Vacular H⁺-ATPase functions as a vacuolar proton pump and is responsible for acidification of intracellular compartments such as the endoplasmic reticulum, Golgi, lysosomes, and endosomes. Previous reports have demonstrated that a 16-kDa subunit (16K) of vacular H⁺-ATPase via one of its transmembrane domains, TMD4, strongly associates with β₁ integrin, affecting β₁ integrin N-linked glycosylation and inhibiting its function as a matrix adhesion receptor. Because of this dramatic inhibition of β₁ integrin-mediated HEK-293 cell motility by 16K expression, we investigated the mechanism by which 16 kDa was having this effect. Using HT1080 cells whose α₁β₁ integrin-mediated adhesion to fibronectin has been extensively studied, the expression of 16 kDa also resulted in reduced cell spreading on fibronectin-coated substrates. A pulse-chase study of β₁ integrin biosynthesis indicated that 16K expression down-regulated the level of the 110-kDa biosynthetic form of β₁ integrin (premature form) and, consequently, the level of the 130-kDa form of β₁ integrin (mature form). Further experiments showed that the normal levels of association between the premature β₁ integrin form and calnexin were significantly decreased by the expression of either 16 kDa or TMD4. Expression of 16 kDa also resulted in a Triton X-100-insoluble aggregation of an unusual 87-kDa form of β₁ integrin. Interestingly, both Western blotting and a pulse-chase experiment showed co-immunoprecipitation of calnexin and 16K. These results indicate that 16K expression inhibits β₁ integrin surface expression and spreading on matrix by a novel mechanism that results in reduced levels of functional β₁ integrin.

Vacular H⁺-ATPase (V-ATPase) is a multi-subunit complex found in all of the eukaryotic cells and consists of the V1 domain, which contains the ATPase activity, and the V0 domain, which contains transmembrane proton channel. V-ATPase is ubiquitously expressed in the various intracellular compartments and is responsible for their acidification (1, 2). The 16-kDa subunit (16K) of the V0 domain is a highly hydrophobic protein, consisting of four transmembrane segments, and it forms a homohexamer that functions as the transmembrane proton channel. The 16K can associate with other proteins through interactions with its fourth transmembrane segment, TMD4. For example, the papillomavirus E5 oncoprotein product associates with the TMD4 of 16K, decoupling 16 kDa from the ATPase V1 and resulting in an elevated pH in the Golgi and facilitation of the oncogenic transformation process. In addition, the complex between bovine papillomavirus E5 and the 16K can associate with the platelet-derived growth factor receptor, leading to a ligand-independent activation of platelet-derived growth factor receptor signaling (3).

β₁ integrin performs a critical function in the anchorage-dependent growth of many cell types and is transcriptionally regulated by a variety of factors during development and oncogenic transformation (4–9). The TMD4 of 16 kDa has also been shown to mediate an association between 16K and β₁ integrin (10). Subsequently, it was shown that the 16K expression in HEK-293 cells caused altered N-linked glycosylation of β₁ integrin, resulting in a significant inhibition of cell migration toward laminin and fibronectin (10, 11). Since altered expression of N-linked glycans on the α₁β₁ integrin has been shown to significantly affect the binding of cell surface α₁β₁ to fibronectin, it was reasonable to hypothesize that the expression of 16 kDa had specific effects on the glycosylation of β₁ integrin, thereby inhibiting its adhesive functions that regulate migration on extracellular matrix.

In this study, we investigated the mechanism of how the 16K expression levels affect the expression and/or the maturation and glycosylation of β₁ integrin during the biosynthesis of the α₁β₁ dimer to ultimately inhibit β₁ integrin-dependent cell adhesion to fibronectin. The results showed that expression of the 16 kDa in human fibrosarcoma HT1080 cells down-regulates surface expression of α₁β₁ integrin dimers, thereby causing reduced cell spreading on fibronectin-coated surfaces. The loss of cell surface α₁β₁ expression was subsequently shown to result from a Triton X-100-insoluble aggregation of β₁ integrin subunits in the endoplasmic reticulum. Initial results suggested that the 16K expression suppressed the maturation of β₁ integrin from its premature form that expressed only high-mannose and not complex glycans. Pulse-chase experiments then showed that premature (110 kDa) β₁ integrin levels in 16K-transfected cells were greatly decreased and that this decrease occurred during the first 15 min of β₁ integrin biosyn-
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thesis. Further experiments using antibodies against calnexin, calreticulin, and BIP showed that calnexin, but not calreticulin or BIP, associated with the premature β1 integrin. When 16K was expressed, the normal association between the premature β1 integrin and calnexin was almost completely abolished. The expression of the TMD4 alone caused the same level of inhibition on β1 integrin association with calnexin. Surprisingly, both 16K and TMD4 were themselves co-immunoprecipitated with calnexin. An examination of the Triton X-100-insoluble fraction of cells transfected with 16K revealed a new form of β1 integrin and the appearance of a Triton X-100-insoluble 87-kDa form of β1 suggest that the decrease of the 110-kDa form was not due primarily to ER-associated degradation (ERAD) but to an induction of the Triton X-100-insoluble form of β1. These results suggested a unique mechanism for the regulation of the cell surface expression of β1 integrin by expression of the V-ATPase 16-kDa subunit.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Materials—HT1080 human fibrosarcoma cells were obtained from ATCC and maintained in DMEM containing 10% fetal bovine serum. Unless otherwise described, all of the transfections were done transiently using Lipofectamine reagent (Invitrogen) in 100-mm dishes. Cells were harvested typically between 24 and 48 h after transfection and used for further experiments. Rat mononuclear antibodies against human αν and β1 integrins, mAb16 and mAb13, respectively, were gifts from Dr. Steven Akiyama (NIEMS, National Institutes of Health). The anti-T7 and HSV-tagged antibodies were purchased from Novagen. Antibodies against calnexin, calreticulin, and BIP were purchased from Stressgen. Agarose-conjugated D-phytohemagglutinin, ConA, Sambucus nigra agglutinin, and Datura stramonium agglutinin lectins were obtained from Vector Laboratories, whereas 18C2, an inhibitor of proteasome activity, was from Biomen. PNGase F was obtained from Sigma, and streptavidin-HRP was from Rockland. NHS-LC-Biotin was purchased from Pierce. anti-ERK2 antibody, anti-actin antibody, Protein G Plus-agarose, and all of the secondary antibodies conjugated to HRP were from Santa Cruz Biotechnology. Goat anti-mouse IgG-Alexa 594 and phallolidin-FITC were all purchased from Molecular Probes. Mammalian expression plasmids, pXJ40-KKO-16K (HSV-tagged 16K), pXJ40-KKO-TMD4 (HSV-tagged TMD4; formerly named α1), and pXJ41-β1-T7 (T7-tagged bovine β1 integrin), were obtained as described previously (11).

Immunoprecipitation and Western Blot Analysis—Cells were lysed in a lysis buffer consisting of 2% Triton X-100, protease inhibitors, and 0.1% SDS. Typically, 100 μg of total protein was used for immunoprecipitation. Precipitates were resolved with SDS-PAGE utilizing 8% gels under non-reducing conditions unless otherwise indicated, transferred to polyvinylidene difluoride membranes, blocked in 2% bovine serum albumin, and probed with antibodies. Following incubation with HRP-conjugated secondary antibodies or streptavidin-HRP, proteins were detected by the ECL reagent (Pierce). Densitometry of the blots was performed using a Bio-Rad Fluor S imager.

Cell Surface Biotinylation and Immunoprecipitation—Cell surfaces were labeled with NHS-LC-biotin as described previously (12). Cells were washed three times with ice-cold PBS in culture dishes. 1 mg/ml NHS-LC-biotin in PBS (1 ml) was added to the cells and incubated at 4 °C for 15 min. Cells were then washed three times in Tris-buffered saline and lysed in lysis buffer. Immunoprecipitation and Western blotting were done as described above with the exception that streptavidin-HRP was directly added with no primary antibody.

Radiolabeling and Autoradiography—Cells were preincubated with Met/Cys-free DMEM (Invitrogen) for 30 min followed by labeling with 100 μCi/ml [35S]methionine/cysteine in 3 ml of DMEM lacking methionine and cysteine but containing 2% dialyzed fetal bovine serum for 15 min. After removing the radioactive medium, regular growth medium was then added and incubated for various times. Labelled cells were then lysed in lysis buffer, and cell lysates were immunoprecipitated with either mAb13 or anti-T7 antibody at 4 °C overnight. For an experiment with MG132, 50 μM final concentration of MG132 in MeSO was added to the cells during both pulse and chase periods.

Real-time PCR Analysis—Cells were transfected with pXJ41-β1-T7 alone (5 μg) or with pXJ41-β1-T7 plus pXJ40-KKO-16K (5 μg of each) and cultured for 24 h. The RNaseasy kit (Qiagen) was used to isolate total RNA. Reverse transcription was done using Superscript III (Stratagene) and random primers using the manufacturer’s protocol. Bio-Rad iCycler and iCycler software were used for real-time PCR reactions and data analysis. Primers were designed using Primer3 software. Sequences of primers used in the experiment were as follows: human αν integrin (forward, 5'-CAGTGGCCATGATGTTGTG-3', and reverse, 5'-CGATGGCCCATCATCATTGAG-3'); human β1 integrin (forward, 5'-ATCCAGAGGCTCCAAAGAT-3', and reverse, 5'-CCCTGATCTTTAATCGAAA-3'); human 16K (forward, 5'-AGTCCATACCCACGTTGTC-3', and reverse, 5'-CACTGGAGGAAGCTCTCTGT-3'); and human GAPDH (forward, 5'-CAGGCCCTCCTATTGC3'-3', and reverse, 5'-ATCCCATGACATCTTCACA-3'). Real-time PCR was performed using the iQ™SYBR Green Supermix (Bio-Rad). The PCR conditions were 1 cycle at 95 °C for 30 s followed by 40 cycles at 95 °C for 10 s, 65 °C for 30 s, 95 °C for 1 min, and 55 °C for 1 min, terminating with 80 cycles at 55 °C for 10 s for melting curve analysis. Standard curves for human αν and β1 integrin primer characterization were generated by a serial dilution of cDNA obtained from the reverse transcription reaction, whereas pXJ40-KKO and pT3-GAPDH were used for standard curve generation for 16K and GAPDH, respectively.

Fluorescent Staining—Chamberslides (Falcon) were coated with 10 μg/ml fibronectin (Sigma) and incubated at 4 °C overnight. Transiently transfected cells were harvested by trypsinization and incubated in serum-free DMEM for 1 h at 37 °C. Approximately 10⁴ cells were added to each well and allowed to spread on fibronectin-coated surfaces for 30 min. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min, and blocked with 1% bovine serum albumin for 30 min at 37 °C. Cells were then incubated with anti-HSV tag antibody (1:10000) and mAb13 (10 μg/ml) at 37 °C for 30 min. Cells were then washed with PBS, the primary antibodies were detected by incubation with either rhodamine-anti-mouse IgG conjugate or rhodamine-anti-rat IgG (1:250) at 37 °C for 30 min. For actin staining, phalloidin-FITC (1:250) was used. After washing with PBS, the coverslips were mounted and images were obtained using an inverted fluorescence microscope (Leica).

RESULTS

Expression of the V-ATPase 16K in HEK-293 cells inhibited significantly β1 integrin-mediated cell adhesion and migration on fibronectin and laminin (11). In addition, the expression of 16K appeared to have an inhibitory effect on the complex N-linked glycosylation of β1 integrin, suggesting that the inhibition of matrix adhesion may have resulted from altered glycosylation of β1 integrin. To test this hypothesis in detail, we used HT1080 cells to examine the effects of 16K expression on β1 integrin-fibronectin interactions as well as the biosynthesis of β1. The predominant β1 integrin expressed in HT1080 cells is αβ1 integrin (12), and these cells have been used to investigate the effects of altered N-linked glycosylation on αβ1 integrin-mediated cell adhesion and migration on fibronectin (12, 13).

16K Expression Delays Cell Spreading and Reduces Cell Surface Expression of αβ1 Integrin—First, we confirmed that 16K expression affected αβ1 integrin-mediated HT1080 cell adhesion and spreading on fibronectin. HT1080 cells were transiently transfected with 16K tagged with the HSV epitope, harvested, preincubated in suspension in serum-free DMEM for 1 h, added to fibronectin-coated chamber slides, and allowed to spread for 30 min. Phallolidin-FITC was used to visualize the actin cytoskeleton of the cells, and anti-HSV was used to detect 16K expression. The results (Fig. 1) showed that cells transfected with vector alone (Mock) showed extensive spreading on the fibronectin substrate. By contrast, cells transfected with 16K-HSV were attached to the substrate but were rounded in appearance due to the inhibition of spreading. Experiments were then performed to examine the effects of 16K expression on β1 integrin cell surface expression using cell surface biotinylation. The results showed a gradual decrease of αβ1 integrin expression on the cell surface as the amount of 16K plasmid used for transfection was increased. Cell surface expression of both the epidermal growth factor receptor and N-cadherin showed no significant decrease after transfection with...
16K plasmid (15 µg), demonstrating a specific inhibitory effect on α5β1 integrin (Fig. 2).

Characterization of Premature and Mature Forms of β1 Integrin—As a first step to understanding the possible effects of 16K expression on β1 integrin cell surface expression, it was necessary to characterize its biosynthesis. Typically, β1 integrin is first expressed as the so-called “premature form” (110 kDa), which dimerizes with α subunits and is transported to the Golgi complex where it then matures into the 130-kDa form that expresses complex N-linked glycans (7, 14, 15). The premature and mature forms can also be distinguished on Western blots by the binding of specific lectins. ConA, which binds high-mannose, hybrid, and diantennary glycans, binds both the premature and mature forms of β1, whereas S. nigra agglutinin, which recognize β1, 6-branched and α2, 6-sialylated glycans, respectively, bind only to the mature β1 form. When T7-tagged bovine β1 integrin (β1-T7) was expressed in HT1080 cells, both the mature and premature forms of the T7-tagged integrin were detected by Western blotting of cell lysates (Fig. 3A). The relatively higher levels of the premature form were probably due to limiting levels of integrin α subunits to allow dimerization with β1, which is a requirement for Golgi processing and complex N-linked glycosylation. Fig. 3A also showed that only the mature (130 kDa) form of β1 was precipitated with a mAb16 against human α5 integrin and the lectin-recognizing complex N-linked glycan modifications, S. nigra agglutinin, but the premature (110 kDa) form was precipitated only by ConA.

Premature Forms of β1 Integrin Were Down-regulated by 16K Expression—When HSV-tagged 16K (16K-HSV) was co-transfected with β1-T7, the expression of the mature β1 form was preferentially decreased. Immunoprecipitation of the lysate of cells transfected with β1 integrin or β1 integrin plus 16K with the antibody specific for the α5 subunit (mAb16) revealed that the mature β1 integrin co-immunoprecipitated with α5 integrin was significantly decreased after 16K expression (Fig. 3B). To determine whether increased 16K expression suppressed the expression of the premature form as well, the amount of 16K plasmid used for transfections was increased successively while keeping the amount of β1-T7 plasmid constant. The results showed that the levels of the premature β1 form were greatly decreased after 16K expression. The levels of α5 integrin and β-actin, however, showed no significant decrease as the expression of 16K increased, demonstrating that the inhibitory effect on β1 expression was specific (Fig. 3C). When cell lysates from cells co-transfected with β1 integrin and 16K-HSV...
were immunoprecipitated with anti-HSV antibody, β1 co-immunoprecipitated with the anti-HSV tag antibody, again demonstrating that 16K associated with β1 integrin. After SDS-PAGE and Western blotting, the β1 that co-immunoprecipitated with 16K migrated similarly to β1 integrin treated with Endo H (data not shown).

Normally, the premature β1 integrin form does not persist for more than 2 h before complete α/β1 dimerization and maturation into the mature 130-kDa form (Fig. 4A). To determine whether 16K expression had similar effects on the endogenous β1 integrin expression and maturation as it had on overexpressed β1, we studied the biosynthesis of the endogenous β1 using metabolic labeling and an anti-β1 integrin antibody. A pulse-chase experiment with cells transfected with 16K plasmid (10 μg) revealed that there was a significant decrease in endogenous premature β1 integrin levels as well, resulting in an overall lower level of α/β1 integrin after 2 h of chase (Fig. 4A). To determine the point when the decrease of exogenously expressed β1 integrin began, we performed another pulse-chase study with β1-T7- and 16K-HSV-co-transfected cells. In these cells, premature β1 integrin levels were already down-regulated by ~80% at the 0-min chase time (Fig. 4B). In cells transfected with β1-T7 alone, there was no significant change of premature β1 integrin level during the 15-min pulse and chase, suggesting that the majority of the premature β1 decrease caused by 16K expression occurred during the 15-min pulse labeling (Fig. 4B). Because the ERAD serves as a central pathway for discarding misfolded or incompletely assembled subunits of oligomeric complexes (16–18), we tested whether the decrease of β1 integrin levels is due to degradation by the conventional proteasome-dependent ERAD pathway using a proteasome-specific inhibitor, MG132. The result showed that the decrease of β1 integrin levels is not mediated by the ERAD pathway (Fig. 4B), although the presence of MG132 (during both pulse and chase) resulted in a slightly increased level of premature β1 integrin. Both this result and the unusually rapid kinetics of the decrease of premature β1 integrin levels (within 15 min) suggested that the decreased β1 integrin level was probably due to degradation by a different mechanism.
The Decreased Premature $\beta_1$ Integrin Levels Resulting from 16K Expression Were Not Due to Transcriptional Down-regulation—Because lowered premature $\beta_1$ integrin levels after 16K expression may be caused by a decrease in the transcription of $\beta_1$ integrin, we studied whether 16K expression could alter transcription of either $\alpha_5$ or $\beta_1$ integrin using real-time PCR analysis. There was no significant difference in the transcription levels of either $\alpha_5$ or $\beta_1$ integrin (Fig. 5), indicating that the decrease in premature $\beta_1$ integrin levels following expression of 16K was not due to transcriptional down-regulation of $\beta_1$ integrin.

Expression of either 16K or the TMD4 of 16K Inhibited Association of the Premature $\beta_1$ Integrin with Calnexin—During the biosynthesis of glycoproteins in the ER, protein folding and oligomeric assembly was mediated by various ER-resident lectin and non-lectin chaperones including calnexin, calreticulin, and BiP (18–20). Both integrin $\alpha$ and $\beta_1$ premature forms associate with calnexin, which is known to result in a dramatically increased ER resident time of unpaired integrin subunits (21, 22). Therefore, the interaction of $\beta_1$ integrin with these chaperones was studied to examine whether 16K expression alters chaperone interactions during folding of premature $\beta_1$ integrin in the ER. Interestingly, co-transfection of $\beta_1$ integrin and 16K caused a marked decrease in the association of the premature $\beta_1$ integrin with calnexin, but there was no association with calreticulin or BiP either before or after expression of 16K (Fig. 6A). Because of the endogenous function of 16K as a component of V-ATPase, we hypothesized that an intracellular pH change resulting from increased expression of the proton channel might be responsible for the effects on the decreased association between calnexin and the premature $\beta_1$. Therefore, as one way to test this hypothesis, since TMD4 serves as the domain of 16 kDa that binds $\beta_1$ integrin (10, 11), we expressed the HSV-tagged TMD4 of 16 kDa (11) to determine whether TMD4 alone could functionally replace the 16K and exert the same level of inhibition on calnexin association with premature $\beta_1$ subunits. The expression of TMD4 alone was sufficient to induce all of the effects of 16K expression on $\beta_1$ integrin reported above, including inhibition of premature $\beta_1$ subunit association with calnexin (Fig. 6B). Furthermore, neither 16K expression nor TMD4 expression showed an effect on $\alpha_5$ integrin association with calnexin, demonstrating the specificity of its interaction with the $\beta_1$ subunit.

Calnexin Interacts with 16K or TMD4—A possible explanation for the inhibition of interaction between the premature $\beta_1$ subunit and calnexin by 16K was that calnexin could also associate with 16K. The results of experiments to test this possibility showed, surprisingly, that both 16K and TMD4 were co-immunoprecipitated with calnexin (Fig. 6B), demonstrating an association between 16K and calnexin. This result was obtained both by immunoprecipitation of lysates of cells overexpressing 16K with anti-calnexin antibody followed by Western blotting with anti-HSV tag antibody and by anti-HSV tag antibody immunoprecipitation followed by Western blotting with anti-calnexin antibody (Fig. 7A). To further confirm this interaction, a pulse-chase study with both 16K-HSV- and TMD4-HSV-expressing cells was performed using an anti-HSV tag antibody (Fig. 7B) and, in both cases, a 90-kDa band was co-immunoprecipitated. This band was no longer co-immunoprecipitated with 16K after 4 h of chase. By contrast, TMD4 was still co-immunoprecipitated with this band after 4 h.

Expression of 16K Caused a Triton X-100-insoluble Non-covalent Aggregation of an 87-kDa Form of $\beta_1$ Integrin—The results presented so far raise a question regarding the fate of the $\beta_1$ integrin forms that were not immunoprecipitated with calnexin (Fig. 6B) after 16K expression. Because the decrease in premature $\beta_1$ integrin levels was not caused by transcriptional down-regulation or ER-associated degradation (Figs. 4 and 5) and because calnexin association with glycoproteins promotes protein solubility, we hypothesized that an association of premature $\beta_1$ integrin with 16K in the ER could result in an insoluble aggregation of the premature $\beta_1$ subunit. This possibility was assessed by comparing the association of calnexin and 16K with samples of cell lysates that were treated with 1% Triton X-100 (Fig. 8A). There was no significant difference in the amount of calnexin co-immunoprecipitated by anti-calnexin, anti-16K, or anti-HSV antibodies, indicating that the association of calnexin with 16K was not due to aggregation of the 16K-calnexin complex. In contrast, a significant decrease in the amount of 16K co-immunoprecipitated by these antibodies was observed in Triton X-100-treated samples, indicating that 16K associated with calnexin in a Triton X-100-insoluble form.
units that would then be excluded from the Triton X-100-soluble fraction of cell lysates used for analysis. To test this hypothesis, cells transfected with β1-T7 alone or β1-T7 plus 16K-HSV were lysed in 1% Triton X-100 and, after assay of crude lysates for protein levels, equal amounts of lysate proteins from both transfectants were centrifuged as before. The Triton X-100-soluble (supernatant) fractions were subjected to SDS-PAGE, whereas the Triton X-100-insoluble pellets were boiled in SDS-loading buffer under reducing or non-reducing conditions and subjected to SDS-PAGE followed by Western blotting with anti-T7 tag antibody. The results showed that when 16K was expressed, a form of β1 integrin of lower apparent molecular mass (~87 kDa) was detected in the Triton X-100-insoluble pellet but not in the Triton X-100-soluble fraction (Fig. 8A). This result suggested that the disappearance of β1 subunits in the soluble fraction of cell lysates after 16K expression was due to an increase in β1 subunit aggregation, which remained in the Triton X-100-insoluble fraction. The aggregated 87-kDa form was detected after boiling the pellet in either reducing or non-reducing SDS sample buffer, demonstrating that the aggregation was not due to disulfide bond formation. By contrast, neither α5 integrin nor the cytosolic protein, ERK2, showed signs of this form of aggregation and insolubility in Triton X-100 (Fig. 8A). Furthermore, the 87-kDa form of β1 was not precipitated with Con A, confirming that the shift of the apparent molecular mass of this form was due to incomplete N-linked glycosylation, whereas both of the molecular mass forms were precipitated with the anti-T7 antibody (Fig. 8B).

In conclusion, our results have demonstrated that the V-ATPase 16K can regulate surface expression of β1 integrin, blocking the β1 biosynthetic pathway by inducing a non-disulfide-dependent aggregation. This effect on biosynthesis thereby inhibits β1 integrin-mediated cell spreading on fibronectin.
DISCUSSION

These studies were initiated to investigate the mechanism by which the expression of the 16-kDa subunit of the vacuolar H^+ ATPase appeared to have a specific inhibitory effect on the expression of complex N-glycans on β1 integrin, which in turn inhibited cell-matrix interactions. Several investigators have demonstrated that N-glycan expression on β1 integrin subunits is required for proper integrin-matrix interactions (13, 23, 24). Moreover, aberrant N-glycosylation of integrins that often occurs during oncogenic transformation results in the modulation of integrin function, cell-matrix interactions, and cell invasiveness (12, 23, 25). Initially, the effects of 16K expression on β1 integrin biosynthesis were investigated, which led to the exploration of the possibility that an association between the 16K and β1 integrin in the ER might be involved in the regulation of β1 subunit maturation and surface presentation. However, during the course of this study, we discovered a novel interaction between calnexin and 16K.

The V-ATPase proton channel consisted of a circular arrangement of six of the 16Ks. Each 16-kDa subunit consisted of four transmembrane helices, each of which consisted of highly hydrophobic amino acids. Approximately 70% 16K total amino acids were predicted to be imbedded in the membrane with very small loop regions, typically ranging from 11 to 19 amino acids in length predicted to lie outside of the membrane. The V-ATPase is unique among the four TM domains of each subunit, because it was predicted by homology to the yeast V-ATPase to be on the exterior of the proton channel where it could easily interact with adjacent protein transmembrane domains. The V-ATPase-HSV used in our experiments consisted of 17 amino acids spanning between the third and the fourth TM regions and 22 amino acids of the fourth transmembrane domain followed by 6 amino acids of the C-terminal region. Based on a recent model (26), the loop preceding the fourth transmembrane domain was found on the cytoplasmic side, whereas the C-terminal 6 amino acids tail was oriented toward the lumen of ER. Consequently, there are probably only relatively small non-membrane-embedded domains that could participate in the interaction of TMD4 with either the lectin domain or stem region of calnexin.

In this study, it is unclear whether calnexin directly associates with non-glycosylated 16K in the ER or whether other proteins are involved in this interaction. However, there are several examples where calnexin seems to show chaperone activity toward highly hydrophobic transmembrane proteins, including CD9 (27, 28), proteolipid protein (29), and CD82 (30), suggesting a possible role of calnexin in the quality control of transmembrane domain assembly. These studies, taken with our current results, raise the possibility that calnexin may interact with 16K to function in the ER quality control of 16K biosynthesis.

Because one of the major functions of calnexin is to keep nascent polypeptides soluble for proper folding and oligomerization assembly by preventing aggregation, the inhibitory effect of 16K expression on the interaction between calnexin and β1 integrin probably promoted the subsequent Triton X-100-insoluble aggregation of the β1 subunit. It is possible that the binding of 16K to premature β1 integrin alters the conformation of the β1 subunit so that it can no longer associate with calnexin, leading to aggregation of β1 integrin in the ER. A lack of inhibition of α5 subunit-calnexin interaction by 16K provided support for the hypothesis in which the binding of 16K to the β1 subunit is a prerequisite for the inhibition of integrin association with calnexin in the ER. The Triton X-100-insoluble β1 integrin that resulted from 16K expression exhibited a molecular mass (87 kDa) less than that of the premature β1 form (110 kDa), and it was not bound by ConA in contrast to the premature form. The reason for the inability of the 87-kDa form to be bound by ConA is unclear, particularly because the mammalian N-glycanase has been reported only to be present in the cytosol (31).

Saliceni et al. (32) recently reported that the LRP-1 (low density lipoprotein-related protein-1) regulated β1 integrin maturation and surface expression. Using LRP-1-deficient mouse embryonic fibroblast cells, these authors demonstrated that LRP-1 deficiency hindered the maturation of β1 integrin and its surface expression, resulting in the accumulation of premature β1 integrin in the ER. Our results demonstrated an additional example of a protein, 16K, whose expression can regulate surface expression of β1 integrin and β1 integrin-mediated cell interactions with the extracellular matrix.

Several questions arise from our results. Does endogenous 16K function in β1 integrin biosynthesis and surface expression? Is there a functional link between a protein involved in vacuolar proton transport and its relatively strong association with a cell adhesion protein such as β1 integrin? Interestingly, a link between another protein that regulates intracellular pH, the NHE1 (a Na^+–H^+ exchanger isofrom), and the modulation of cell-matrix interactions has recently been established. Schwartz et al. (33) demonstrated that many anchorage-dependent cells showed an alteration of their intracellular pH (pHi) upon cell spreading on matrix; however, NIH3T3 cells transformed with src or ras showed no pHi change when allowed to spread (33). More recently, several groups have reported that cell spreading on extracellular matrix caused a consistent pHi change that was shown to be mediated by the activation of NHE1 via various signal transduction pathways, including RhoA, Cdc42, and PIP2, and that pHi change upon cell spreading was required for stress fiber formation (34–36). Moreover, studies have revealed that the E subunit of the V-ATPase domain associated with mSos1 resulting in enhanced guanine nucleotide exchange activity of Rac1, whereas the C subunit of the V1 domain mediated the binding of the V-ATPase to the actin cytoskeleton. Both of these effects are relevant to cell spreading and membrane protrusion (37, 38), suggesting the involvement of V-ATPase in various aspects of actin cytoskeleton rearrangement. In light of these studies along with the reported ability of the TMD4 deletion mutant of 16K to induce NIH3T3 cell anchorage-independent growth (39), our results suggested that there are probably additional factors involved in the pHi regulation of cell spreading of anchorage-dependent cells and that pHi regulation of both cytosol and vacular systems may play an important role for preventing loss of the anchorage-dependent phenotype.

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