mTORC1 activation.

In this study the inhibition of EGF-induced PRAS40 phosphorylation at Thr-246 was decreased by both bafilomycin and chloroquine. Amino acid deficiency has been observed to decrease PRAS40 phosphorylation (57). A number of observations suggested that cell surface amino acid transporters may play a role in amino acid-dependent mTORC1 activation (71–73). However, in our studies preincubation of primary-cultured hepatocytes with bafilomycin for 30 min resulted in a decrease of the intracellular concentration of essential amino acids despite amino acid concentrations in the incubation medium that were ~10 times higher than the corresponding intracellular amino acid levels. Combined with the fact that EGF did not stimulate an increase in leucine uptake nor did bafilomycin inhibit uptake, we propose that the inhibition of V-ATPase by bafilomycin affects intracellular essential amino acid levels primarily by an intracellular process rather than by the modulation of amino acid transporter function at the plasma membrane.

It has been previously noted that the intracellular level of amino acids can be refractory to changes in the level of extracellular amino acids (74). Studies in rats (75, 76) have shown that ethanol and fasting can produce significant changes in the levels of amino acids and related compounds in plasma and various tissues. However, the changes in tissue amino acid levels were found to be highly tissue-specific and generally unrelated to changes seen in the plasma. These observations indicate that individual tissues have strong regulatory mechanisms essential to maintaining intracellular amino acid homeostasis. By using inhibitors of protein synthesis and autophagy Beugnet et al. (34) showed that the regulation of mTOR was responsive to the levels of intracellular amino acids, which were critically determined by the intracellular rates of protein synthesis and degradation. In our study the reduction in essential amino acid levels after the inhibition of vacuolar acidification occurred despite a large excess of extracellular amino acids. Thus, in cultured hepatocytes, intracellular amino acid levels largely reflected a balance between protein synthesis and protein degradation. We, therefore, suggest that bafilomycin/chloroquine blocked EGF-induced mTORC1 activation by inhibiting lysosomal proteolysis, leading to reduced intracellular levels of essential amino acids. The fact that only the levels of essential amino acids decreased may reflect the total dependence of intracellular essential amino acid levels on recycling in the absence of inflow from the extracellular space. This possibility is supported by the observation that cycloheximide prevented the bafilomycin-induced decrease in intracellular levels of essential amino acids and correspondingly prevented the inhibition by bafilomycin of mTORC1 activation by EGF.

A recent study in Caenorhabditis elegans showed that EGF signaling alters protein homeostasis by increasing ubiquitin proteasome system activity and polyubiquitination while decreasing protein aggregation (77). Interestingly inhibition of proteasome-dependent proteolysis by MG132 in our study also inhibited EGF-stimulated activation of p70S6K, and this effect was dramatically reversed by cycloheximide, again emphasizing a role for proteolysis in maintaining intracellular amino acid levels, thus sustaining mTORC1 activation.

In previous studies it was observed that Rag GTPase heterodimers are activated by amino acids, enabling their interaction with Raptor to promote the translocation of mTORC1 to lysosomes where its association with Rheb leads to its activation (64, 65, 78, 79). This likely involves late endosomes as well, as blocking early to late endosomal trafficking prevented the interaction between mTOR and Rheb in the latter compartment (80).

In our work we did not observe mTOR recruitment to endosomes. We speculate that this might reflect a difference between the significant amino acid deprivation in the studies of Sancak et al. (65) compared with the relatively milder level of intracellular amino acid deprivation effected by inhibiting vacuolar acidification (Fig. 5B). Indeed, using immunofluorescence microscopy, we found in cultured HepG2 cells that amino acid deprivation promoted a highly dispersed distribution of mTOR that returned to a punctuate pattern after amino acid supplementation (data not shown). The localization of endogenous Rheb was not demonstrated by Sancak et al. (65). However, using rat liver fractionation, we showed that EGF induced the translocation of endogenous Rheb, but not mTOR, to late endosomes/lysosomes. Notably, preventing vacuolar acidification with chloroquine significantly inhibited both this recruitment and mTORC1 activation but did not affect mTOR levels in the endosome preparations. Perhaps mTOR dissociates from endosomes/lysosomes only under more extreme amino acid deprivation. Interestingly we observed an EGF-dependent decrease in RagA/C coincident with the recruitment of
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Rheb to our endosome preparations (supplemental Fig. S6). A decrease in lysosome-bound Rag consequent to mTORC1 activation was previously observed by others (65). We suggest that this reflects the displacement of the Rag heterodimer consequent to binding to and activation of mTOR by Rheb (Fig. 7D).

Our observations suggest that EGF induces an increase of lysosomal proteolysis that releases essential amino acids from lysosomes, thus, sustaining cytosolic amino acid levels and contributing to mTORC1 activation (Fig. 7D). During preparation of this manuscript, Zoncu et al. (81 proposed that the V-ATPase complex senses amino acids accumulating in the lysosomal lumen leading to mTORC1 translocation and signaling (81). This model, which places the active V-ATPase downstream of amino acids, could explain some of our observations; however, other findings argue extra-lysosomal amino acids play an important role. Thus, we observed no or minimal effect of the V-ATPase inhibitor bafilomycin on cycloheximide induced mTOR activation, suggesting that this activation was evoked by extra-lysosomal amino acids that accumulate in the presence of cycloheximide. Furthermore the acidotropic agent chloroquine, which has no inhibitory effect on V-ATPase activity (82), mimicked the effect on mTORC1 of inhibiting V-ATPase by bafilomycin. This strongly suggests that vacuolar acidification promoted by V-ATPase recruitment but not V-ATPase activity itself facilitates mTORC1 activation by EGF.

In our study we found that Rheb, not mTORC1, was recruited to late endosomes and lysosomes by EGF. Because inhibiting acidification by chloroquine blunted Rheb recruitment, then it is possible that the recruitment of Rheb is mediated by EGF-induced acidification in concert with Rheb activation (i.e. conversion to Rheb\(^{GTP}\)) via TSC2 inhibition. Because EGF induced vacuolar acidification would appear to assure intracellular amino acid sufficiency and growth factor-dependent mTORC1 activation is strongly dependent on the presence of essential amino acids (59, 83), it is possible that the amino acid availability is sensed by Rheb and promotes Rheb translocation to late ENs/lysosomes to activate mTORC1 signaling. One should also consider the possibility of cell/tissue-specific mechanisms involved in mTORC1 activation as exemplified perhaps by our studies in rat liver versus those in cultured HEK 293 cells (81).

In summary this work is the first to show that EGF induces the recruitment of V₁ subunits to the V₀ domain to generate increased V-ATPase holoenzyme leading to increased vacuolar acidification. We propose that this leads to increased lysosomal proteolysis that assures an adequate supply of intracellular amino acids that would otherwise be depleted by augmented protein synthesis under the anabolic response to growth stimulation (84). At the same time, the EGF-induced vacuolar acidification is accompanied by Rheb recruitment. The abrogation of vacuolar acidification (viz. by bafilomycin) resulted in a decline of intracellular essential amino acid levels leading to an inhibition of EGF induced mTORC1 and mitogenesis. Thus, in addition to activating TORC1 via both increased Akt and Erk activation, EGF-induced vacuolar acidification may reflect a mechanism for maintaining adequate intracellular amino acid levels and Rheb targeting essential for the continuing activation of mTORC1 (85) (Fig. 7D).

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