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Hypoxia Inhibits Protein Synthesis through a 4E-BP1 and Elongation Factor 2 Kinase Pathway Controlled by mTOR and Uncoupled in Breast Cancer Cells†

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Hypoxia is a state of low oxygen availability that limits tumor growth. The mechanism of protein synthesis inhibition by hypoxia and its circumvention by transformation are not well understood. Hypoxic breast epithelial cells are shown to downregulate protein synthesis by inhibition of the kinase mTOR, which suppresses mRNA translation through a novel mechanism mitigated in transformed cells: disruption of proteasome-targeted degradation of eukaryotic elongation factor 2 (eEF2) kinase and activation of the regulatory protein 4E-BP1. In transformed breast epithelial cells under hypoxia, the mTOR and S6 kinases are constitutively activated and the mTOR negative regulator tuberous sclerosis complex 2 (TSC2) protein fails to function. Gene silencing of 4E-BP1 and eEF2 kinase or TSC2 confers resistance to hypoxia inhibition of protein synthesis in immortalized breast epithelial cells. Breast cancer cells therefore acquire resistance to hypoxia by uncoupling oxygen-responsive signaling pathways from mTOR function, eliminating inhibition of protein synthesis mediated by 4E-BP1 and eEF2.

Hypoxia severely decreases ATP levels in cells (18). To survive, cells downregulate high-energy processes including protein synthesis. Hypoxia is also a key feature of the solid tumor microenvironment, and overcoming translational regulation during hypoxia is thought to be an important step in progression to malignant disease (6, 19, 28, 32). Alterations in the protein synthesis machinery, particularly in initiation and elongation factors, can facilitate overexpression of proteins required for cellular transformation, promotion of angiogenesis, and inhibition of apoptosis, among others (38). Neither the mechanism of translational inhibition in nontransformed cells during hypoxia nor the process by which transformed cells circumvent this block is well understood.

Regulation of protein synthesis involves a number of different possible mechanisms acting on initiation and/or elongation steps of mRNA translation. Recruitment of mRNA to ribosomes in mammalian cells involves interaction of the 5′m^{7}GpppN (cap) structure on the mRNA with initiation factor eIF4E, also known as cap-binding protein. eIF4E is a component of the cap-initiation complex, a group of interacting proteins that bridge mRNA, the ribosome, and the initiation machinery to initiate protein synthesis. The cap-initiation complex contains the scaffold protein eIF4G, which binds eIF4E; the ATP-dependent RNA helicase known as eIF4A; a multisubunit factor known as eIF3 that binds the 40S ribosomal subunit; and the poly(A)-binding protein (PABP), which stimulates translation (15).

Three related eIF4E-binding proteins (4E-BP1, -2, and -3) inducibly regulate the formation of the cap-initiation complex and control cap-dependent mRNA translation. 4E-BP1, the major member of this family, binds eIF4E and competitively inhibits its association with eIF4G, preventing formation of the cap-initiation complex and reducing cap-dependent mRNA translation (reviewed in reference 15). Hyperphosphorylation of 4E-BP1 carried out by mTOR inhibits 4E-BP1 binding to eIF4E, thereby promoting protein synthesis (reviewed in reference 16). The elongation step of protein synthesis is also subject to inhibition by phosphorylation of eukaryotic elongation factor 2 (eEF2) at position Thr-56. eEF2 mediates ribosomal translocation during peptide chain elongation. eEF2 is phosphorylated by eEF2 kinase (eEF2K), which is both inhibited and activated by phosphorylation at a number of sites (5). Both mTOR and the p70 ribosomal S6 kinase (p70^{S6k}) inhibit eEF2K by phosphorylation, in turn upregulating eEF2 activity and protein synthesis (4, 46). mTOR activity in hypoxia may also be regulated in part through AMP-activated kinase (AMPK) and p70^{S6k} (Fig. 1A). AMPK acts as an ATP energy-sensing signal protein (48). AMPK downregulates protein synthesis (3, 21) by decreasing the activity of ribosome-associated kinase p70^{S6k} (3, 26), which inhibits eEF2K activity (3). AMPK can also activate the tuberous sclerosis complex 2 (TSC2) protein, which with TSC1 inhibits mTOR (Fig. 1A) (24). mTOR inhibition during hypoxia can also occur in an AMPK-independent manner, involving transcriptional upregulation of the hypoxia gene *REDD1* (*r*egulated in *d*evelopment and *D*NA *d*amage *r*esponse) and the TSC1/2 complex (6, 12).

Many nontransformed cells undergo hypoxia inhibition of protein synthesis, whereas highly transformed cells are largely resistant (19, 49). Although general translation is downregulated during hypoxia, mRNAs important for adaptation to hypoxia, such as hypoxia-inducible factors 1α and -β (HIF-1α/β),

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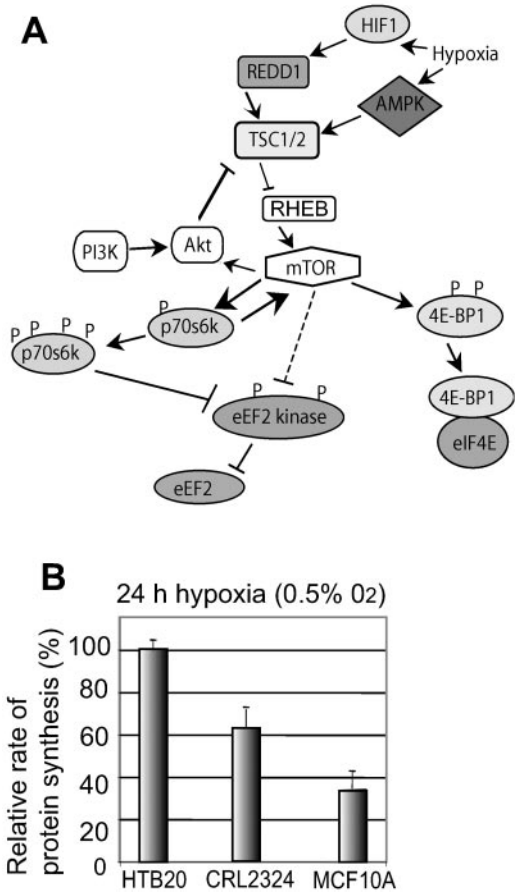

FIG. 1. (A) Diagrammatic representation of the mTOR signaling pathway involved in translational control. Arrows indicate activation, bars indicate inhibition, and dotted lines indicate uncertainty as a primary or established mechanism. Figure based on references 35 and 23. (B) Protein synthetic rates following 24 h of hypoxia. Cells were grown for 24 h under atmospheric oxygen (normoxic) or hypoxic (0.5% O_2) conditions. Cultures were labeled for 1 h with [^{35}S]methionine, lysates were prepared, and rates of protein synthesis were determined by protein-specific activity derived by trichloroacetic acid precipitation and scintillation counting of samples containing equal amounts of protein. The results are the means with standard deviations derived from at least three independent experiments performed in duplicate. Data were normalized to the mean value of normoxic HTB20 cells.

vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), continue to be translated (22, 44). Translation in these cases is often cap independent due to the presence of an internal ribosome entry site (IRES) in the 5′-noncoding region of the mRNA (18, 44, 45). An IRES typically bypasses cap-dependence in recruiting ribosomes to the mRNA, utilizing direct binding of translation factors eIF4G and eIF3 that interact with ribosome subunits (34). Studies in nontransformed and immortalized cells demonstrate a significant decrease in cap-dependent translation rates (>50%) during hypoxia (27, 44) commensurate with reduced ATP levels (17). However, many studies which report translation regulation during hypoxia were actually conducted under anoxic conditions (0.00 to 0.02% O_2) or with combined multiple stresses such as hypoxia plus serum starvation (1, 2, 25, 29, 45). Hypophosphorylation (activation) of 4E-BP1 is reported during hypoxia (1.5% O_2) with combined serum starvation in highly transformed human embryonic kidney cells (HEK293), as well as during mild hypoxia (5.0% O_2) in serum-starved rat hepatocytes (1a, 45). However, activation of 4E-BP1 cannot be sufficient to explain the inhibition of protein synthesis, because hypoxia induces a greater inhibition of global translation than treatment with only rapamycin (45), a potent inhibitor of mTOR known to fully activate 4E-BP1. There has not been a systematic evaluation of the effect of transformation on protein synthetic activity under hypoxic conditions or the mechanism of inhibition or resistance. We therefore characterized the mechanisms of translational regulation that occur during hypoxia (0.5 to 1.0% O_2) in breast epithelial cell lines and how regulation is altered with increasing transformation.

MATERIALS AND METHODS

Antibodies and cells. Rabbit polyclonal antiserum to eIF4G was previously described (13). Mouse monoclonal anti-eIF4A antibody was provided by W. Merrick (Case Western Reserve University, Cleveland, OH). Other antisera were from commercial sources and include mouse monoclonal anti-FLAG antibody (Sigma), rabbit polyclonal anti-eIF2α P (Ser51) antibody (Biosource), rabbit polyclonal anti-eIF2α antibody (Santa Cruz Biotechnology), and horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibodies (Amersham). The enhanced chemiluminescence (ECL) system (Amersham) was used for detection. All other antibodies were from Cell Signaling Technology. All cell lines used for these studies were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were MCF10A, CRL2324, HTB20, HTB25, HTB121, and CRL-1902. Cells were grown under the guidelines of the American Type Culture Collection in their recommended media.

Hypoxia treatments. Hypoxic culture conditions (0.5% O_2) were achieved in a custom-designed hypoxic incubator by continuous infusion of a preanalyzed gas mixture (95% N_2, 5% CO_2) (Reming BioInstruments, Redfield, NY). All experiments were performed with exponentially growing cells plated at approximately 40% cell density and then made hypoxic 18 to 24 h later. Hypoxic medium was preequilibrated for 6 h in the hypoxia chamber. Normoxic cells used for comparison were grown and treated under atmospheric oxygen in parallel.

[^{35}S]methionine incorporation assay. Cells were labeled with 50 μCi of [^{35}S]methionine per ml (Easytag Express protein labeling mix; Dupont/NEN) in Dulbecco's modified Eagle's medium (DMEM) without cold methionine for 1 h, washed twice with phosphate-buffered saline (PBS), and lysed in 0.5% NP-40 lysis buffer (0.5% NP-40, 50 mM HEPES, pH 7.0, 250 mM NaCl, 2 mM EDTA, 2 mM sodium orthovanadate, 25 mM glycerophosphate, and 1 tablet of protease inhibitor [Roche] per 10 ml) at 4°C. Lysates were clarified by centrifugation for 10 min at 13,000 × *g*. Specific activity of methionine incorporation was determined by trichloroacetic acid precipitation onto GF/C filters and liquid scintillation counting. Labeling of hypoxic samples was performed within the hypoxic chamber with preequilibrated media.

Western immunoblot analysis. Following treatments, cells were washed twice in ice-cold PBS, lysed in 0.5% NP-40 lysis buffer at 4°C, and clarified by centrifugation at 13,000 × *g* for 10 min. Protein concentrations were determined for each sample by Bradford assay (Bio-Rad, Hercules, CA). To determine the total levels and phosphorylation status of specific proteins, equal amounts of protein from NP-40 lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by protein immunoblotting with specific antibodies. The phosphorylation status of 4E-BP1 was determined by SDS-15%-PAGE, whereas 8% gels were used to determine total 4E-BP1 levels. The phosphorylation status of $p70^{S6k}$, TSC2, Akt, mTOR, eIF4E, eEF2, and eIF2α was determined by first immunoblotting the membrane with phospho-specific antibody and then stripping the membranes using Restore Western blot stripping buffer (Pierce), followed by reprobing the membranes with non-phospho-specific antibodies.

eIF4G immunoprecipitation and immunoblot analysis. Equal amounts of protein from NP-40 lysates were precleared for 1 h at 4°C with 30 μl of protein A-Sepharose (Santa Cruz Biotech) and then incubated overnight with the indicated antiserum (preimmune serum or anti-eIF4G C-terminal fragment) at 4°C. Protein A-Sepharose was added, and incubation was continued for 1 h at 4°C

before precipitates were washed four times with 1 ml NP-40 lysis buffer, boiled in SDS-sample buffer, and analyzed by SDS-PAGE and immunoblotting.

Analysis of eIF4E and 4E-BP1 interaction. Equal amounts of protein from NP-40 cell lysates were incubated with 7-methyl GTP-Sepharose 4B (30 μl of settled bed volume) overnight at 4°C. Pelleted beads were washed four times with 1 ml NP-40 lysis buffer and resuspended in 0.7 ml of NP-40 lysis buffer plus 1 mM GTP for 1 h at 4°C. Following a final four washes with 0.75 ml of NP-40 lysis buffer, the beads were suspended in sample buffer and boiled, and the bound proteins were analyzed by SDS-PAGE and immunoblotting. Unbound 4E-BP1 was precipitated by adding 1 ml of 100% ethanol for 20 min at −80°C, and the precipitate was recovered by centrifugation at 14,000 × *g* for 10 min. The pellet was solubilized in 1× SDS-sample buffer, heated to 37°C for 20 min before boiling, and analyzed by SDS-PAGE and immunoblotting.

Retroviral expression studies. Constitutively active FLAG-GADD34 C-terminal protein fragment (A1) cloned into pBABE-puro, a retrovirus expression vector, was provided by D. Ron (NYU Medical School, New York, NY) (33). HEK293 cells at 80% confluence in 10-cm-diameter dishes were transfected with 5 μg each of pVPack-VSV-G and FLAG-GADD34 pBABE-puro or pBABE-puro as a control with the use of Lipofectamine Plus (Invitrogen). Retrovirus-containing supernatants were harvested after 48 h, passed through a 0.22-μm filter, and frozen at −80°C until use. Target cells were subsequently infected by the addition of retrovirus-containing supernatants to the medium along with Polybrene (8 μg/ml). Target cells were selected 24 h following infection by the addition of puromycin (1.5 μg/ml). Cells shown by Western blotting to express high levels of FLAG-GADD34 following puromycin selection were released from selection for 48 h before treatment with hypoxia.

RNAi expression. For RNA interference (RNAi), interfering RNAs were delivered either directly by transfection of small interfering RNAs (siRNAs) or by transduction of cells with lentivirus short hairpin RNA (shRNA) expression vectors. Double-stranded siRNAs were designed following the procedure described by Elbashir et al. (14), directed to either the 5′- or 3′-untranslated regions. Target sequences were aligned with the human genome database in a BLAST search to eliminate those with significant homology to other genes. Lyophylized siRNA duplexes were synthesized (QIAGEN Corp.), dissolved in siRNA suspension buffer (l00 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) to a final concentration of 1 μg/μl, and stored at −20°C until use. Universal negative (nonsilencing) control siRNA was purchased from QIAGEN. To suppress 4E-BP1 or eEF2K expression, MCF10A cells were transfected with 5 μg of siRNA in six-well plates, 24 h after plating at 30% density, using Oligofectamine reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. siRNA transfections were repeated 24 h later; the next day, cells were split and allowed to recover for 48 h. This process was then repeated twice more. Once transfected cells demonstrated a significant decrease in protein levels of targeted genes by immunoblot analysis, they were subjected to hypoxia. For lentivirus expression of shRNAs, target sequences were inserted into pLK0.1 downstream of the U6 promoter. To produce virus containing the shRNA-generating cassette, 293GP cells were transfected with 4 μg each of pCI-VSV-G, pCMVΔ8.2R′, and pLK0.1 with the Fugene (Roche). Target cells were infected in the presence of Polybrene (4 mg/ml) and selected with puromycin at 3.0 μg/ml for 48 h.

RESULTS

Hypoxia inhibition of protein synthesis is inversely related to cellular transformation. We first determined the effect of hypoxia (0.5% O_2) on protein synthesis in immortalized and increasingly transformed human breast epithelial cells of invasive ductal carcinoma derivation. Spontaneously immortalized human breast epithelial cells (MCF10A) were originally cloned and immortalized from breast epithelium of a patient diagnosed with fibrocystic disease and possess normal karyotype and differentiation markers (43). Human carcinoma cell line CRL2324 was derived from a primary infiltrating ductal carcinoma without lymph node involvement (stage I, grade 3) (50). Human carcinoma cell line HTB20 was derived from a primary invasive ductal carcinoma with multiple distant metastases (stage IV) (50). Other cell lines of similar transformation state were also utilized for confirmatory evaluation, as described in Materials and Methods. These cell lines were selected to meet criteria regarded as valid models for evaluating the pathobiology of ductal carcinoma (8). Resistance of protein synthesis to hypoxia was measured by comparing the ratio of specific activity of [^{35}S]methionine incorporation into protein in cells that had been cultured under 24 h of hypoxia compared to normoxic conditions. Studies showed that cell lines reached their respective maximal levels of inhibition by 16 to 20 h of hypoxia (data not shown). Under identical conditions, normoxic translation rates of MCF10A cells were approximately 40 to 50% and those of CRL2324 cells were 70% that of HTB20 cells (see data in the supplemental material). In immortalized MCF10A cells, hypoxia reduced the level of protein synthesis 70 to 80%, whereas partially transformed CRL2324 cells were reduced about 30%, and fully transformed HTB20 cells were virtually unaffected (Fig. 1B). Cells exposed to 24 h of hypoxia but returned to normoxia for 1 h before [^{35}S]methionine labeling rapidly recovered protein synthetic rates to the level of normoxic cells (within 2 to 3 h), and rates of uptake of [^{35}S]methionine into methionyl-tRNA were similar in normoxic and hypoxic cells (data not shown). There was also no decrease in cell viability for any of the cell lines during 24 h of hypoxia, as determined by dye exclusion assay and lactate dehydrogenase release assay (see data in the supplemental material). Importantly, analysis of other cell lines with comparable states of transformation provided similar results. For example, highly transformed metastatic tumor-derived HTB25, MCF-7, and HTB121 cells, equivalent in transformation to HTB20 cells, were also resistant to inhibition of protein synthesis during hypoxia, and primary tumor stage II-derived CRL1902 cells were inhibited by approximately 30%, similar to the transformed stage I-derived CRL2324 cells (data not shown). Thus, MCF10A, CRL2324, and HTB20 cells were used as representative of a panel of breast epithelial cells of increasing transformation.

Hypoxia activates 4E-BP1 and inactivates eEF2 through a novel mechanism uncoupled by transformation. The steady-state level of translation factors and their phosphorylation status, when indicative of activity, were examined in each cell line following 24 h of hypoxia. There was no change in the overall abundance of eIF4A between normoxic and hypoxic samples for each of the cell lines tested (Fig. 2A). eIF4A is therefore an appropriate control for protein loading. Levels of eIF4E in each cell line were unchanged by hypoxia treatment but were increased to two- to threefold more abundant in highly transformed HTB20 cells (Fig. 2A). The eIF4E binding inhibitor, 4E-BP1, was found to shift to the hypophosphorylated (activated) form during hypoxia in MCF10A cells which undergo translation inhibition, whereas 4E-BP1 was maintained in the hyperphosphorylated form in partial or fully transformed cells (Fig. 2A). Analysis of 4E-BP1 by low-resolution SDS-8% PAGE (to avoid separation of the phosphorylated species) demonstrated no change in overall abundance under hypoxia in any of the cell lines (Fig. 2B). Thus, either the activity of mTOR or its ability to target 4E-BP1 during hypoxia is uncoupled by transformation. eIF4G levels were unchanged by hypoxia but were fivefold more abundant in highly transformed HTB20 cells (Fig. 2B) and increased in other transformed cell lines (data not shown). eEF2 levels in all cell lines were unaltered by hypoxia but increased threefold with transformation (Fig. 2C). In immortalized MCF10A cells, eEF2 was shifted to

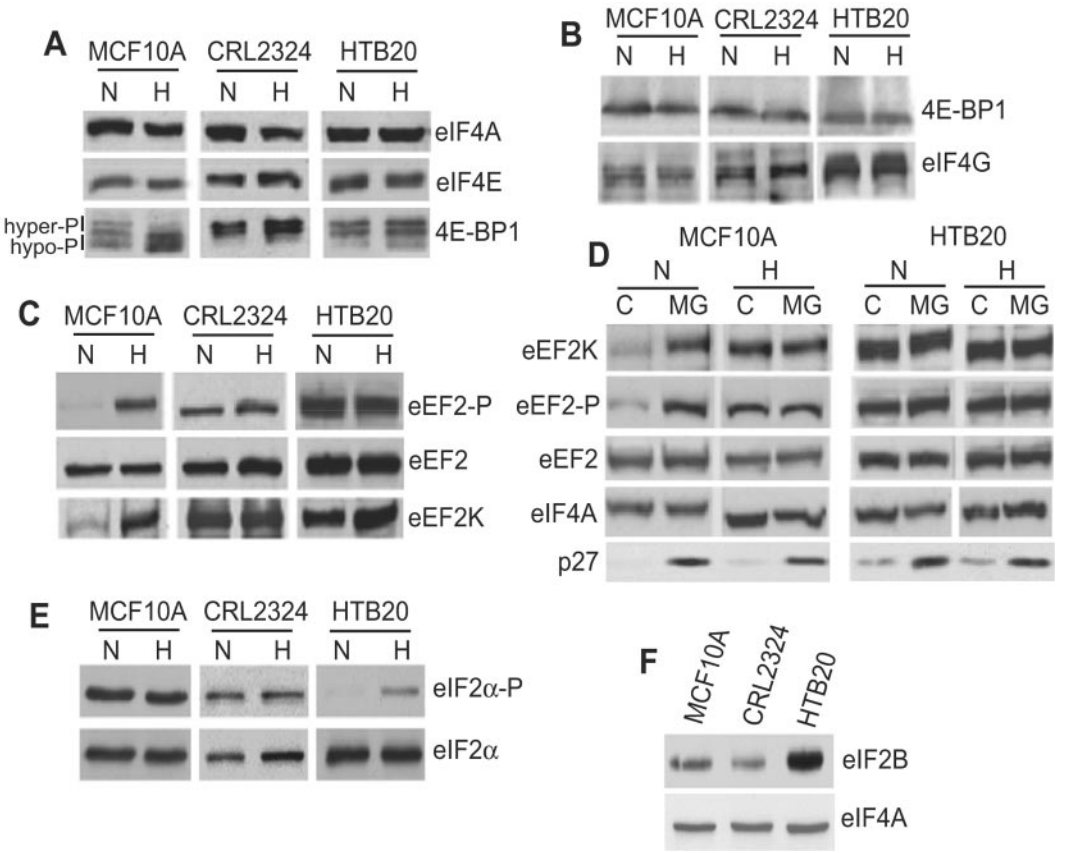

FIG. 2. Effect of hypoxia on abundance and phosphorylation of key translation and regulatory factors. MCF10A, CRL2324, and HTB20 cells were subjected to hypoxia (0.5% O_2) for 24 h (H) or in parallel were grown under normoxia (N) as a control. Cells were collected and lysed in 0.5% NP-40 buffer, normalized for soluble protein content, and resolved by SDS-PAGE, and proteins were identified by immunoblot analysis with specific antisera as shown. Representative immunoblots are shown. (A) Immunoblot analysis of equal amounts of total protein (50 μg) of eIF4A, eIF4E, and high-resolution separation of hyperphosphorylated (hyper-P) and hypophosphorylated (hypo-P) forms of 4E-BP1, resolved by SDS-15% PAGE. (B) Low-resolution SDS-8% PAGE analysis of 30 μg of cell lysates showing total eIF4G and 4E-BP1 levels, using extracts prepared as described above. (C) Immunoblot analysis of total eEF2K, Thr56 phosphorylated eEF2 (eIF2-P), and total eEF2 protein levels in 100 μg of protein lysates, prepared as described above. (D) Immunoblot analysis of total and phosphorylated forms of eEF2, total EF2K, eIF4A, and p27 cell cycle regulator as a control from 30 μg of lysate, prepared as described above, under hypoxic and normoxic conditions, with and without prior treatment of cells with proteasome inhibitor MG132 (MG) or control (C) vehicle. (E) Immunoblot analysis of serine 51 phosphorylated eIF2α and total eIF2α protein levels, prepared as described above. (F) Immunoblot analysis of total eIF2B protein levels in 30 μg lysate: only samples from normoxia are shown. Data were quantified by densitometry of autoradiograms from at least three independent experiments; representative results are shown.

the Thr56 phosphorylated (inactive) form during hypoxia, consistent with translation inhibition (Fig. 2C). A slight increase in eEF2 Thr56 phosphorylation was found in hypoxic CRL2324 cells, which are only partially inhibited in protein synthesis, and there was no increase in phosphorylation in HTB20 cells, which are almost fully resistant to translation inhibition (Fig. 2C). Increased eEF2 phosphorylation in hypoxic MCF10A cells was associated with a 5- to 10-fold increase in eEF2K protein levels (Fig. 2C) without an increase in eEF2K mRNA levels (data not shown), suggesting that the kinase may be stabilized against decay during hypoxia by phosphorylation. As it has been reported that EF2K can be targeted by the ubiquitin-proteasome pathway (1), the proteasome inhibitor MG132 was added to normoxic MCF10A cells (Fig. 2D). Addition of MG132 to normoxic MCF10A cells resulted in a 5- to 10-fold increase in eEF2K levels but had no effect on hypoxic MCF10A or HTB20 cells. Control studies showed that MG132 increased the abundance of cell cycle kinase inhibitor p27, an established target of proteasome decay, which was not stabilized by hypoxia (Fig. 2D). Inhibition of proteasome function with MG132 did not alter eEF2K mRNA levels (data not shown). These results suggest that under normoxic conditions, eEF2K is rapidly degraded in the proteasome in nontransformed cells, its turnover is selectively blocked during hypoxia, and it is constitutively lost with increased transformation. Thus, the increased phosphorylation of eEF2 involves a mechanism not previously described whereby hypoxia stabilizes eEF2K. eEF2K was constitutively stabilized in transformed cells, and the level of eEF2 phosphorylation was elevated independent of hypoxia (Fig. 2C), but surprisingly, without inhibiting translation activity. Since eEF2 levels increase significantly with transformation, this likely provides a large pool of nonphosphorylated elongation factor which potentially provides resistance to hypoxia inhibition of protein synthesis. In

this regard, phosphorylated eEF2 cannot participate in elongation and it does not act as a dominant inhibitor. Finally, eIF2α protein levels and Ser51 inactivating phosphorylation did not change with hypoxia in any of the cell lines tested, apart from a slight increase in eIF2α Ser51 phosphorylation in HTB20 cells after 24 h of hypoxia (Fig. 2E). The increased abundance of the eIF2α GTP recycling factor known as eIF2B in HTB20 cells (Fig. 2F) likely overrides translation inhibition by eIF2α phosphorylation, as shown in other systems (11). Under conditions of anoxia (<0.1% O_2), prolonged serum starvation (24 h), or treatment with thapsigargin, increased eIF2α phosphorylation was observed, which demonstrates that these cells can respond to severe stress by activating this pathway (data not shown).

Hypoxia rapidly disrupts cap-initiation complexes through 4E-BP1 activation. We investigated whether the activation of 4E-BP1 during hypoxia contributes to the downregulation of protein synthesis in MCF10A cells. Association of 4E-BP1 with eIF4E was determined by purification of eIF4E complexes from normoxic and hypoxic cell lysates by 7-methyl GTP-Sepharose chromatography and immunoblot analysis. Extracts used for analyses were normalized to equal levels of eIF4E, as shown in Fig. 3A. In normoxic MCF10A cells, there was only slight interaction between 4E-BP1 and eIF4E and there were overall lower levels of eIF4G, as expected. Hypoxia promoted strong interaction of 4E-BP1 with eIF4E, whereas there was only a modest increase in eIF4E–4E-BP1 interactions in transformed cells. Cell lysates normalized for roughly similar amounts of eIF4G were also subjected to immunoprecipitation with anti-eIF4GI antibody (Fig. 3B); proteins were resolved by SDS-PAGE and detected by immunoblot analysis to determine the association of eIF4E with eIF4G. The association of eIF4G with eIF4E was significantly reduced in hypoxic MCF10A cells, whereas there was little change in transformed cells. These data confirm that in nontransformed cells inhibition of cap-dependent translation during hypoxia occurs rapidly and involves 4E-BP1 sequestration of eIF4E, but with transformation the pathway is poorly responsive to hypoxic stress.

Inhibition of eIF2α phosphorylation has no effect on hypoxia-induced inhibition of protein synthesis. Under conditions of severe hypoxia/anoxia (<0.02% O_2), and in combination with serum starvation, eIF2α undergoes phosphorylation (2, 25). Although we did not observe any significant change in overall levels of eIF2α phosphorylation during hypoxia in any of the cell lines, there is a high basal level that is maintained in MCF10A cells. We therefore utilized a retrovirus vector to stably express the GADD34 C-terminal fragment which dephosphorylates eIF2α at Ser51 (33) or a vector control in MCF10A cells. Stable expression of FLAG-GADD34 C-terminal fragment was demonstrated by immunoblot analysis with FLAG antibody (Fig. 4A, top panel). Complete inhibition of eIF2α phosphorylation was observed only in cells expressing the GADD34 C terminus, compared to untreated cells (Fig. 4A, middle panel). Despite complete inhibition of eIF2α phosphorylation, hypoxia still downregulated translation (Fig. 4B). These data demonstrate that eIF2α phosphorylation does not play a role in protein synthesis inhibition by hypoxia in the absence of other stresses.

Selective gene silencing of 4E-BP1 and eEF2K blocks hypoxia inhibition of protein synthesis. The functional role of

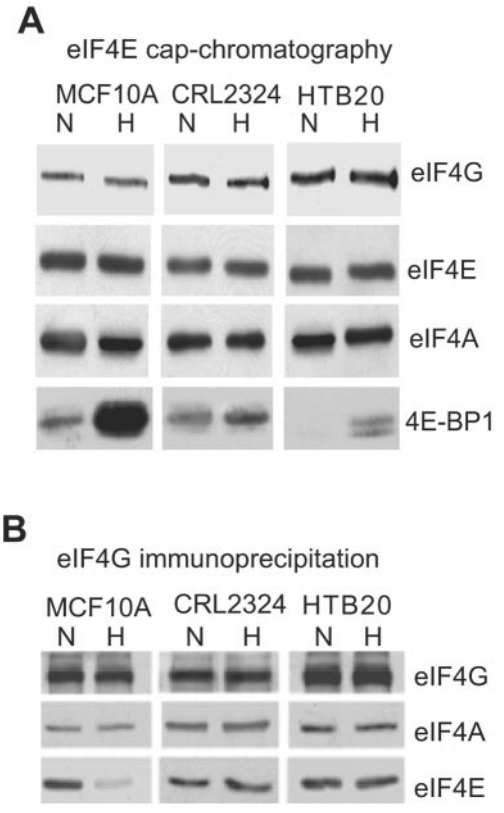

FIG. 3. Analysis of eIF4E interaction with 4E-BP1 and eIF4G during hypoxia. (A) Equal amounts of NP-40 lysates (300 μg) from MCF10A, CRL2324, and HTB20 cells under normoxic (N) or hypoxic (H) conditions were subjected to m^7GTP-Sepharose cap-chromatography, recovered by elution with m^7GTP, and resolved by SDS-15% PAGE, followed by immunoblot analysis with antisera as indicated. (B) Equal amounts of NP-40 lysates (300 μg) from MCF10A, CRL2324, and HTB20 cells cultured under normoxia (N) or hypoxia (H) were subjected to immunoprecipitation with anti-human eIF4GI antibodies. Immunoprecipitates were resolved by SDS-15% PAGE, and proteins were detected by immunoblot analysis as indicated. Data are representative of three independent experiments, which were quantified by densitometry of autoradiograms.

4E-BP1 was directly tested in protein synthesis inhibition during hypoxia in sensitive MCF10A cells by a loss-of-function analysis using siRNA knockdown of the mRNA. An siRNA targeted to the 4E-BP1 mRNA 3′-untranslated region was designed, as well as a nonsilencing (NS) siRNA, as described previously (14). MCF10A cells were transfected by multiple rounds of either 4E-BP1 siRNA or NS siRNA, cell lysates were produced 48 h after the last transfection, and the levels of 4E-BP1 protein expression were determined by immunoblot analysis. Selective knockdown achieved a >95% specific reduction in 4E-BP1 protein levels (Fig. 5A), which did not increase for up to 96 h (data not shown). Depletion of 4E-BP1 increased protein synthesis during hypoxia to 60% of normoxic cells, whereas control cells treated with NS siRNA were fully sensitive (Fig. 5B, compare to Fig. 4B). The association of 4E-BP1 with eIF4E was determined by recovery of eIF4E using 7-methyl GTP-Sepharose chromatography. Very little 4E-BP1 was found bound to eIF4E in 4E-BP1-silenced MCF10A cells under both normoxia and hypoxia (Fig. 5C). Thus, 4E-BP1

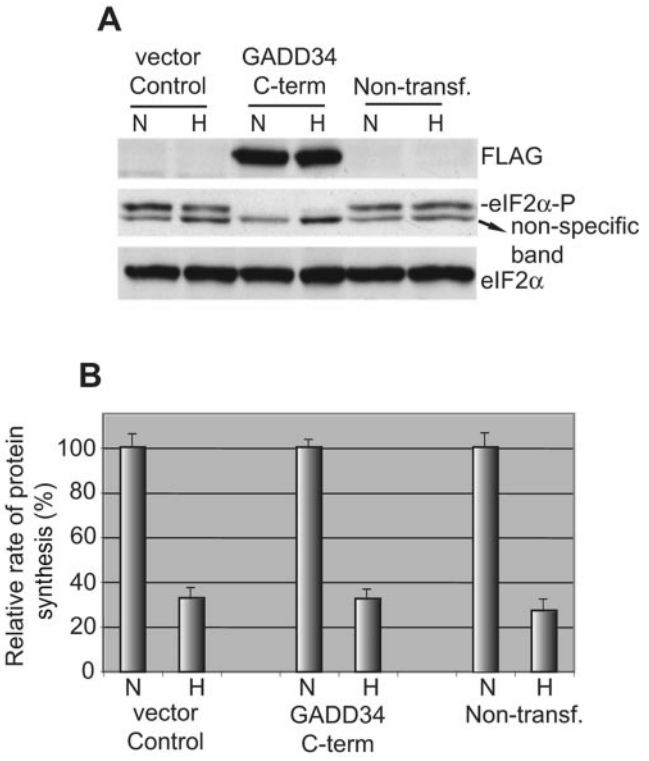

FIG. 4. Inhibition of eIF2α Ser51 phosphorylation in MCF10A cells has no effect on translation inhibition during hypoxia. (A) Immunoblot analysis of protein extracts from MCF10A cells expressing the FLAG-GADD34 C-terminal fragment, which constitutively dephosphorylates eIF2α, pBABEpuro vector control, or parental MCF10A cells. Antibodies specific for total and Ser51 phosphorylated eIF2α were used. A cross-reactive nonspecific protein is identified. Nontrans., nontransfected.(B) Relative protein synthesis activity during hypoxia was determined by [^{35}S]methionine labeling cells for 1 h, followed by trichloroacetic acid precipitation, determination of specific activity per mg of protein, and scintillation counting of samples. Samples were normalized to normoxic vector control set at 100%.

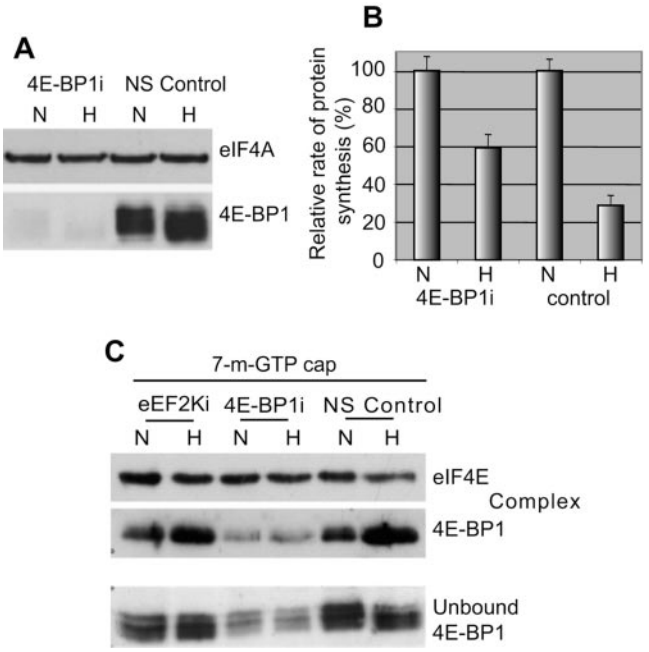

FIG. 5. Selective silencing of 4E-BP1 by siRNA in immortalized MCF10A cells partially prevents protein synthesis inhibition during hypoxia. MCF10A cells were transfected four times with either 4E-BP1 siRNA (4E-BP1i) or a nonsilencing (NS) control siRNA. Forty-eight hours following the last transfection, cells were cultured for 24 h under either normoxic (N) or hypoxic (H) (0.5% O_2) conditions. (A) Equal amounts of protein lysates from cells were resolved by SDS-PAGE, and immunoblot analysis was carried out with antibodies specific for 4E-BP1 or eIF4A. eIF4A was used as a loading control. (B) Total protein synthesis was determined by [^{35}S]methionine labeling cells for 1 h, followed by trichloroacetic acid precipitation of equal amounts of protein and scintillation counting of samples to determine protein specific activities. Results represent an average of three independent experiments, normalized to the normoxic control. (C) m^7GTP (cap) chromatography was carried out using equal amounts (300 μg) of protein extracts from cells transfected with control nonsilencing or specific siRNA for eEF2K or 4E-BP1; bound proteins were eluted and compared by immunoblot analysis to unbound 4E-BP1. The 4E-BP1 blots were overexposed to visualize the low levels of protein remaining following knockdown.

activation is an important mediator of protein synthesis inhibition during hypoxia but cannot fully account for the effect.

We therefore examined the importance of eEF2 phosphorylation in inhibition of protein synthesis during hypoxia. MCF10A cells were transfected with an eEF2K siRNA or NS siRNA. Cell lysates were produced 48 h after the last of multiple rounds of transfection, and the level of eEF2K protein expression was determined by immunoblot analysis (Fig. 6A). eEF2K protein levels were decreased ~90% in cells transfected with eEF2K siRNA and unaltered by NS siRNA. The reduction in eEF2K levels (eEF2Ki) in MCF10A cells diminished eEF2 phosphorylation by approximately fourfold during hypoxia compared to NS control cells, without affecting levels of eEF2 or eIF4A (Fig. 6A). The strong reduction in eEF2K levels decreased the sensitivity of cells to hypoxia inhibition of protein synthesis by half (Fig. 6B). siRNA knockdown of eEF2K did not alter interaction of 4E-BP1 with eIF4E during hypoxia (Fig. 5C). Inhibition of eEF2 phosphorylation therefore conferred partial resistance of protein synthesis to hypoxia. We suspect that the activation of eEF2K likely slows the rate of ribosome elongation through increased inactivation of elongation factor eEF2 during hypoxia, to better match mRNA translation to reduced energy availability. To achieve simultaneous silencing of 4E-BP1 and eEF2K expression, lentivirus vectors were developed that express shRNAs directed to the 3′-untranslated region of each mRNA, since repeated cotransfection of multiple target siRNAs was toxic to cells. MCF10A cells were infected with shRNA expression vectors and cultured for 2 days, and levels of 4E-BP1 and eEF2K were determined by immunoblot analysis (Fig. 6C, siRNA target proteins in upper panel). 4E-BP1 and eEF2K protein levels were individually reduced six- to eightfold by specific targeting, whereas the nonsilencing vector alone had no effect. Rates of protein synthesis were determined under normoxic and hypoxic conditions by [^{35}S]methionine incorporation. Simultaneous knockdown of 4E-BP1 and eEF2K prevented hypoxia inhibition of protein synthesis (Fig. 6D), to roughly the sum of each depletion, and nearly restored full protein synthesis activity. Hypoxia inhibition of protein synthesis is therefore mediated by activa-

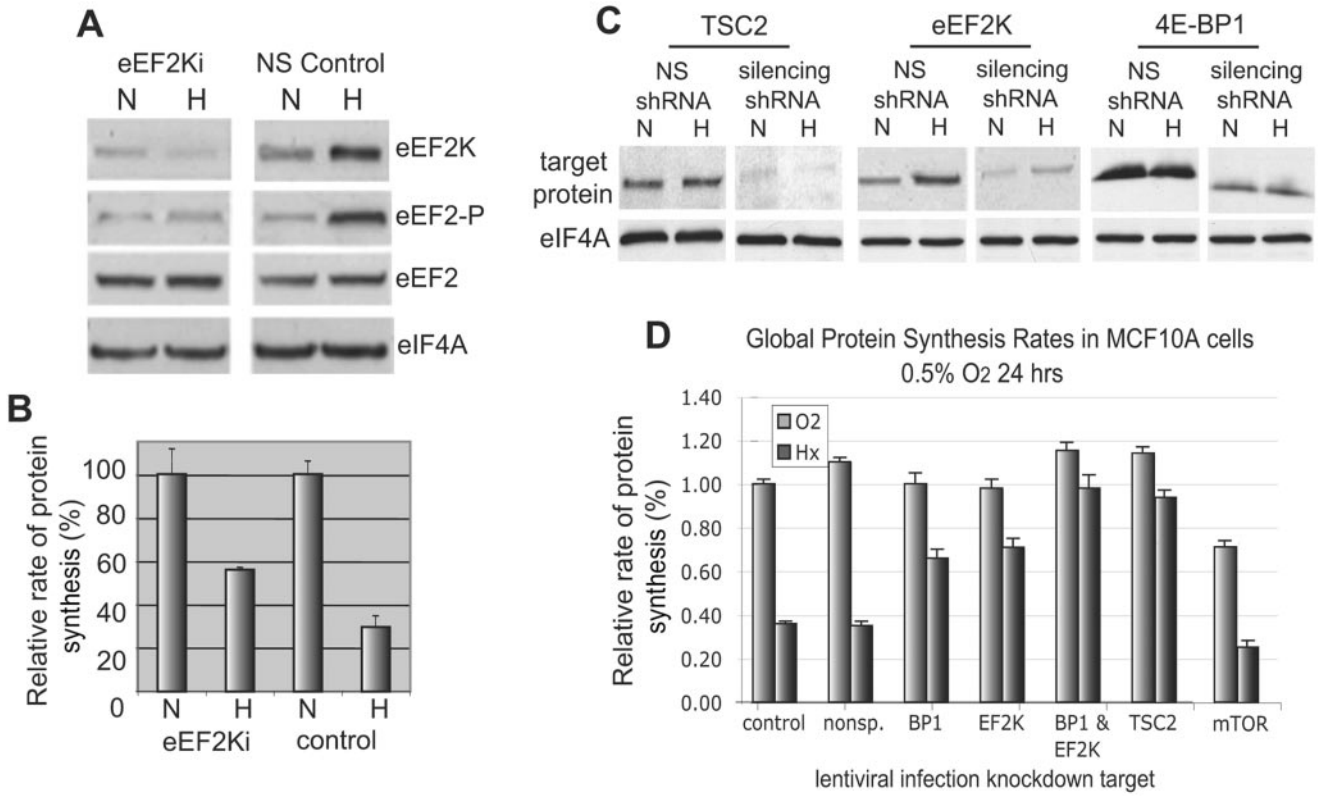

FIG. 6. RNAi-mediated knockdown of eEF2K and 4E-BP1, or TSC2, in immortalized MCF10A cells confers resistance to hypoxia-mediated protein synthesis inhibition. (A) MCF10A cells were transfected four times with siRNAs as indicated or a nonsilencing (NS) control siRNA, and protein levels were determined by immunoblot analysis for eEF2K, eEF2, eIF4A, and Thr56 phospho-eEF2. (B) Total protein synthesis activity during hypoxia and normoxia was determined by [^{35}S]methionine incorporation in vivo for 1 h, followed by trichloroacetic acid precipitation, scintillation counting of samples, and determination of specific activity of incorporation into protein. Data were derived from triplicate studies. Values were normalized to the normoxic control at 100%. (C) shRNA lentivirus vectors were developed to specifically knock down expression of TSC2, eEF2K, mTOR, and 4E-BP1 proteins or to express a nonspecific control siRNA. Cells were stably transformed with vectors and subjected to normoxia or hypoxia, and equal amounts of protein lysates were analyzed by immunoblot analysis as shown. (D) Total protein synthesis activity during hypoxia and normoxia in shRNA lentivirus-transformed MCF10A cells was determined by [^{35}S]methionine incorporation, as described above.

tion of 4E-BP1 and eEF2K, which is uncoupled from hypoxia in transformed cells.

Since both 4E-BP1 and eEF2K are controlled by mTOR activity, we determined where hypoxia signaling and translational regulation are uncoupled with transformation in breast carcinoma cells. To examine this issue in more depth, shRNA gene-silencing lentivirus vectors were developed to inhibit expression of TSC2 and reduce the negative regulatory function of the TSC1/2 complex on mTOR in hypoxia-sensitive MCF10A cells. Knockdown of TSC2 protein expression to 20% that of controls (Fig. 6C; further knockdown was lethal), significantly uncoupled inhibition of protein synthesis from hypoxia in immortalized breast epithelial cells, increasing levels to within 20% that of normoxic cells (Fig. 6D). Silencing of mTOR (shown in Fig. 7B) had the opposite effect, causing some inhibition of protein synthesis under normoxia and severe inhibition during hypoxia (Fig. 6D). Thus, these data suggest that TSC2 acts on mTOR as a major control point to inhibit protein synthesis during hypoxia, by acting on EF2K and 4E-BP1. We were not able to test the effect of AMPK gene silencing or inhibition as it proved to be highly toxic in immortalized cells (data not shown).

Transformation uncouples TSC1/2-mTOR from translation inhibition by hypoxia. AKT kinase-mediated phosphorylation positively regulates mTOR activity through inhibition of TSC2 (a negative regulator of mTOR; Fig. 1A). Phosphorylation of mTOR at Ser2448 correlates in some studies with activation by growth factors (37, 41), although this has been challenged (10, 20). Nevertheless, we monitored Ser2448 phosphorylation to determine whether it correlates with decontrol of this pathway in transformation. In addition, eEF2K can be directly inhibited by mTOR phosphorylation and indirectly inhibited by mTOR through $p70^{S6k}$ phosphorylation (Fig. 1A). We therefore examined the abundance and activity of Akt, mTOR, TSC2, and $p70^{S6k}$ in immortalized and transformed breast epithelial cells under normoxia and hypoxia using site-specific phosphorylation as a surrogate for activity (Fig. 7A). Hypoxia downregulated Akt activation of phosphorylation at Ser473 by three- to fourfold without affecting Akt abundance in immortalized MCF10A cells. Hypoxia had no effect on Akt Ser473 phosphor-

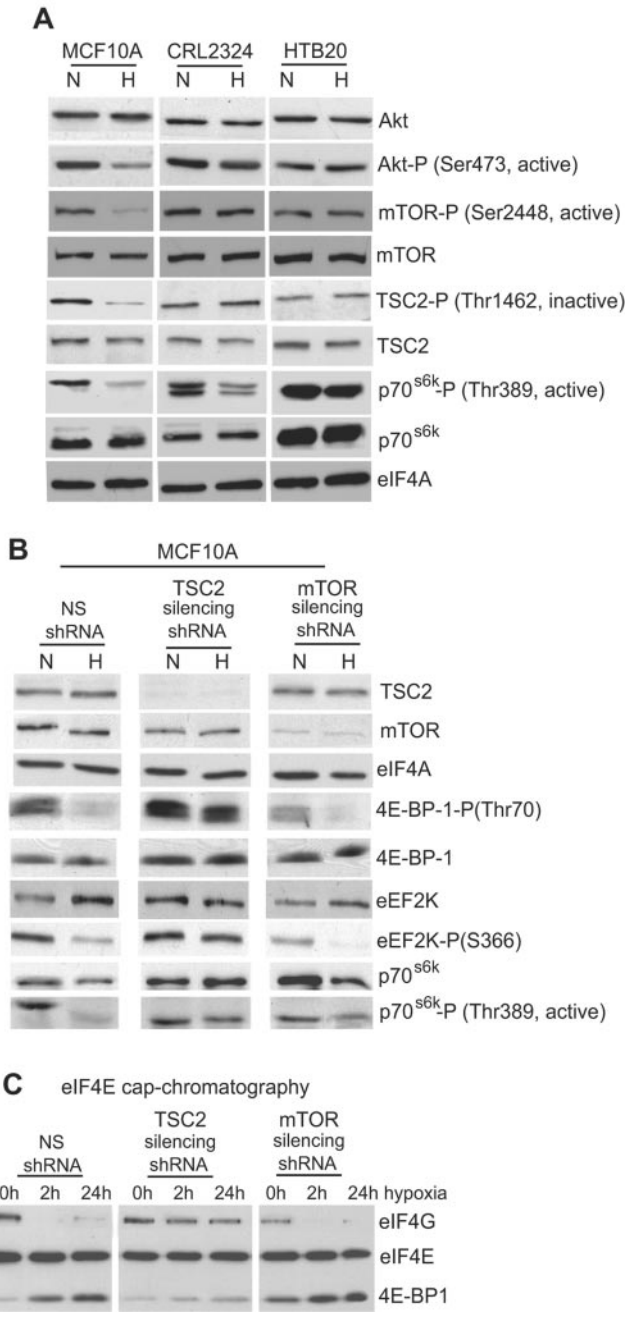

FIG. 7. Levels and phosphorylation state of oxygen signaling pathway proteins in normoxic and hypoxic cells. MCF10A, CRL2324, and HTB20 cells were cultured for 24 h under either normoxic (N) or hypoxic (H) (0.5% O_2) conditions. (A) Equal amounts of protein lysates from cells were resolved by SDS-PAGE, and immunoblot analysis was carried out with antibodies specific for total proteins and phospho-specific forms as shown. (B) Selective silencing of TSC2 or mTOR was carried out using shRNA lentivirus vectors under normoxic or hypoxic conditions in MCF10A cells. Equal amounts of protein lysates were analyzed as shown by immunoblot analysis using protein-specific and protein phospho-specific antibodies. (C) m^7GTP-Sepharose cap chromatography was carried out using equal amounts of protein lysates from normoxic (0 h hypoxic) and hypoxic MCF10A cells that have undergone lentivirus-mediated gene silencing for TSC2, mTOR, or control nonsilencing shRNA. Proteins were eluted and identified by immunoblot analysis.

ylation in transformed cells, which maintained equal levels of the protein and its activating phosphorylation (Fig. 7A). Activated (phosphorylated) AMPK downregulates mTOR activity by phosphorylating TSC2 at Thr1227, which then impairs mTOR function (24), whereas activated Akt inhibits TSC2 by phosphorylation at Thr1462 (31). TSC2 protein levels were unaffected by hypoxia in all cell lines, but its phosphorylation (inactivation) at the Akt site (Thr1462) was blocked only in immortalized hypoxic MCF10A breast epithelial cells, not in transformed cells. These data demonstrate that transformation uncouples hypoxia signaling at the control of TSC2. Downregulation of mTOR and $p70^{S6k}$ activity by hypoxia in nontransformed cells is consistent with the decrease in mTOR activity in these cells (shown by loss of Akt activating phosphorylation). These data therefore suggest that $p70^{S6k}$ is a likely kinase that controls eEF2K during hypoxia, consistent with a previous report on $p70^{S6k}$ activity (24). We note that $p70^{S6k}$ abundance was unchanged in cells by hypoxia, although its level in highly transformed HTB20 cells increased by >10-fold. Hypoxia strongly inhibited $p70^{S6k}$ phosphorylation in nontransformed MCF10A cells and moderately did so in partially transformed CRL2324 cells, also consistent with inhibition of mTOR activity. We do not know why immunoblotting of the Thr384-phosphorylated form of $p70^{S6k}$ identifies a doublet in CRL2324 cells. Inhibition of mTOR, activation of 4E-BP1, and inhibition of eEF2 by activation of eEF2K are therefore all acting in hypoxia-sensitive cells via mTOR (and indirectly by $p70^{S6k}$), control of which is uncoupled by transformation.

We therefore silenced TSC2 or mTOR in hypoxia-sensitive MCF10A cells (Fig. 7B) using lentivirus vector shRNAs. The effects on eEF2K, $p70^{S6k}$, and 4E-BP1 activity by surrogate phosphorylation were investigated. As expected in hypoxia-resistant HTB20 cells, silencing of TSC2 had only a slight effect on 4E-BP1, which remained hyperphosphorylated during hypoxia, and had no effect on $p70^{S6k}$ phosphorylation (see data in the supplemental material). We were not able to investigate mTOR silencing in transformed cells which proved to be cytotoxic. Silencing of mTOR in MCF10A normoxic cells significantly reduced the phosphorylation (inactivation) of eEF2K under normoxia and hypoxia and inactivating phosphorylation of 4E-BP1 at Thr70 (Fig. 7B). These results demonstrate that mTOR is a key regulator of both eEF2K and 4E-BP1 activity during hypoxia. mTOR silencing reduced $p70^{S6k}$ phosphorylation by about half, possibly indicating that $p70^{S6k}$ phosphorylation of mTOR is a stronger direction of the pathway or that there is another aspect to the regulation of mTOR phosphorylation of $p70^{S6k}$ which remains poorly described. Knockdown of TSC2 resulted in continued phosphorylation of 4E-BP1 at Thr70 during hypoxia (albeit somewhat reduced), consistent with its critical role in inhibition of mTOR during hypoxia. It is possible that residual TSC2 protein is responsible for the inability to fully restore 4E-BP1 phosphorylation during hypoxia. With TSC2 silencing, eEF2K remained phosphorylated under hypoxia, as did $p70^{S6k}$ at Thr389, again consistent with the importance of TSC2 and mTOR in mediating translation-inhibiting signals during hypoxia. As shown earlier, hypoxia rapidly mediates dissociation of eIF4E from eIF4G through 4E-BP1 sequestration of eIF4E. Silencing of TSC2 also impaired 4E-BP1 sequestration of eIF4E from eIF4G during hypoxia

and dissociation of cap-initiation complexes (Fig. 7C). As expected, mTOR silencing had the opposite effect, strongly reducing eIF4E-eIF4G interaction commensurate with a strong increase in 4E-BP1 sequestration of eIF4E.

The protein REDD1 has been shown to be a transcriptional target of the hypoxic response that is upregulated in response to hypoxia and acts as a negative regulator of mTOR, probably by acting on the TSC1/2 complex (6, 12, 42). REDD1 also prevents apoptosis mediated by HIF-1 (40). Consequently, REDD1 activity is also likely uncoupled from the TSC1/2 complex with transformation or it is no longer upregulated. To distinguish between these two possibilities, we analyzed the level of REDDl mRNA by quantitative real-time reverse transcription-PCR under normoxic and hypoxic conditions for the different cell lines. Hypoxia increased REDD1 levels by 2.6-fold in immortalized MCF10A cells, almost 2-fold in partially transformed CRL2324 cells, and approximately 2.5-fold in highly transformed HTB20 cells (see data in the supplemental material). Thus, these data indicate that REDD1 is induced in cells regardless of transformation, and they further support the conclusion that TSC2/mTOR is the point of convergence for hypoxia signaling which is disrupted by transformation.

DISCUSSION

In this study, we investigated the mechanisms underlying suppression of protein synthesis during hypoxia in immortalized breast epithelial cells and characterized how breast cancer cells circumvent this process. It was previously shown that hypoxia inhibits global translation (30), but the detailed mechanism for regulation of translation during hypoxia has been understudied and largely investigated using either combined multiple stresses which have distinct effects individually or under extreme hypoxia/anoxia (0.02% O_2) in highly transformed cells. We therefore characterized translation inhibition during physiologically relevant hypoxia (0.5% O_2) in immortalized human breast epithelial cells, as well as in progressively more transformed human breast carcinoma tumor cell lines, to examine proposed mechanisms of translation inhibition and the means of acquired resistance.

Translation in immortalized cells was inhibited by 60 to 70% under hypoxia, whereas transformed cells demonstrated progressive resistance with increased transformation (Fig. 1B). We found no significant increase in the phosphorylation of eIF2α during hypoxia, nor was there any role in hypoxia inhibition of translation (Fig. 2 and 4). While it is widely accepted that eIF2α phosphorylation plays a significant role in translation inhibition during oxidative stress following hypoxia concomitant with activation of an unfolded protein response, in our studies it cannot be responsible for suppression of protein synthesis during hypoxia. It should be noted that while our paper was under review, Liu et al. (29) showed that hypoxia plus serum deprivation can activate 4E-BP1 and eEF2K, in addition to stimulating eIF2α phosphorylation. Thus, in combination with our results, eIF2α phosphorylation seems to be a component of serum deprivation rather than hypoxia.

A large shift in 4E-BP1 to the dephosphorylated (active) form, concomitant with sequestration of eIF4E, was observed in immortalized cells, but not in increasingly transformed cells during hypoxia (Fig. 2A and 3). Significant knockdown (>90%) of 4E-BP1 by siRNA in immortalized cells partially prevented translation inhibition during hypoxia (Fig. 5), suggesting that an additional mechanism contributes to hypoxia-mediated translation inhibition. While some studies have shown that mTOR kinase is suppressed during hypoxia, leading to 4E-BP1 activation (1), this alone is not sufficient to account for translation inhibition because the effect is not fully recapitulated by treatment with rapamycin (see data in the supplemental material), which also activates 4E-BP1. The only other alteration in the translational machinery observed in hypoxia-sensitive cells was a significant increase in eEF2 phosphorylation (Fig. 2). Hypoxia was found to stabilize eEF2 kinase against proteasome degradation, resulting in increased accumulation of inactivating eEF2 phosphorylation (Fig. 2D). The selective silencing of eEF2K using siRNA partially prevented hypoxia inhibition of protein synthesis (Fig. 6D) and, when combined with 4E-BP1 depletion by siRNA, can account for the mechanism by which nontransformed cells respond to hypoxia at the level of translation inhibition. Transformed cell lines overexpress eEF2 by two- to threefold, which may be a mechanism by which they overcome reduced rates of elongation during hypoxia, as phosphorylated eEF2 cannot participate in elongation and does not act as a dominant inhibitor.

The data presented here demonstrate that translation suppression is mediated by the mTOR pathway during hypoxia and is uncoupled from oxygen sensing and signaling pathways with transformation. It was previously shown that hypoxia regulation of mTOR does not require HIF1α and does not correlate with AMPK phosphorylation (activity) (1a). It has also been shown that downregulation of mTOR function during hypoxia requires the TSC1/2 complex (6). Our data demonstrate that transformation of breast cancer cells promotes the constitutive activation of mTOR and $p70^{S6k}$ during hypoxia (Fig. 7A). These and other data presented suggest that the uncoupling of hypoxia responsiveness occurs at the junction of TSC1/2 with mTOR, resulting in a minimal ability to activate (dephosphorylate) 4E-BP1 and stimulate eEF2K activity. Gene silencing of TSC2, a negative regulator of mTOR, prevented hypoxia inhibition of mTOR, as well as 4E-BP1 activation and disassembly of cap-initiation complexes, only in immortalized cells and had no effect in transformed cells. Collectively, these data indicate that during hypoxia, transformation uncouples the ability of TSC1/2 to block mTOR function. These data are consistent with the findings that TSC2 deficiency increases HIF1α levels and hypoxia responsiveness (6, 7). In fact, loss of TSC1/2 complex function is associated with growth advantage of hypoxic cells and likely contributes to tumor progression (6, 9). Our results suggest that at least part of the tumor suppressor function of the TSC1/2 complex lies in its suppression of protein synthesis, and release of breast cancer cells from hypoxia-mediated translation inhibition is likely another important step in the progression to malignancy. In this regard, loss of TSC1/2 complex function strongly promotes VEGF-A protein levels, resulting in increased angiogenesis (39).

It remains to be determined whether the contribution of deregulated translation in transformation increases cap-dependent or cap-independent (IRES) mediated mRNA translation mechanisms, as both have been reported. Considerably more work needs to be focused on determining the relative contri-

butions of deregulated cap-dependent and cap-independent translation in hypoxia and cancer progression. mRNAs which are crucial for hypoxia responsiveness (HIF1α), antiapoptotic responses (Bcl2), angiogenesis (VEGF), and cell cycle control (p27), among others, are reported to bifunctionally translate via both mechanisms (47). Uncoupling of protein synthesis regulation pathways to permit unrestricted mRNA translation under hypoxia could be achieved by either mechanism. Indeed, studies have demonstrated that events which promote transformation, such as overexpression of eIF4E and activation of Ras or Akt, result in a large recruitment of specific mRNAs into polyribosomes, which include both cap-dependent and cap-independent mRNAs (36). Studies also need to resolve the importance of translation elongation regulation in hypoxia control and cancer progression. Our results disclose a novel mechanism for elongation control, whereby eEF2K is normally rapidly degraded in a proteasome-dependent manner but is blocked during hypoxia. Although suppression of elongation is at first impression antithetical to translation of any mRNAs during hypoxia, it makes good sense for cells to slow the rate of elongation during hypoxia to match translation to the much lower level of energy production.

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