Hypoxia Stimulates Carcinoma Invasion by Stabilizing Microtubules and Promoting the Rab11 Trafficking of the α6β4 Integrin

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Abstract

Hypoxia plays a key role in tumor cell survival, invasion, and metastasis. Here we show that hypoxia increases tumor cell invasion by the modulation of Rab11, an important molecule for vesicular trafficking, especially membrane protein recycling and translocation of proteins from *trans*-Golgi network to plasma membrane. Dominant-negative Rab11 dramatically decreased hypoxia-induced invasion of MDA-MB-231 breast carcinoma cells without affecting cell apoptosis. Hypoxia-induced Rab11 trafficking is regulated by microtubule stability, as evidenced by the findings that hypoxia increases Glu tubulin and that colchicine blocks Rab11 trafficking and invasion. Inhibition of GSK-3β activity by hypoxia seems to be central to microtubule stabilization and invasion. In fact, expression of a dominant-negative GSK-3β was sufficient to stimulate invasion in normoxia. One target of Rab11-mediated trafficking that contributes to invasion is the integrin α6β4. Hypoxia induced a significant increase in α6β4 surface expression but it had no effect on the surface expression of α3β1. This increase is dependent on Rab11 and stable microtubules. In summary, we identify vesicle trafficking as a novel target of hypoxic stimulation that is important for tumor invasion. (Cancer Res 2005; 65(7): 2761-9)

Introduction

Breast epithelial cells, similar to other epithelia, are able to survive and differentiate because of the tissue architecture and growth factor milieu present in the mammary gland (1–3). This rich environment, however, is progressively lost during malignant transformation, especially as malignant cells become invasive and metastatic. Such cells must acquire mechanisms that maintain their survival and promote their invasion outside the confines of the mammary gland. As argued recently, cancer progression is an evolutionary process that selects for cells that exhibit the capacity for survival, among other properties (4, 5). Moreover, and perhaps more importantly, tumor cells must survive in environmental conditions not present in normal tissue. One of the most formidable barriers to their survival is hypoxia (6). The oxygen tension within many solid tumors is substantially less than that in adjacent normal tissue, presumably because of poor vascularization (6). Although hypoxia will kill most normal cells and some tumor cells, it also provides a strong selective pressure for the survival of the most aggressive and metastatic cells. In fact, clinical studies have clearly shown that the low pO_2 tension within a neoplastic lesion is an independent prognostic indicator of poor outcome and correlates with an increased risk to develop distant metastasis independently of therapeutic treatment (6–8).

An important property of tumor cells that are able to survive in hypoxia is that they exhibit an enhanced propensity to invade (9), a finding that underscores the hypothesis that hypoxia facilitates tumor progression. An understanding of the mechanisms by which hypoxia stimulates invasion would provide insight into the nature of invasion itself. Indeed, such studies to date have substantiated the importance of growth factor receptors such as Met and protease receptors such as urokinase-type plasminogen activator receptor in invasion (10, 11). These and other studies have highlighted the ability of hypoxia to increase the expression of these proteins at the level of transcription (12, 13). This conclusion is consistent with the well-documented influence of hypoxia on gene activation (14, 15). Surprisingly, however, other potential mechanisms by which hypoxia may facilitate invasion have not been considered.

In this study, we evaluated the hypothesis that hypoxia affects the trafficking of specific proteins, which are involved in the invasive process, to the cell surface. This novel hypothesis is rooted in the emerging view that cell migration may involve the recycling of adhesion receptors and other components of the migratory apparatus (16). To this end, we focused our efforts on the Rab family of small G proteins, which consist of more than 30 members that regulate intracellular vesicle trafficking. Among the various Rabs, Rab11 is of potential relevance because it is a key regulator of the dynamics of recycling endosomes (17, 18) and it has been implicated in the plasma membrane recycling of several proteins that could be important for invasion including β1 integrins and the epidermal growth factor (EGF) receptor (19, 20). Rab11 is also involved in transport of molecules from recycling endosomes to *trans*-Golgi network and from *trans*-Golgi network to plasma membrane (17, 21, 22). The data we report here implicate a key role for Rab11 in the hypoxia-stimulated invasion of breast carcinoma cells. Moreover, we elucidate a mechanism for this phenomenon that involves the inhibition of glycogen synthase kinase 3β (GSK-3β) activity by hypoxia and a consequent increase in microtubule stability, and the microtubule-dependent trafficking of Rab11-containing vesicles. Importantly, we also identified one surface receptor critical for invasion that is a target of this pathway, the α6β4 integrin.

Materials and Methods

Cells and reagents. MDA-MB-231 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University. An integrin α6 antibody (ZMD.192) for immunoblot analysis was purchased from Zymed (South San Francisco, CA); an integrin α6 antibody (GoH3) for immunofluorescence was obtained from Immunotech (Westbrook, ME); and an integrin α6 antibody (2B7) for fluorescence-activated cell sorting

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(FACS) analysis was prepared in our lab (23). A detyrosinated α-tubulin antibody was purchased from Synaptic Systems and a Rab11 antibody was obtained from Santa Cruz Biotech (Santa Cruz, CA). Colchicine was obtained from Sigma (St. Louis, MO).

Transfections. Wild-type Rab11 (pEGFP-Rab11-WT) and GDP-locked Rab11 (pEGFP-Rab11-S25N) constructs were kindly provided by Dr. Robert Lodge. A kinase-inactive GSK-3β construct was a gift from Dr. James R. Woodgett. Cells were transfected with LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer.

Hypoxia experiments. For experiments that involved hypoxic culture conditions, cells were grown in low-glucose DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum. At the initiation of these experiments, the cells were washed twice with PBS and serum-free medium was added. After 6 hours, cells were incubated in either a normoxic environment (standard tissue culture incubator) or in a hypoxic chamber maintained at 3% O_2.

Apoptosis assays. Immediately following incubation in either normoxia or hypoxia, cells were washed once with serum-containing medium, once with PBS, once with annexin V-FITC buffer [10 mmol/L HEPES-NaOH (pH 7.4) 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$], and then incubated for 15 minutes at room temperature with 5 μg/mL annexin V-FITC (Biosource International, Camarillo, CA). After washing once with annexin V buffer, the samples were resuspended in the same buffer. Immediately before analysis, 5 μg/mL propidium iodide (Biosource International) was added to distinguish apoptotic cells from necrotic cells and the cells were analyzed using a flow cytometer. For the detection of apoptosis of GFP-transfected cells, the same methods were employed except that annexin V-phycoerythrin without propidium iodide was used.

Analysis of protein expression. Subsequent to incubation in normoxia or hypoxia, cells were analyzed for their expression of specific proteins by either FACS or immunoblotting. For FACS analysis, cells were washed twice with ice-cold PBS containing 0.2% bovine serum albumin (BSA). Aliquots of cells were incubated for 1 hour at 4°C with antibodies in the PBS/BSA solution. The cells were washed thrice with PBS/BSA and then incubated with secondary antibodies coupled to R-phycoerythrin for 1 hour at 4°C. After washing thrice with PBS/BSA, the cells were resuspended in PBS and analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ).

For immunoblot analysis, the cells were extracted in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1.0% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mmol/L Tris (pH 8.0)]. For integrin α6, samples were resuspended in a nonreducing sample buffer [5×: 60 mmol/L Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.1% bromophenol blue] and for Rab11, samples were resuspened in reducing buffer [5×: 60 mmol/L Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mmol/L 2-mercaptoethanol, 0.1% bromophenol blue]. These were boiled for 5 minutes and electrophoresed by SDS-PAGE. Proteins were then transferred to Hybond-enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Blots were blocked with TBST buffer [25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% Tween 20] containing 5% nonfat dried milk and probed with primary antibodies overnight and secondary antibodies coupled to peroxidase for 1 hour. Blots were developed using the enhanced chemiluminescence system (Amersham Biosciences).

Analysis of RNA expression. Total cellular RNA was purified from cultured cells using RNeasy mini kit (Qiagen, Valencia, CA) following the protocol of the manufacturer. For reverse transcription-PCR (RT-PCR) analysis, a one-step RT-PCR kit (Qiagen) was used.

Invasion assays. The upper chambers of Transwells (Corning Costar) were coated with 0.5 μg of Matrigel (Collaborative Research, Bedford, MA) that had been diluted with cold water and allowed to air dry. Subsequently, the coated Transwell membranes were incubated with DMEM for 1 hour and 5×10^4 cells were plated in the upper chambers. The lower chambers contained 5% NIH-3T3 conditioned medium. The inserts were incubated for 24 hours at 37°C in either a normoxic or hypoxic environment. The cells that had invaded the lower surface of the membrane were fixed with methanol and stained with 0.2% crystal violet in 2% ethanol. The number of cells that had invaded was quantified by counting random fields using a light microscope equipped with a reticle.

Indirect immunofluorescence microscopy. Cells were plated at low density and incubated overnight at atmosphere with 5% CO_2 at 37°C in either normoxia or hypoxia. Subsequently, the cells were fixed immediately with a buffer containing 2% paraformaldehyde, 100 mmol/L KCl, 200 mmol/L sucrose, 1 mmol/L EGTA, 1 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 mmol/L PIPES at pH 6.8 for 15 minutes, and then treated with a buffer containing 0.2% Triton X-100, 100 mmol/L KCl, 200 mmol/L sucrose, 1 mmol/L EGTA, 1 mmol/L $MgCl_2$, and 10.8 mmol/L PIPES at pH 6.8 for 2 minutes. The cells were rinsed with PBS thrice and incubated with a blocking solution containing 3% BSA and 5% serum in PBS for 15 minutes. The cells were then incubated with specific antibodies for 90 minutes. The cells were rinsed thrice and incubated with a secondary antibody conjugated to fluorescein (minimal cross-reaction interspecies; Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking buffer for 1 hour. Cells were rinsed with PBS thrice and mounted with a mounting buffer [1% propylgallate in a mixture of glycerol and PBS (8:2)] and examined using a confocal microscope.

Results

Rab11 is involved in hypoxia-stimulated invasion. Invasive and metastatic carcinoma cells have the propensity to survive autonomously in stressful microenvironments such as hypoxia (14). Indeed, MDA-MB-231 breast carcinoma cells exhibit no significant increase in apoptosis as determined by annexin V-FITC staining when exposed to hypoxia (3% O_2) for 24 hours in the absence of serum (Fig. 1*A*). Such conditions, however, stimulate the relative rate of invasion of these cells ~2.5-fold in comparison with cells maintained in normoxia (Fig. 1*B*). To assess the hypothesis that Rab11 is involved in hypoxia-stimulated invasion, cells were transfected with either a GFP control vector (pEGFP) or a GDP-bound inactive Rab11 vector (pEGFP-Rab11-S25N). The transfection efficiency for both vectors was ~50% to 55% as determined by GFP expression. Cells that expressed the control vector exhibited increased invasion in hypoxia that mimicked the data shown in Fig. 1*B* (Fig. 1*C*). Expression of the inactive Rab11 vector, however, resulted in a drastic decrease in invasion in hypoxia (Fig. 1*C*). The relatively low level of invasion in normoxia was also inhibited by expression of inactive Rab11 but the degree of inhibition was substantially less than that observed in hypoxia (Fig. 1*C*). Of note, expression of the inactive Rab11 vector had no effect on cell viability or apoptosis (data not shown). These data argue for the involvement of Rab11 in invasion, an involvement that is amplified in hypoxia.

The data shown in Fig. 1 suggest that hypoxia alters either the expression or function of Rab11. To evaluate these possibilities, we examined the effect of hypoxia on the expression of Rab11 mRNA by RT-PCR and protein by immunoblotting. The data obtained indicate that hypoxia does not influence Rab11 expression either at 24 hours, the time period for the invasion assays, or longer time periods (48 hours; Fig. 2*A*). Given that Rab11 is involved in protein recycling and transport to the plasma membrane, we assessed the effect of hypoxia on the localization of Rab11 by expressing a GFP-tagged wild-type Rab11 vector in MDA-MB-231 cells. In cells maintained in normoxia, Rab11 concentrates in vesicles in the perinuclear region and it seems that relatively little Rab11 is present near the plasma membrane or dispersed throughout the cytoplasm (Fig. 2*B*). Hypoxic exposure for 24 hours, however, caused Rab11-containing vesicles to disperse throughout the cytoplasm and proximal to the plasma membrane (Fig. 2*C*). These findings are consistent with the notion that hypoxia stimulates the ability of Rab11 to mediate the transport of vesicles to the plasma membrane.

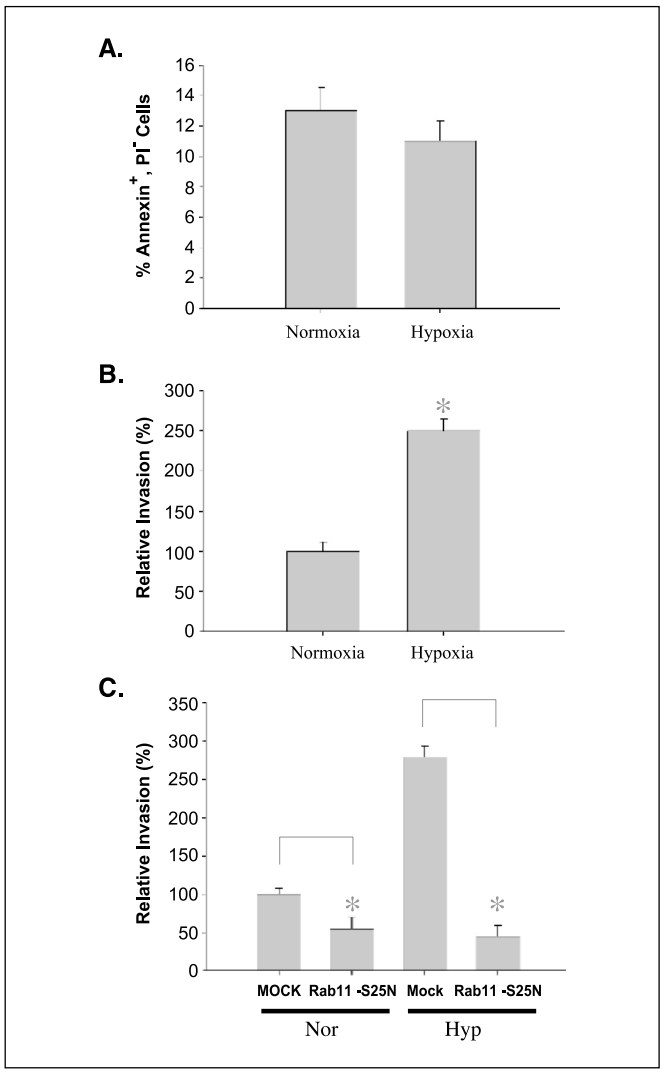

Figure 1. Rab11 is involved in the hypoxia-stimulated invasion of MDA-MB-231 cells. *A,* apoptosis in normoxic or hypoxic culture conditions was assessed using annexin V-FITC as described in Materials and Methods. *B,* the ability of cells to invade Matrigel during 24 hours of incubation in either normoxia or hypoxia was evaluated. *C,* cells were transfected with either pEGFP or pEGFP-Rab11-S25N, and after 24 hours their ability to invade Matrigel was determined as described in *B*. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test.

Hypoxia stabilizes microtubules. Based on the reports that Rab11 trafficking is regulated by microtubules (24–26) and that microtubules are involved in vesicle trafficking (27), we examined the hypothesis that hypoxia alters Rab11 localization by altering or stabilizing microtubules. For this purpose, MDA-MB-231 cells were transfected with a GFP-wild-type Rab11 vector, maintained in either normoxia or hypoxia for 24 hours, and then stained with a tubulin antibody. As shown in Fig. 3*A*, cells maintained in normoxia exhibited a relatively sparse and disorganized pattern of microtubules and perinuclear localization of Rab11. Cells maintained in hypoxia, however, displayed an extensive network of microtubules that were thicker and more aligned than those observed in normoxia and a dispersed localization of Rab11. Of note, Rab11-containing vesicles were seen adjacent to the elongated microtubules (Fig. 3*A*). The dependence of Rab11 localization on microtubule structure is evidenced by the finding that disruption of microtubules using colchicine in cells maintained in hypoxia resulted in the aggregation of Rab11-containing vesicles in the perinuclear region (Fig. 3*B*).

More definitive evidence to support the hypothesis that hypoxia stabilizes microtubules was obtained using an antibody that recognizes detyrosinated (Glu) tubulin, an indicator of microtubule stability (28–30). As seen in Fig. 3*C*, little, if any, Glu tubulin staining was detected in cells maintained in normoxia. In contrast, Glu tubulin staining was clearly evident in cells maintained in hypoxia (Fig. 3*C*). Finally, to validate the importance of microtubules for invasion, invasion assays were done in the presence of colchicine (Fig. 3*D*). This microtubule disrupting agent decreased invasion dramatically (Fig. 3*D*) but it did not have a significant effect on the apoptosis of these cells (Fig. 3*D*).

Hypoxia stabilizes microtubules by a mechanism that involves activation of Akt and inhibition of glycogen synthase kinase 3β. Microtubule stability is regulated by GSK-3β, the activity of which is inhibited by Akt. These findings lead to the hypothesis that one mechanism by which hypoxia stabilizes microtubules is to stimulate Akt activation and inhibit GSK-3β. This hypothesis was evaluated by assessing the effect of hypoxia on the activities of these kinases. As shown in Fig. 4*A*, hypoxia increased the phosphorylation of Akt on serine 473 as determined by immunoblotting with a phosphospecific antibody. This increased phosphorylation was evident at 24 hours of hypoxic exposure and was sustained for an additional 24 hours. A similar induction of GSK-3β phosphorylation was observed, an event that triggers the inactivation of this kinase.

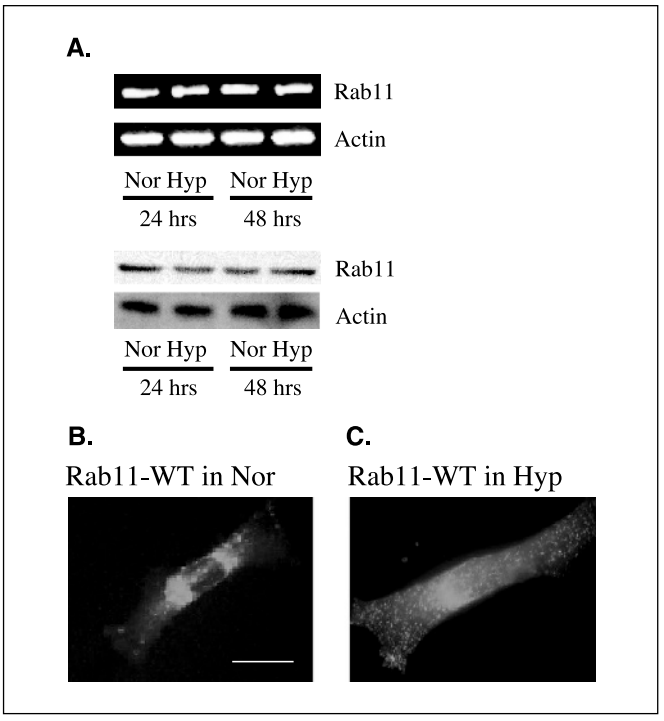

Figure 2. Hypoxia regulates the localization of Rab11. *A,* mRNA and protein levels of Rab11 were detected using RT-PCR and Western blot analysis, respectively. RNA and protein loadings were normalized using the signal obtained with actin. *B* and *C,* cells were transfected with wild-type Rab11 (pEGFP-Rab11-WT). After 12 hours, the cells were incubated either in normoxia (*B*) or hypoxia (*C*) and examined using a confocal microscope. Bar, 20 μm.

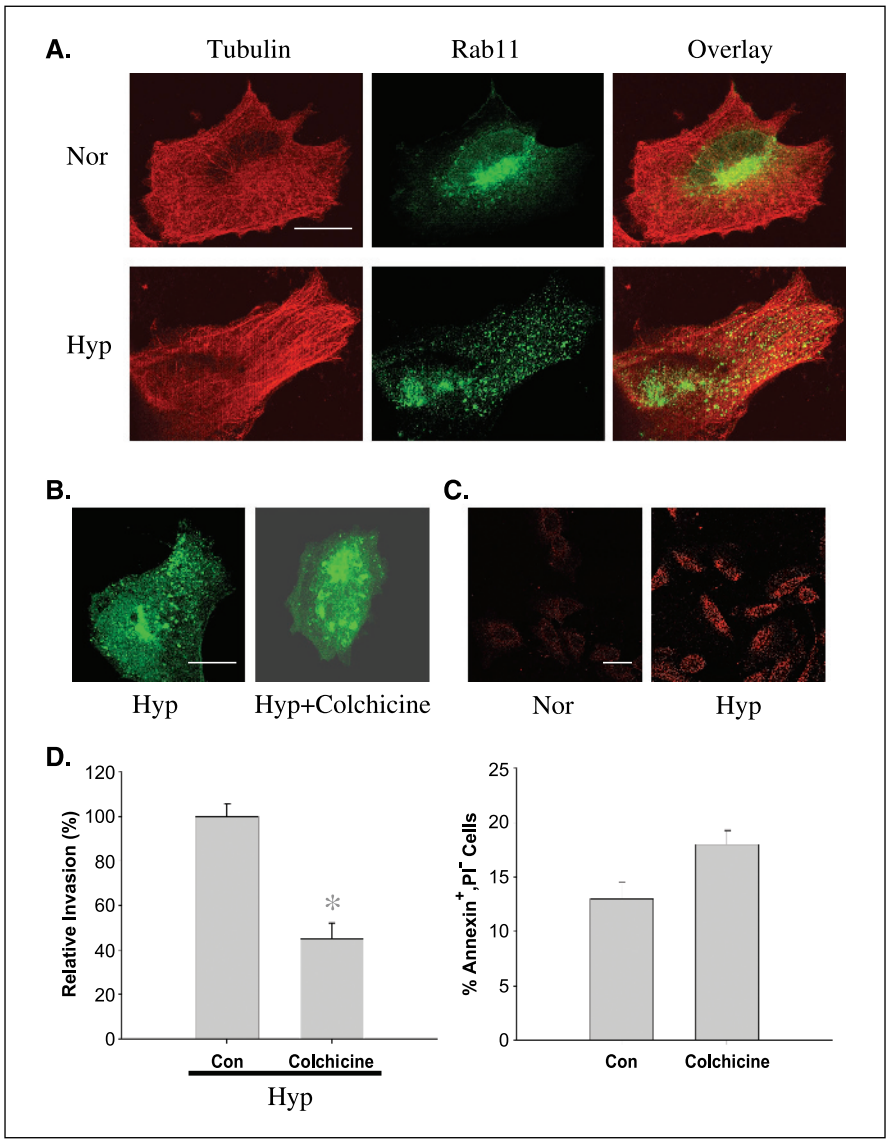

Figure 3. Hypoxia induces stabilization of microtubules. *A,* cells were transfected with wild-type Rab11 (pEGFP-Rab11-WT) for 12 hours and then incubated either in normoxia or in hypoxia for an additional 24 hours. The cells were stained with an α-tubulin antibody and subjected to confocal microscopy. Bar, 20 μm. *B,* cells were transfected with wild-type Rab11 (pEGFP-Rab11-WT) for 12 hours, treated with colchicine, and incubated in hypoxia for 24 hours. The localization of GFP-Rab11 was visualized by confocal microscopy. Bar, 20 μm. *C,* cells were incubated in normoxia or hypoxia for 24 hours and stained with a detyrosinated tubulin antibody. Bar, 50 μm. *D,* invasion assay was done for 24 hours in hypoxia in either the absence or presence of 100 nmol/L colchicine in the upper chambers. Apoptosis was measured using annexin V-FITC methods.

To evaluate the contribution of Akt and GSK-3β to hypoxia-induced microtubule stabilization, we used LY294002 to inhibit phosphatidylinositol 3-kinase, which is upstream of Akt, and the GSK-3β inhibitor, SB415286. Treatment of cells maintained in hypoxia with LY294002 resulted in a dramatic disruption of microtubules (Fig. 4*B*). Conversely, SB415286 treatment of cells maintained in normoxia resulted in increased microtubule organization (Fig. 4*B*). Additional evidence to support the involvement of GSK-3β in hypoxia-induced stabilization of microtubules was obtained using the Glu tubulin antibody. As shown in Fig. 4*C*, the induction of Glu tubulin expression at 24 hours of exposure to hypoxia parallels the increase in phospho-Akt expression. Moreover, inhibition of phosphatidylinositol 3-kinase with LY294002 prevented the induction of Glu tubulin expression and, conversely, inhibition of GSK-3β with SB415286 increased Glu tubulin expression substantially (Fig. 4*C*). These data support the hypothesis that the inhibition of GSK-3β activity in cells maintained in normoxia would increase their rate of invasion. Indeed, as shown in Fig. 4*D*, expression of a kinase-dead GSK-3β resulted in an ~2-fold increase in the relative rate of invasion of MDA-MB-231 cells.

Hypoxia increases surface expression of integrin α6β4 through a Rab11-dependent pathway. The foregoing data argue for a mechanism of hypoxia-stimulated invasion that involves inhibition of GSK-3β activity, increased microtubule stability, and microtubule-dependent trafficking of Rab11-containing vesicles. A key question that emerges from this mechanism is the identification of surface proteins of which expression is regulated by Rab11 and that function in invasion. In this regard, we focused on specific integrins as potential targets of Rab11 trafficking for several reasons. Integrins mediate the invasion of cells through basement membranes and there is evidence that trafficking of β1 integrins can be regulated by Rab11 (19). We chose to study the two integrins that are the primary receptors for basement membranes on epithelial and carcinoma cells: α3β1 and α6β4. Both of these integrins are expressed on MDA-MB-231 cells, and, with respect to the α6 integrins, these cells express mostly α6β4 and little, if any, α6β1 (31, 32). Initially, we assessed whether hypoxia increased the surface expression of these integrins using flow cytometry. As shown in Fig. 5*A*, hypoxia increased the surface expression of the α6 and β4 subunits significantly, but it

did not have a significant effect on the expression of the α3 subunit. Moreover, the time course for the hypoxia-induced increase in α6 expression (Fig. 5*B*) mirrored the invasion and biochemical data shown previously.

The potential regulation of α6 integrin surface expression by Rab11 was investigated using the inactive, GDP-bound form of Rab11. Expression of this construct in MDA-MB-231 cells prevented the hypoxia-induced increase in α6 surface expression (Fig. 5*C*). Importantly, expression of this construct had no significant effect on the surface expression of the α3β1 integrin (Fig. 5*D*), a finding that indicates that α3β1 surface expression is not regulated by Rab11 in these cells. To exclude the possibility that hypoxia increases total expression of the α6 integrin, we examined α6 mRNA by RT-PCR (Fig. 5*E*) and protein expression by immunoblotting (Fig. 5*F*) in cells maintained either in normoxia or hypoxia. No differences in expression were evident under these conditions.

Additional insight into the relationship between α6 integrins and Rab11 was obtained by immunostaining cells maintained in normoxia or hypoxia. In normoxia, α6 and Rab11 exhibited an intense colocalization in vesicles in the perinuclear region (Fig. 6). Hypoxia resulted in a striking redistribution of α6 to the plasma membrane and a dispersal of Rab11 containing vesicles throughout the cytoplasm (Fig. 6). In addition, some of these cytoplasmic vesicles exhibited α6 staining. These staining patterns are consistent with a model in which hypoxia stimulates the Rab11-dependent transport of α6-containing vesicles to the plasma membrane. In contrast, there was no indication of a colocalization of Rab11 with α3 integrin either in normoxia or hypoxia, and hypoxia had no effect on the plasma membrane localization of α3 (Fig. 6). These immunostaining data substantiate the flow cytometry data shown in Fig. 5.

Discussion

The hypoxic microenvironment of solid tumors selects for the survival of aggressive, highly invasive cells that have the propensity to metastasize (8). Although the mechanisms used by tumor cells

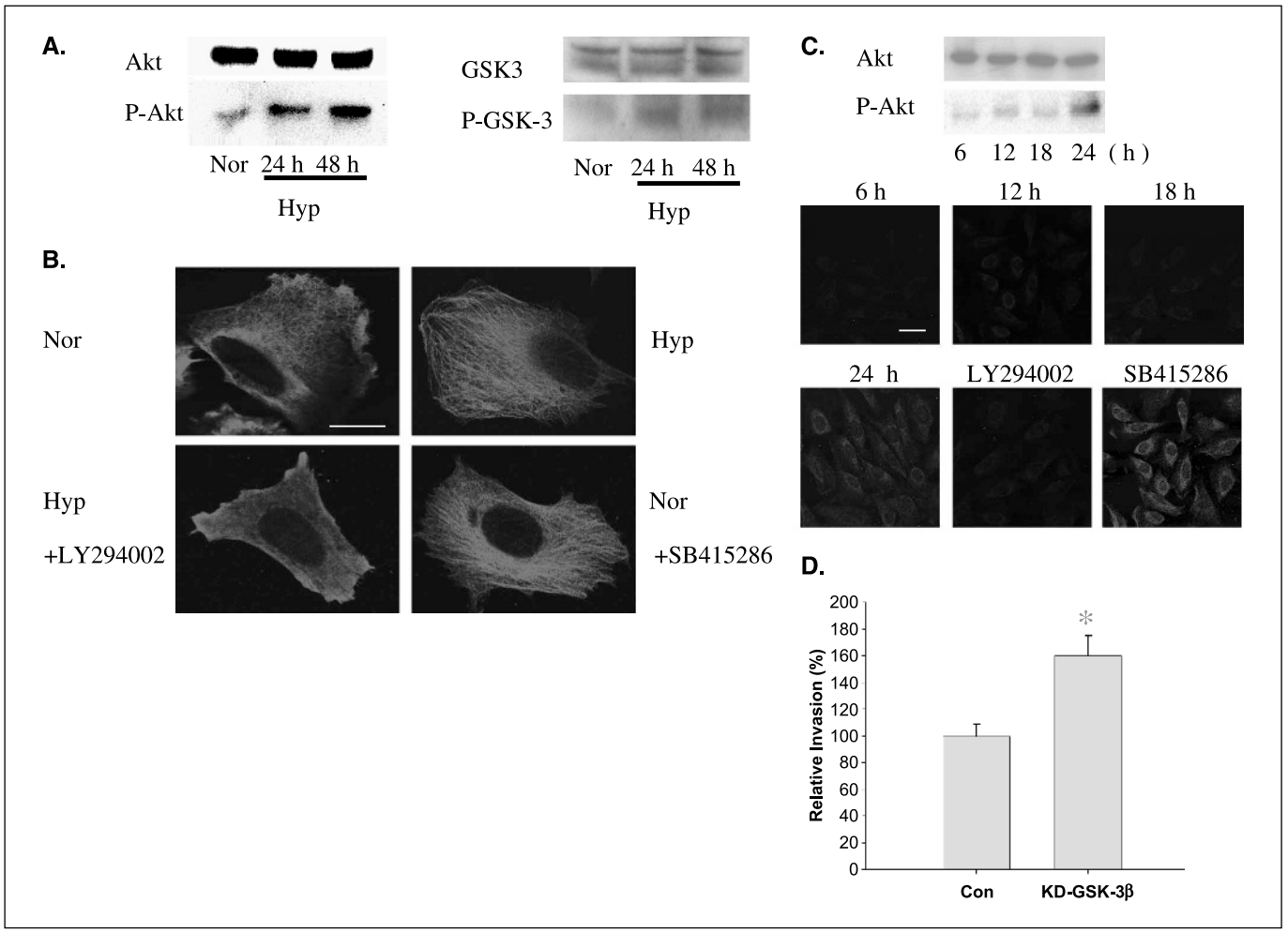

Figure 4. Hypoxia regulates microtubules through a phosphatidylinositol 3-kinase/GSK-3β pathway. *A,* cells were incubated either in normoxia or in hypoxia for indicated times. Expression of Akt, phospho-Akt, GSK-3, and phospho-GSK-3β was detected by immunoblot analysis. *B,* cells were treated with either 20 μmol/L of LY294002 and incubated for 24 hours in hypoxia or with 40 μmol/L of SB415286 and incubated for 6 hours in normoxia. Subsequently, the cells were stained with a tubulin antibody and analyzed by confocal microscopy. Bar, 20 μm. *C,* cells were incubated in hypoxia for the indicated times. Expression of Akt and phospho-Akt was detected by immunoblot analysis and detyrosinated tubulins were stained and subjected to confocal microscopy. To assess the effects of phosphatidylinositol 3-kinase and GSK-3β on detyrosinated tubulin expression, 20 μmol/L of LY294002 or 40 μmol/L of SB415286 were added and the cells were incubated for 24 hours in hypoxia. Bar, 50 μm. *D,* cells were transfected with a dominant-negative GSK-3β vector. After 24 hours, cells were trypsinized and subjected to an invasion assay in normoxia.

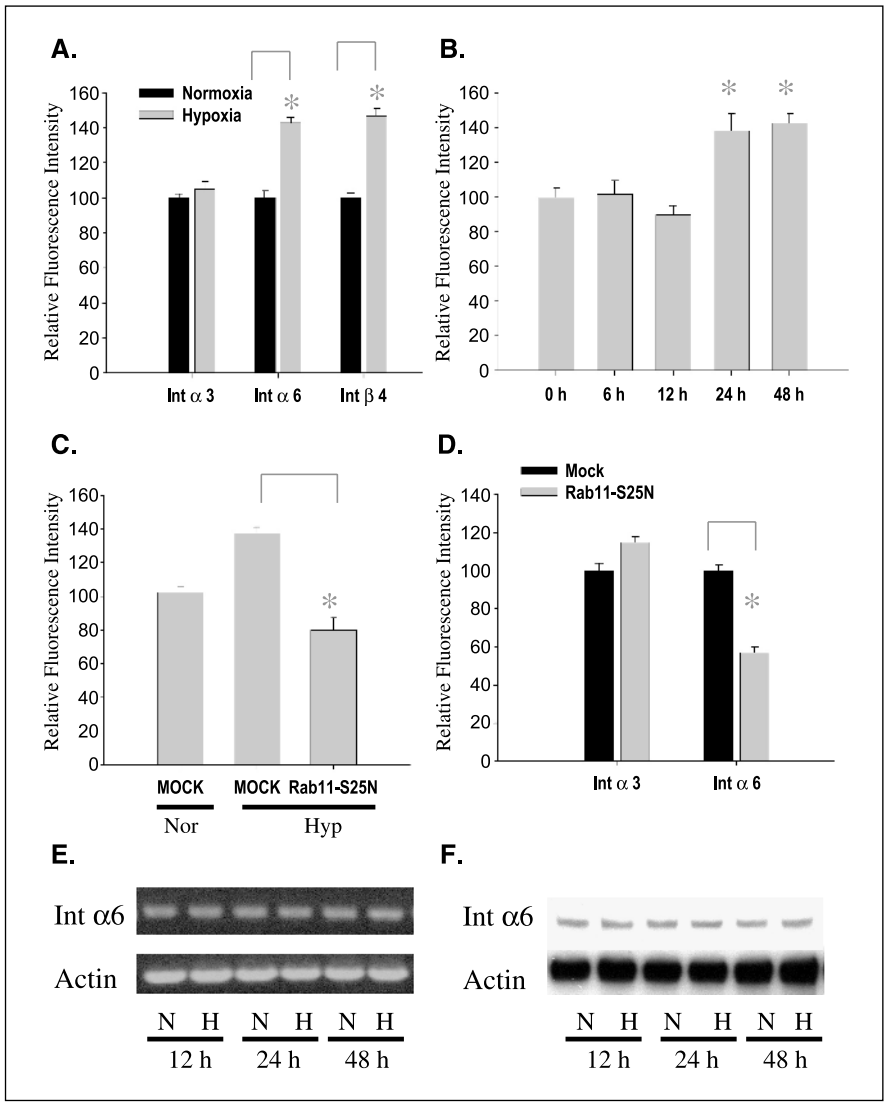

Figure 5. Hypoxia increases surface expression of integrin α6β4. Analysis of integrin surface expression was done by flow cytometry. Relative fluorescence intensity was calculated from mean channel fluorescence of 5,000 cells. *Columns,* mean of three independent experiments; *bars,* SD. Statistically significant differences in integrin expression were assessed using Student's *t* test ($P < 0.01$) and are denoted with an asterisk (*). *A,* cells were incubated either in normoxia and hypoxia for 24 hours and surface expression of integrin subunits α3, α6, and β4 was measured. *B,* cells were incubated in hypoxia for the indicated times and expression of integrin α6 was evaluated. *C,* pEGFP and GDP-locked Rab11 (pEGFP-Rab11-S25N) were transfected into the cells and integrin α6 expression was assessed after incubation in normoxia or hypoxia. Data reflect integrin α6 expression only in GFP-positive cells. *D,* cells were transfected with either pEGFP or GDP-locked Rab11 (pEGFP-Rab11-S25N) and incubated in hypoxia. Cell surface integrins levels were measured; data reflect integrin α6 expression only in GFP-positive cells. *E* and *F,* cells were incubated in either normoxia or hypoxia for indicated times and expression of integrin α6 was detected by either RT-PCR (*E*) or immunoblot analysis (*F*). RNA and protein loading were normalized using the signal obtained with α-actin.

to survive in hypoxia are known in reasonable detail, less is known about how hypoxia facilitates the migration and invasion of such cells. In this direction, the data reported here reveal that microtubule stabilization and the consequent effects of this stabilization on Rab11-mediated vesicle transport are novel targets of hypoxic stimulation that are linked to invasion. Moreover, we identified one target of Rab11-mediated vesicle transport, the integrin α6β4, of which surface expression is regulated by hypoxia and that functions to mediate invasion.

Previous studies on the mechanism by which hypoxia stimulates tumor invasion have focused largely on the ability of hypoxia-inducible factor 1 to stimulate the transcription of proteases and protease receptors that have potential to facilitate invasion (33, 34). In addition, a recent study highlighted the ability of hypoxia to enhance the transcription of the Met receptor and promote hepatocyte growth factor–dependent invasion in hypoxic areas of tumors (12). To our knowledge, ours is the first study that identifies Rab GTPase-mediated vesicle transport as a target of hypoxic stimulation. In simple terms, these findings imply that the tumor microenvironment can alter the transport and recycling of cell surface proteins, a process that would provide a dynamic link between the microenvironment and invasion. Although the established function of Rab11, the Rab GTPase that we have implicated in invasion, is recycling of plasma membrane proteins (17, 18), it is also involved in transport of molecules from recycling endosomes to the *trans*-Golgi network (21) and from the *trans*-Golgi network to plasma membrane (17, 21, 22). In this context, it has been shown recently that phosphatidylinositol 4-kinase β, one of the isoforms of phosphatidylinositol 4-kinase, interacts with GTP-bound Rab11 to regulate vesicular transport from the Golgi complex to the plasma membrane (35). This role of Rab11 in transport from the Golgi complex to the plasma membrane is substantiated by our finding that hypoxia facilitated the transport of vesicles containing the α6 integrin from the perinuclear region to the plasma membrane. Thus, it seems likely that hypoxia increases recycling, as well as translocation of newly synthesized and endocytosed vesicles, from the Golgi complex to the plasma membrane.

Although microtubules have been implicated in Rab11 vesicle transport (24–26), the ability of hypoxia to stabilize microtubules had not been considered. This result, however, is consistent with numerous studies that have implicated microtubules in cell

migration (36-39). Reorganization of the microtubule network and regulation of its dynamics are essential for cell polarization toward the direction of movement. More specifically, the formation of a subset of unusually stable microtubules by capping microtubule plus ends contributes to this polarization (29, 40, 41). Stable microtubules increase cell migration because they serve as tracks for directed membrane and organelle transport to the leading edge of the cell and they regulate adhesion and contraction. These stable microtubules have a long half-life (>1 hour) compared with dynamic microtubules with a half-life of 5 to 10 minutes (42). Detyrosinated tubulin (Glu tubulin) is a form of stabilized tubulin that accumulates in the microtubules of migrating cells (28–30). Our finding that hypoxia increases the content of Glu tubulin in carcinoma is consistent with a role for stabilized tubulin in hypoxia-induced migration and invasion. Interestingly, these data also agree with the observation that tubulin detyrosination occurs in breast cancers with poor prognosis (30).

The finding that hypoxia inhibits the activity of GSK-3β is intriguing in light of the potential contribution of this kinase to tumor invasion. GSK-3β is a key regulator of numerous signaling pathways including G-protein coupled receptors and cellular response to Wnt signaling (43). Of relevance to our study are the reports that GSK-3β phosphorylates a number of microtubule-associated proteins, such as microtubule-associated protein (MAP2C), MAP1B, tau, adenomatous polyposis coli, and cytoplasmic linker protein–associating protein 2 (44–48). The fact that their phosphorylation by GSK-3β decreases their ability to stabilize microtubules provides a mechanism that links hypoxia-mediated inactivation of GSK-3β to microtubule stabilization. Indeed, our data indicate that active GSK-3β impedes the invasion of carcinoma cells. This conclusion is consistent with the recent reports that inhibition of GSK-3β induces the transcription and stabilization of Snail, a transcription factor that represses E-cadherin transcription and, as a consequence, promotes an epithelial-to-mesenchymal transition (49, 50). Moreover, it is well established that loss of E-cadherin expression is associated with more invasive and aggressive tumors and that the epithelial-to-mesenchymal transition is a useful paradigm for studying tumor invasion (51). Based on our results, it will be informative to test the hypothesis that microtubule stabilization is a critical component of the epithelial-to-mesenchymal transition.

The fact that the α6β4 integrin seems to be a target of Rab11-mediated trafficking adds to the numerous reports that have implicated this integrin in the migration and invasion of carcinoma cells (1, 52, 53). Interestingly, the α3β1 integrin, which has also been implicated in epithelial and carcinoma cell migration, does not seem to be subject to regulation at the level of trafficking by hypoxia. These observations agree with an earlier study that had shown that α6β4 but not α3β1 participates in endocytotic recycling (54). In addition, it was argued in this study that the determinants of integrin recycling are integrin α chains and not β chains. This point is substantiated by the finding that the cytoplasmic domain of the α6 subunit is responsible for the trafficking of the α6β4 and α6β1 integrins (55). A more recent study provided evidence that integrin β1 is transported through a Rab11-positive recycling compartment (19). Taken together, it will be informative to determine which specific β1 integrins are subject to Rab11 trafficking and whether such trafficking is regulated by hypoxia. Nonetheless, our finding that α6β4 is regulated by Rab11 and that this regulation facilitates invasion reinforces a central role for this integrin in carcinoma progression.

Although our study highlights α6β4 as one target of Rab11-mediated trafficking that facilitates invasion, it is more than likely

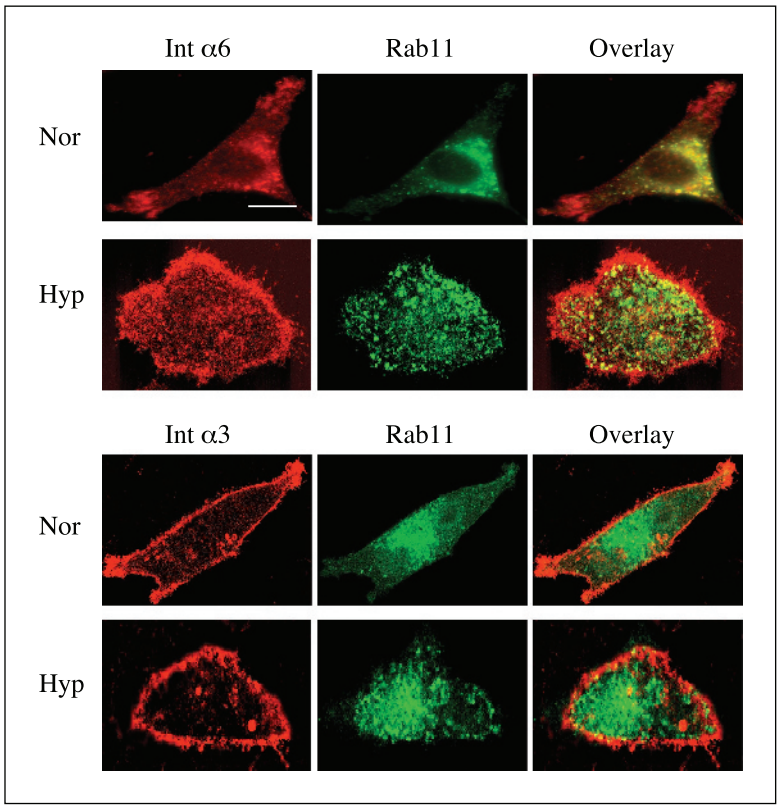

Figure 6. Rab11 regulates trafficking of integrin α6. Cells were transfected with wild-type Rab11 (pEGFP-Rab11-WT) for 12 hours and then incubated in either normoxia or hypoxia for 24 hours. The localization of the integrin α6 and α3 subunits was detected by indirect immunofluorescence microscopy as described in Materials and Methods. Bar, 20 μm.

that several other proteins are also targets. Of interest for further study in this direction are invasion-related proteins such as EGF receptor, protein kinase C-α, and CXC chemokine receptors, all of which can be regulated by Rab11 (20, 56-58). Moreover, given that EGF receptor signaling and protein kinase C-α have been shown to promote the invasive function of α6β4 (59-63), it is tempting to speculate that hypoxia may enhance a synergism among these proteins, which facilitates invasion.

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