Enhancement of Endothelial Cell Migration and In Vitro Tube Formation by TAP20, A Novel β5 Integrin–Modulating, PKCθ-Dependent Protein

Shaoqing Tang, Yunling Gao, and J. Anthony Ware

Cardiovascular Division, Department of Medicine, Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

Abstract. Migration, proliferation, and tube formation of endothelial cells are regulated by a protein kinase C isoenzyme PKCθ. A full-length cDNA encoding a novel 20-kD protein, whose expression was PKCθ-dependent, was identified in endothelial cells, cloned, characterized, and designated as theta-associated protein (TAP) 20. Overexpression of TAP20 decreased cell adhesion and enhanced migration on vitronectin and tube formation in three-dimensional culture. An antiintegrin αvβ5 antibody prevented these TAP20 effects. Overexpression of TAP20 also decreased focal adhesion formation in αvβ3-deficient cells. The interaction between TAP20 and β5 integrin cytoplasmic domain was demonstrated by protein coprecipitation and immunoblotting. Thus, the discovery of TAP20, which interacts with integrin β5 and modulates cell adhesion, migration, and tube formation, further defines a possible pathway to angiogenesis dependent on PKCθ.

Key words: TAP20 • integrin • PKCθ • endothelial cells • migration

INTEGRIN receptors mediate the interactions between cells and extracellular matrix (ECM),[1] including endothelial cell (EC) adhesion and migration, which are required events for angiogenesis (Folkman and Shing, 1992; Hynes, 1992; Gumbiner, 1996; Clark and Hynes, 1997). Integrins are not only involved in cell adhesion but also signal transduction. Signaling initiated through integrin clustering and ligation with ECM-derived ligands has been termed outside-in signaling, because it mobilizes intracellular messengers. In contrast, in response to cytoplasmic signals, integrin conformation can be changed, resulting in modulation of their ligand-binding affinity, which is termed inside-out signaling (Schwartz et al., 1995). Integrins in the β1, β2, β3, and β7 families have been shown to exist in different ligand-binding affinity states (Faull et al., 1993; Crowe et al., 1994; Shattil et al., 1998). Integrin affinity modulation is initiated by intracellular signals that change the affinity of integrins for their ligands (Schwartz et al., 1995; Hughes and Pfaff, 1998). This process involves the propagation of conformation changes from the cytoplasmic domains to the extracellular ligand-binding sites, resulting in an alteration of ligand-binding affinity. The expression and analysis of recombinant integrins in the cells demonstrate the importance of the cytoplasmic domains for integrin affinity modulation (Chen et al., 1992; Crowe et al., 1994; O'Toole et al., 1994, 1995; Wang et al., 1997). The cytoplasmic signaling pathways regulating integrin affinity are poorly understood; however, several recent studies indicate that the Ras family, Rho family, and phosphoinositide 3-kinase (PI3-kinase) are involved in the regulation of integrin affinity (Laudanna et al., 1996; Zhang et al., 1996; Hughes et al., 1997; Marte and Downward, 1997). There is increasing evidence to suggest that integrin functions are modulated by intracellular proteins that interact with integrin cytoplasmic domains (Schwartz et al., 1995). Since integrin cytoplasmic tails are crucial in the modulation of integrin affinity, proteins that interact directly with the integrin cytoplasmic tails are excellent candidates for modulators of integrin affinity. Interactions of integrins with the cytoskeleton via talin and α-actinin (Clark and Brugge, 1995; Lauffenburger and Horwitz, 1996), and with the focal adhesion components, focal adhesion kinase (FAK) and paxillin have been studied. These interactions may depend upon ligand binding to regulate integrin affinity by stabilizing an active conformation. However, recent evidence suggests that interaction of the integrin cytoplasmic tail with the cytoskeleton can lock integrin in an inactive state (Kucik et

Address correspondence to Shaoqing Tang, Albert Einstein College of Medicine, Forchheimer Building G-42, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: (718) 430-2365. Fax: (718) 430-8989. E-mail: tang@aecom.yu.edu

1. *Abbreviations used in this paper:* EC, endothelial cell(s); ECM, extracellular matrix; EGFP, enhanced GFP; FAK, focal adhesion kinase; FN, fibronectin; HUVEC, human umbilical vein EC(s); GFP, green fluorescent protein; GST, glutathione *S*-transferase; LN, laminin; PKC, protein kinase C; RCE, rat capillary endothelial cell(s); TAP, theta-associated protein; VEGF, vascular endothelial growth factor; VN, vitronectin; wt, wild-type.

al., 1996; Stewart et al., 1998). A serine/threonine kinase ($pp59^{ILK}$) can bind to β1, β2, and β3 tails and disrupt cell adhesion, indicating a possible role in integrin modulation (Hannigan et al., 1996). Binding of RACK1, the receptor for activated protein kinase C (PKC), to the β1, β2, and β5 cytoplasmic tails appears to depend upon stimulation with phorbol esters (Liliental and Chang, 1998), indicating that RACK1 could act as a link between PKC and integrins.

Integrin affinity modulation is a cell type– and integrin-specific process; therefore, the interactions of integrins with the abundant and integrin-nonspecific proteins discussed above may not definitively explain integrin affinity regulation. Recently, several proteins interacting with specific integrin subunit cytoplasmic tails have been identified. β3-endonexin, a novel 111-residue polypeptide that is capable of binding the cytoplasmic tail of the β3 integrin subunit was identified by a yeast two-hybrid screening strategy (Shattil et al., 1995; Eigenthaler et al., 1997; Kashiwagi et al., 1997). This polypeptide does not bind other integrin tails, including those of β1, β2, and α_{IIb}. Transient coexpression of integrin receptor $\alpha_{IIb}\beta3$ and β3-endonexin in CHO cells revealed that β3-endonexin can modulate the affinity state of $\alpha_{IIb}\beta3$ in a manner that is structurally specific and subject to metabolic regulation. A β2 integrin cytoplasmic domain binding protein, cytohesin-1, has been found to increase αLβ2-mediated cell adhesion (Kolanus et al., 1996). Ca^{2+}- and integrin-binding protein (CIB) interacts with the integrin α_{IIb} tail (Naik et al., 1997). Finally, ICAP-1 (integrin cytoplasmic domain–associated protein-1), interacts specifically with the β1 integrin cytoplasmic domain (Chang et al., 1997). The effects of CIB and ICAP-1 on integrin activation are not known.

Modulation of integrin function, in particular that of the vitronectin (VN) receptors αvβ3 and αvβ5, is an important process controlling angiogenesis. In vivo angiogenesis models have defined two distinct angiogenic pathways: a pathway using the αvβ3 integrin that mediates angiogenesis induced by fibroblast growth factor 2 or by tumor necrosis factor α; and a pathway initiated by vascular endothelial growth factor (VEGF), transforming growth factor α, or PKC that is linked to integrin αvβ5 (Friedlander et al., 1995). Our previous study (Tang et al., 1997) revealed that selective inhibition of a specific member of the PKC family, PKCθ, prevents EC migration, proliferation, and tube formation in vitro, thus raising the possibility that regulation of EC function by PKCθ is accomplished by modulation of αvβ5 function. One mechanism by which PKCθ might regulate αvβ5 function is by controlling the expression of one or more integrin regulatory proteins.

Materials and Methods

Molecular Cloning

RNA display was performed using an RNAmap Kit (GenHunter) according to the manufacturer's instructions. The resulting PCR products were cloned into plasmid pBluescript for sequencing analysis. To screen the rat PC12 cDNA λgt11 library (Clontech) for full-length theta-associated protein (TAP) 20, both the 5′ and 3′ insert screening amplimers of λgt11 as the forward primers and a TAP20 3′ end sequence ACCATAAGAATGCAGACAAGA as the reverse primer were used for PCR reaction with pfu DNA polymerase (Stratagene). The PCR products were cloned into the pBluescript plasmid (Stratagene) and sequenced with T7 and T3 primers.

Plasmids and Cell Culture

To explore the function of TAP20 in mammalian cells, we constructed a TAP20 + green fluorescent protein (GFP) expression plasmid. The EGFP (enhanced GFP) gene from plasmid pEGFP-C1 (Clontech) including its 5′ end cytomegalovirus (CMV) promoter to 3′ end polyA signal region, was inserted into the EcoRI-KpnI restriction sites (non-multicloning sites) to create a GFP/pRc plasmid. TAP20 cDNA was then cloned into the HindIII-XbaI restriction sites in the plasmid GFP/pRc. Thus, in this vector the TAP20 and EGFP genes are controlled by separate CMV promoters. To localize TAP20 protein in the cell, we cloned the TAP20 cDNA into the BglII-SmaI restriction sites in plasmid pEGFP-C1 for expression of a GFP-TAP20 fusion protein in mammalian cells.

ECV304 cells (a human bladder carcinoma cell line, distributed by American Type Culture Collection) were cultured in M199 medium supplemented with 10% FBS and antibiotics (GIBCO BRL), MV3 cells were cultured in DMEM medium with 10% FBS and antibiotics, and human umbilical vein ECs (HUVEC) were cultured in M199 with 20% newborn calf serum, and 5% human serum, at 37°C in a humidified 5% CO^2 atmosphere. Monolayers of cells were transfected with either control vector or TAP20 construct using lipofectin (GIBCO BRL) or GenePorter (Gene Therapy System) for HUVEC. 1 d after transfection of GFP-containing plasmids, cells were harvested and suspended in Hanks' buffer (137 mM NaCl, 5.4 mM KCl, 5.6 mM dextrose, 4.2 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 0.44 mM KH_2PO_4, pH 7.4), and were sorted by GFP fluorescence using a FACScan flow cytometer (Becton Dickinson). The sorted cells were recultured in the complete medium. Expression of TAP20 was confirmed by Western analysis using the anti-TAP20 antibody. The sorted cells were tested in the cell adhesion, migration, and tube formation experiments.

Northern Transfer Analysis

Total RNA was prepared using Trizol (GIBCO BRL). For Northern transfer analysis, 20 μg of total RNA was subjected to electrophoresis on a 1.5% formaldehyde-agarose gel and transferred to Gene-Screen Plus membrane according to the manufacturer's recommendations. The blot was hybridized with random-primed TAP20 cDNA probes at 65°C for 3 h in Quik-Hyb solution (Stratagene), and was washed under high stringency conditions before autoradiography.

Immunoblotting

Immunoblotting was performed as follows: cell lysates were prepared by addition of 1 ml of lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, 30 ml/ml aprotinin) per 10^7 cells. Samples were run on a 15 or 10% SDS polyacrylamide gel and electrophoretically transferred to Immobilon-P membranes (Millipore). The membranes were then hybridized with indicated antibodies (anti-αv polyclonal antibody; Santa Cruz Biotechnology), PKCθ, β1, and β3 mAb (Transduction Laboratories), and anti-β5 polyclonal antibody (Chemicon), in PBS containing 5% dry milk and detected via ECL (Amersham Pharmacia Biotech). The affinity-purified rabbit polyclonal anti-TAP20 antibody was produced by QCB Inc.

Adhesion Assay

Cell adhesion was performed as described previously (Giancotti and Ruoslahti, 1990; Leavesley et al., 1992) with minor modifications. In brief, 96-well polystyrene plates (Costar) were coated with 10 μg/ml laminin (LN), fibronectin (FN), or VN in PBS. Cells were harvested, suspended in medium M199 (GIBCO BRL) with 2% BSA at a concentration of 100,000 cells/0.1 ml, and 50,000 cells were added to each well, with antibody as indicated. After incubation at 37°C for 1 h, nonadherent cells were removed with gentle washing. The cells in wells that were not washed were used as the standard (100%). Quantitation of cell attachment was determined by staining the cells with 0.5% crystal violet in 20% methanol, washing cells, and then eluting the dye with 0.1 M sodium citrate, pH 4.2, and measuring the absorbance at 596 nm. The integrin-blocking antibodies were purchased from Chemicon.

Cell Migration Assay

The cell monolayer was briefly treated with trypsin to lift the cells, which were pelleted and washed once with PBS and resuspended in M199 medium containing 0.1% BSA to a final concentration of 500,000 cells/ml.

Cells were added to 3 μm FALCON Cell Culture FluoroBlok Inserts (Becton Dickinson) at a density of 50,000 cells/insert. M199 (0.8 ml) with 10% FBS and VEGF (10 ng/ml) was used as a chemoattractant in the lower wells. The inserts were incubated for 4 h at 37°C. The membranes of the inserts were then mounted on glass slides and coverslips. Cells migrated through the FluoroBlok Inserts were quantitated by counting the fluorescent cells with a Nikon Optiphot-2 fluorescence microscope with a COHU video camera (COHU Electronics) and NIH Image software for Macintosh computer.

In Vitro Tube Formation

Matrix gel (MATRIGEL Basement Membrane matrix; Becton Dickinson) plates were prepared in 12-well plates following the manufacturer's instructions. After transfecting and sorting, HUVEC (~80% confluent) were treated with trypsin, and 5×10^4 cells were seeded on the top of plates with complete M199 medium. The photographs were taken under a 100× light microscope, monitoring with a COHU video camera, and captured with a Scion 7 video card (Scion Image) in a PowerMac computer.

Focal Adhesion

24 h after transfection with GFP alone, TAP20 + GFP, or GFP-TAP20 plasmids, human MV3 (van Muijen et al., 1991, a gift from Dr. E. Danen, NIH) cells were plated on 4-well glass chamber slides (Nalge Nunc) coated with VN (10 μg/ml) (Sigma Chemical Co.). Cells were incubated for the indicated times in DMEM containing 10% BSA, fixed with 4% paraformaldehyde and 0.1% Triton X-100 in PBS for 5 min, and then incubated with 4% paraformaldehyde in PBS for an additional 20 min. Focal adhesions were visualized by incubating first with mouse mAb against vinculin (1 μg/ml; Chemicon), paxillin (0.5 μg/ml; Transduction Laboratories), or FAK (2 μg/ml, Transduction Laboratories), and then with Cy-3–conjugated goat antibody to mouse IgG (dilution 1/600, Jackson ImmunoResearch Laboratories). Cells were viewed on a Nikon Optiphot-2 fluorescence microscope and COHU video camera as described above.

Glutathione S-Transferase Coprecipitation

The TAP20 cDNA was inserted into BamHI and XhoI cloning sites in pGEX4T1 (Amersham Pharmacia Biotech). Cytoplasmic domains of integrin β3 (amino acid sequence: KLLITIHDRKEFAKFEEERARAKWFTANNPLYKEATSTFTNITYRGT) or β5 (amino acid sequence: KLLVTIHDRREFAKFQSERSRARYEMASNPLYRKPISTHTVDFTFNKFNKSYNGTVD) were synthesized by the PCR method using the pfu enzyme and inserted into the BamHI and XhoI cloning sites. Glutathione *S*-transferase (GST) or GST fusion proteins were expressed in *Escherichia coli* BL21 cells and immobilized on glutathione Sepharose beads (Amersham Pharmacia Biotech) following the manufacturer's instructions. To precipitate protein from EC lysate, the GST-TAP20 coated beads were incubated with the cell lysate in PBS for 20 h at 4°C. To precipitate TAP20 with GST-integrin tail fusion protein, the GST-TAP20 beads were first incubated in thrombin containing buffer for 16 h at room temperature to release the TAP20 protein. The TAP20-containing solution was then incubated with GST-integrin tail fusion protein beads for 20 h at 4°C. After three washes with PBS, the proteins bound to the beads were eluted in 10 mM glutathione buffer and analyzed by Western blotting. The antibodies used included: GST polyclonal antibody (Amersham Pharmacia Biotech), αv polyclonal antibody (Santa Cruz Biotechnology), β1 mAb and β3 mAb (Transduction Laboratories), and β5 polyclonal antibody (Chemicon).

Results

Cloning of TAP20

We investigated the expression of genes regulated by PKCθ with the mRNA display method (Liang and Pardee, 1992) using mRNAs from clonal populations of rat capillary endothelial cells (RCE), in which either the kinase-negative PKCθ (PKCθ-kn, a dominant negative inhibitor) or a constitutively active form of PKCθ (PKCθ-ca) were overexpressed (Tang et al., 1997). An RNA of ~0.8 kb

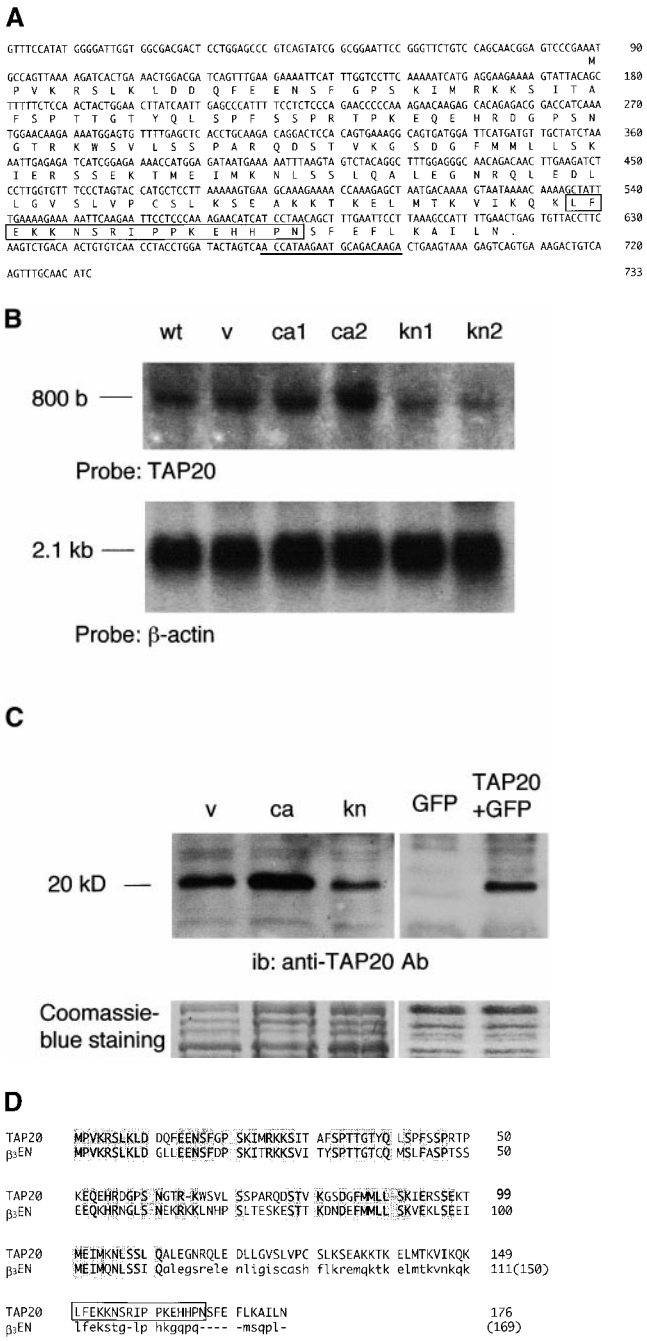


Figure 1. Molecular cloning and analysis of TAP20. (A) Nucleotide and deduced amino acid sequence of TAP20. The deduced amino acid sequence (single letter code) is shown under the nucleotide sequence. The solid line indicates the sequence used as the reverse primer to screen the rat PC12 library. The box indicates the sequence of peptide used as an immunogen to induce TAP20 antibody formation. (B and C) Differential expression of TAP20 in RCE with varying PKCθ activity levels. Total RNA (B) and RCE lysate (C) were prepared from wt RCE (wt) or cells stably expressing control plasmid (v), PKCθ-ca (ca1 and ca2), or PKCθ-kn (kn1 and kn2). The full-length TAP20 cDNA was used as probe on Northern blot (B), and the TAP20 antibody was used for Western analysis (C). (D) Amino acid sequence comparison of TAP20 with β3-endonexin. Identical amino acids are shown by the letters in gray boxes. Lower case letters indicate additional 59 amino acids of the longer form of β3-endonexin, and the boxed sequence indicates the immunogen peptide for TAP20.

whose expression depended upon the presence of active PKCθ was identified. Northern transfer analysis showed that this 0.8-kb mRNA is highly expressed in PKCθ-ca RCE but is dramatically suppressed in PKCθ-kn RCE (Fig. 1 B). The partial sequence of this gene obtained from differential display was used to screen a rat PC12 cDNA library by the PCR method. The cDNA generated from the PCR was cloned, and the nucleotide sequence was determined (Fig. 1 A). The open reading frame encodes a novel protein of 175 residues with a calculated molecular mass of ~20 kD. An antibody raised against the COOH terminus of this putative protein recognized a protein of 20 kD (Fig. 1 C), as assessed by immunoblotting of an RCE cell lysate, which confirmed that the expression of this protein depended upon functional PKCθ, as suggested by the Northern transfer analysis. In the PKCθ kinase-negative RCE, TAP20 expression was significantly decreased at the protein level. This anti-TAP20 antibody also recognized a 20-kD band in TAP20 transfected human cells (Fig. 1 C and Fig. 2), suggesting that TAP20 can be expressed as a 20-kD, full-length protein in the cells. Accordingly, this novel PKCθ-associated protein was designated TAP20. By searching the GeneBank database, we found that a partial 3′ end sequence of TAP20 had been reported previously (Kerr et al., 1994), and the first 110 amino acid residues of TAP20 share 55% homology with human β3-endonexin (Shattil et al., 1995; Eigenthaler et al., 1997; Kashiwagi et al., 1997), a 111-residue polypeptide that interacts with the β3 integrin subunit (Fig. 1 D). TAP20 also has an additional 66-residue COOH terminus. Thus, although the differences suggest that TAP20 may have functional properties that differ from those of β3-endonexin, we hypothesized that TAP20 is involved in integrin-mediated cell functions that are regulated by PKCθ.

Expression of TAP20 and TAP20 Fusion Proteins in Human Cell Lines

The immunoblotting with the anti-TAP20 antibody that recognizes an epitope at the COOH terminus of the protein suggests that TAP20 is expressed as a full-length protein in RCE. To explore the function of TAP20 in human cells, TAP20 cDNA was cloned into the pRc/CMV vector and a cDNA encoding GFP was inserted into a separate region of the same plasmid; thus, these two cDNAs were controlled by separate CMV promoters (Fig. 2 A). 1 d after transfection, human ECV304 or HUVEC cells were sorted by GFP fluorescence using FACS®. Cells with GFP expression were recultured for 1–2 d before experiments. Cells were then harvested for cell adhesion, migration, and tube formation experiments, and also for the protein expression test (Fig. 2, B and C). Blotting with anti-TAP20 antibody showed that the antibody does not detect antigen in the 20-kD range in wild-type (wt) or GFP transfected cells. The expression of TAP20 in unsorted TAP20 + GFP cells was 30–70% of the sorted cells, depending on the transfection efficiency. Furthermore, the expression level of PKCθ, integrin αv, β3, and β5 was not changed in the TAP20 + GFP expressed ECV cells compared with the untransfected or GFP transfected cells (Fig. 2 B).

To investigate the TAP20 effect on focal adhesion and to further characterize TAP20 distribution in the cells, ei-

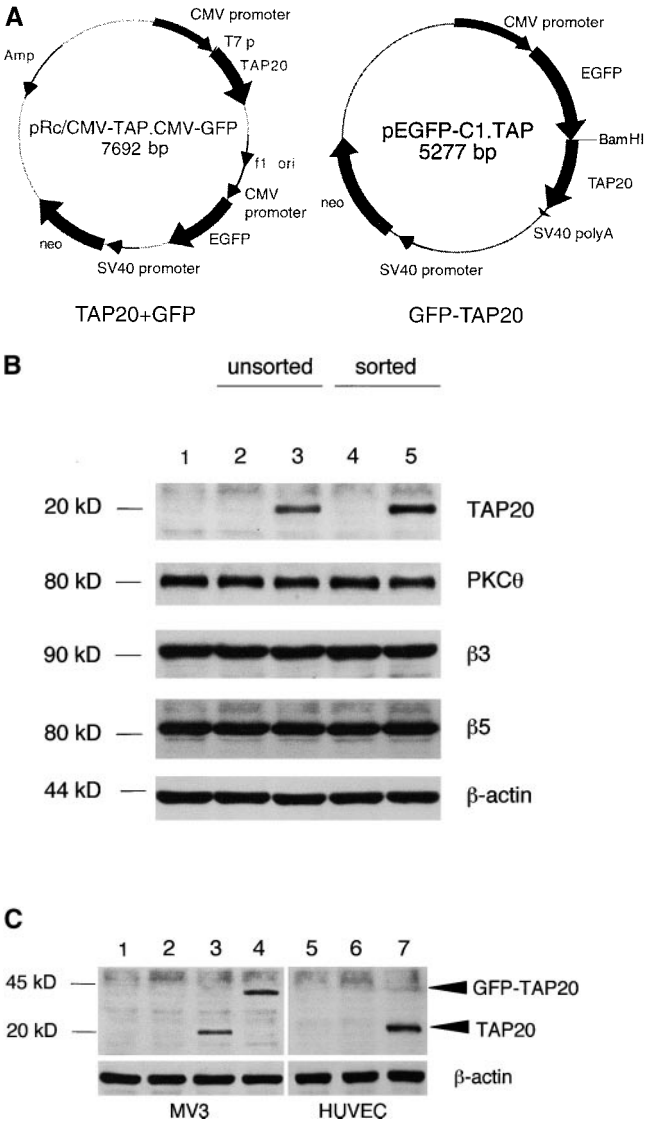


Figure 2. Expression of TAP20 in human cell lines. (A) Plasmid constructs. The EGFP gene with its 5′ end CMV promoter to 3′ end polyA signal region was inserted into pRc/CMV to create a GFP/pRc plasmid (GFP). TAP20 cDNA then was cloned into the multicloning site in the GFP/pRc vector. Thus, TAP20 and EGFP cDNAs were controlled by separate CMV promoters. This construct is designated as TAP20 + GFP. TAP20 cDNA was also cloned into plasmid pEGFP-C1 for expression of a GFP-TAP20 fusion protein in mammalian cells, and designated as GFP-TAP20. (B) Expression of TAP20 in human ECV cells. 1 d after transfection, cells were sorted by GFP fluorescence using FACS®. Cells with GFP expression were recultured for 1–2 d before preparing cell lysates. The lysate of unsorted cells was prepared 2–3 d after transfection. Anti-TAP20, anti-PKCθ, anti-β3, and anti-β5 antibodies were used to detect protein expression. Lane 1, wt untransfected cells (wt); lanes 2 and 4, GFP/pRc transfected cells (GFP); lanes 3 and 5, TAP20 + GFP/pRc transfected cells (TAP20 + GFP). (C) Expression of TAP20 or fusion protein GFP-TAP20 in human MV3, and expression of TAP20 in HUVEC cells. Lysates of transfected MV3 and sorted HUVEC cells were prepared as described above for immunoblotting, and anti-TAP20 antibody was used to detect protein expression. Lane 1 and 5, wt untransfected cells (wt); lanes 2 and 6, GFP/pRc transfected cells (GFP); lanes 3 and 7, TAP20 + GFP/pRc transfected cells (TAP20 + GFP); and lane 4, GFP-TAP20/pRc transfected cells (GFP-TAP20).

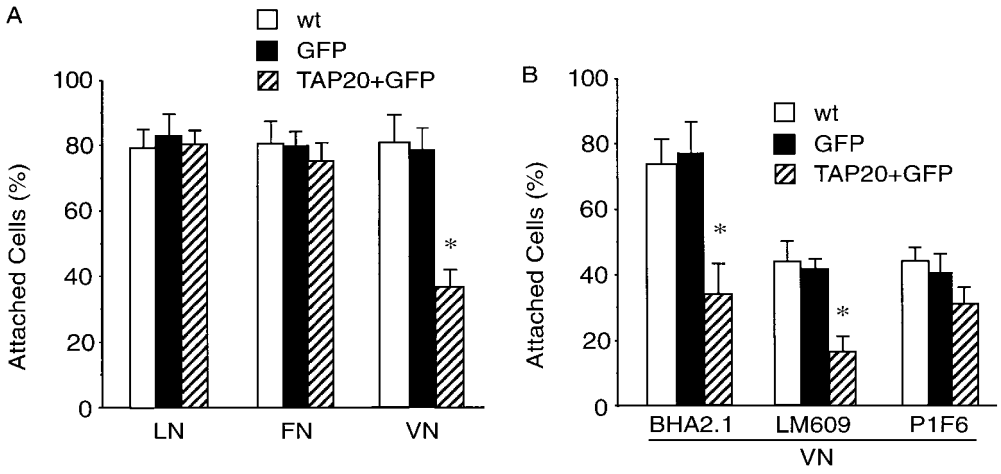


Figure 3. Inhibition of cell adhesion by TAP20. Transfected cells were sorted with GFP fluorescence and recultured for 1–2 d. Cells were briefly treated with trypsin, washed with PBS, and resuspended in M199 medium with 2% BSA. Cells (50,000/well) were allowed to adhere to a 96-well plate coated with LN, FN, or VN (A). Cells attached to VN were further investigated with monoclonal antiintegrin antibodies (10 μg/ml) as indicated: the anti-αvβ3 antibody, LM609; anti-αvβ5, P1F6, and as a control for integrin ligation, an anti–α2β1 integrin antibody, BHA2.1 (B). The attached EC were quantified by the dye staining method. Attached cells are expressed as the percentage of the seeded cells. The values are means from four separate experiments. Error bars indicate standard deviation; asterisk indicates $P < 0.01$, TAP20 transfected ECV (TAP20 + GFP) versus either wt or GFP alone transfectants, by *t* test.

ther the TAP20 + GFP plasmid or the fusion protein GFP-TAP20 plasmid was transfected into MV3 cells, a β3 integrin–deficient cell line. The transfection efficiency varied from 20–40% in different experiments. 2 d after transfection, the cells were harvested and tested for TAP20 expression. Similar to ECV cells, the anti-TAP20 antibody did not detect antigen in the 20-kD range in nontransfected or GFP transfected MV3 cells, whereas a 20-kD band in the TAP20 + GFP transfected cells and an ~45-kD band in the GFP-TAP20 transfected cells were recognized, which indicated the expression of TAP20 and GFP-TAP20 fusion protein, respectively (Fig. 2 C).

TAP20 Reduces EC Adhesion on VN

Transfected ECV cells were sorted by GFP fluorescence using a FACScan flow cytometer and recultured for 1–2 d. Adhesion of TAP20 + GFP expressing cells to VN, but not to FN and LN, was significantly attenuated (Fig. 3 A) compared with that of either group of control cells, i.e., the wt cells or the cells expressing GFP only (a mean of 33.7 ± 5.1% of the added TAP20 + GFP cells adhered to VN, in contrast to 73.5 ± 7.5% [wt] and 75.0 ± 6.3% [GFP] of the control cells, $P < 0.01$). This result indicates that TAP20 affects cell adhesion by modulating the VN receptors, i.e., the αvβ3 and/or αvβ5 integrins. To further investigate the TAP20 effect on VN receptors, antiintegrin antibodies were added to cells on VN-coated plates to block function of the VN receptors. As a control to rule out nonspecific effects of integrin ligation, an anti–α2β1 integrin (LN receptor) antibody, BHA2.1 was tested. The results obtained with the anti–VN receptor antibodies were compared with those with anti-α2β1 antibody (Fig. 3 B). The anti–α2β1 integrin antibody BHA2.1 did not significantly affect cell adhesion on VN (comparing the BHA2.1 group of bars on Fig. 3 B with the VN group of bars on Fig. 3 A). Furthermore, adhesion of TAP20 + GFP cells on VN was reduced in the presence of BHA2.1 (31.6 ± 7.7% of added cells) to an extent similar to that with cells on VN plates without BHA2.1 antibody (Fig. 3 A; VN group of bars). In the experiments with the anti–VN receptor antibodies, the effects of the anti-αvβ3 antibody LM609 were compared with those of the control antibody BHA2.1. The degree of adhesion in the BHA2.1 group was used as basal (100%) for comparison with that of the LM609 and P1F6 groups in Fig. 3 B. LM609 caused a 50.3% reduction of adhesion of TAP20 + GFP cells, and also caused a 39.4% reduction in the wt cells and a 43.4% reduction in GFP cells. The difference in the magnitudes of the reductions between BHA2.1 and LM609 was not statistically significant among the three groups ($P > 0.5$). In contrast, the anti-αvβ5 antibody P1F6 compared with the control antibody BHA2.1 caused only a 7.4% reduction of adhesion of the TAP20 + GFP cells, but caused significant reduction in both groups of the control cells (38.0 [wt] and 45.6% [GFP]). The magnitude of the reductions between BHA2.1 and P1F6 was significantly less in the TAP20 + GFP cells than that seen in either group of the control cells ($P < 0.01$). These observations suggest that in the TAP20 transfectants, the αvβ3 integrin remains functional as a VN receptor, which can be blocked by the anti-αvβ3 antibody LM609. On the other hand, the VN receptor function of αvβ5 integrin was attenuated by TAP20 overexpression. Thus, blockage of αvβ5 in TAP20 transfectants by anti-αvβ5 antibody P1F6 did not further reduce cell adhesion on VN significantly, whereas the adhesion of the control cells with normal αvβ5 function was dramatically decreased by this anti-αvβ5 antibody.

TAP20 Inhibits Focal Adhesion Formation

The results of adhesion with the integrin-blocking antibodies suggested that TAP20 affects cell function through the αvβ5 integrin. To further clarify the role of TAP20 on αvβ5 integrin, we next examined the effect of TAP20 on focal adhesion formation in MV3 cells (van Muijen et al., 1991), which are αvβ3-negative human melanoma cells and therefore have the αvβ5 integrin as the principal surface receptor for VN. Thus, when the cells are cultured on VN-coated plates, the focal adhesion formation is caused chiefly by the interaction of αvβ5 integrin with VN. We first investigated the cellular localization of TAP20. After

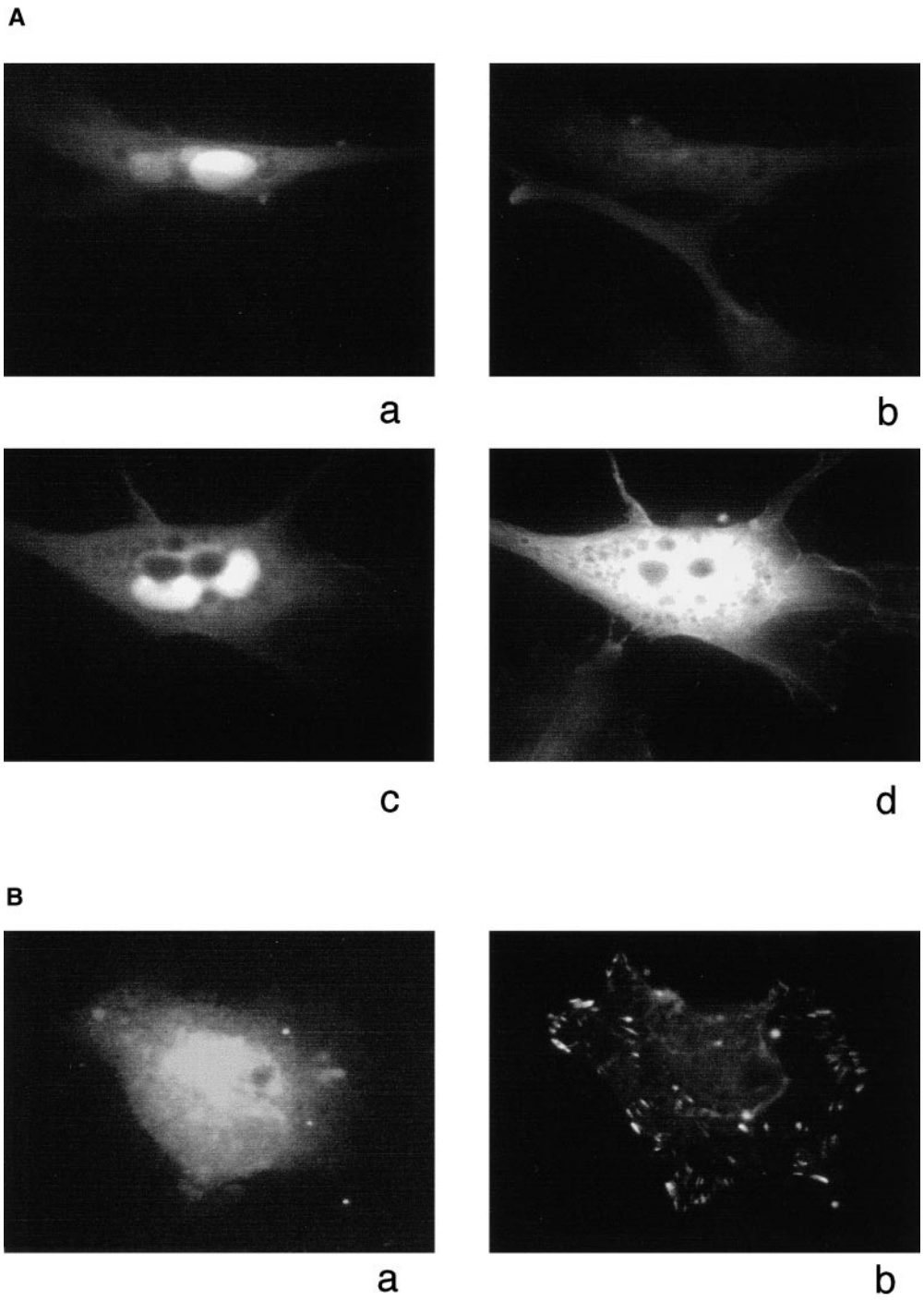
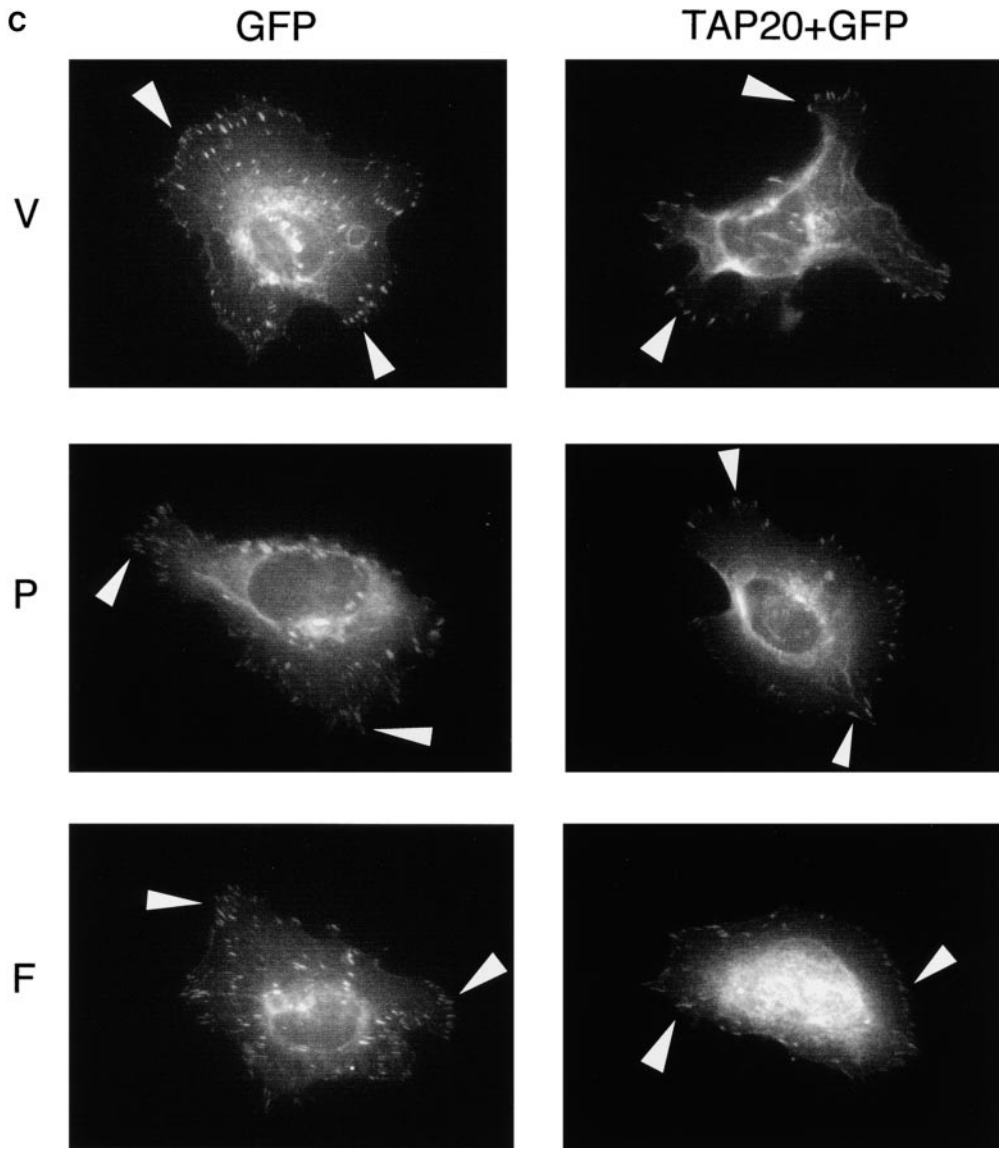


Figure 4 (continues on facing page).

16 h of adhesion to VN, MV3 cells expressing either GFP alone (Fig. 4 A, panels a and b) or TAP20 + GFP (Fig. 4 A, panels c and d) were stained with anti-TAP20 antibody that was visualized with a Cy-3–conjugated secondary antibody (Fig. 4 A, panels b and d). The strong staining in Fig. 4 A, panel d, suggests that TAP20 exists in transfected cells as a full-length protein. The TAP20 fluorescence was observed diffusely in the cytoplasm and the nucleus, which was also demonstrated by GFP fluorescence of the cells expressing the GFP-TAP20 fusion protein (Fig. 4 B, panel a). When these cells were stained with antifocal adhesion component antibodies (antivinculin shown in Fig. 4 B, panel b) to visualize the focal adhesions, no strong colocalization of GFP-TAP20 with focal adhesions was observed, indicating a possibility that TAP20 might dissociate from or interrupt focal adhesion during the cell adhesion process. To characterize the effect of TAP20 on focal adhesions, MV3 cells were transfected with either control GFP plasmid or TAP + GFP plasmid and plated on VN-coated chamber slides. GFP transfectants formed numerous focal adhesions, as demonstrated by antibodies against vinculin, paxillin, or FAK (Fig. 4 C). In TAP20 + GFP transfected



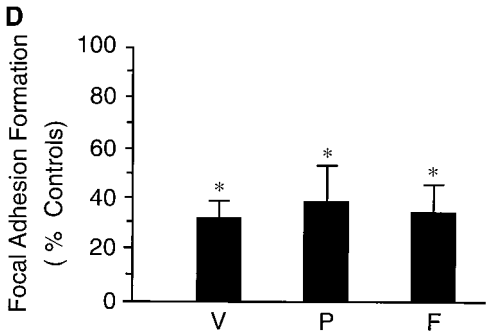


Figure 4. TAP20 modulation of focal adhesion formation on MV3 cells. 24 h after transfection with the plasmids encoding GFP, TAP20 + GFP, or the fusion protein GFP-TAP20, respectively, human MV3 cells were seeded on 4-well glass chamber slides coated with VN (10 μg/ml) and incubated in a 37°C incubator for 16 h. After fixation, cells were immunostained with antibodies. (A) Cells transfected with GFP (panels a and b) or TAP20 + GFP (panels c and d) were stained with anti-TAP20 antibody. Cells were visualized with GFP fluorescence (panels a and c) and Cy-3 fluorescence (panels b and d). Cells expressing TAP20 were strongly stained. The weak stain in TAP20-negative cells may be caused by nonspecific staining by the antibody. (B) Cells expressing the GFP-TAP20 fusion protein were visualized with GFP fluorescence (panel a) or were stained with antivinculin antibody and visualized with Cy-3 fluorescence (panel b). (C) Cells expressing GFP (left panels) or TAP20 + GFP (right panels) were stained with antibodies against vinculin (V), paxillin (P), or FAK (F). Arrowheads indicate focal adhesions. (D) Focal adhesions were quantified by counting four cells for each staining. A relative value was determined in which the number of focal adhesions in TAP20 + GFP cells is expressed as a percentage of those in control (GFP alone) cells, which were normalized at 100%. Data are expressed as the mean ± S.D. (error bars). Asterisk indicates $P < 0.01$, TAP20 + GFP transfectants (TAP20 + GFP) versus GFP alone transfected cells by *t* test.

cells, the number of focal adhesions was significantly reduced by 60–70% (Fig. 4 D), compared with those in wt or GFP alone transfected MV3 cells.

TAP20 Enhances EC Migration on VN and Tube Formation on Matrix Gel

Next, we monitored the effects of TAP20 on cell migration with a modified Boyden chamber assay (Fig. 5 A). Transfected HUVEC cells were sorted by GFP fluorescence using a FACScan flow cytometer and recultured for 1–2 d. 50,000 cells were seeded on each chamber. There was no significant difference in migration on an FN-coated membrane between the untransfected cells, GFP sorted GFP-expressing cells, and TAP20 + GFP expressing cells. Migration of cells on a VN-coated plate was markedly en-

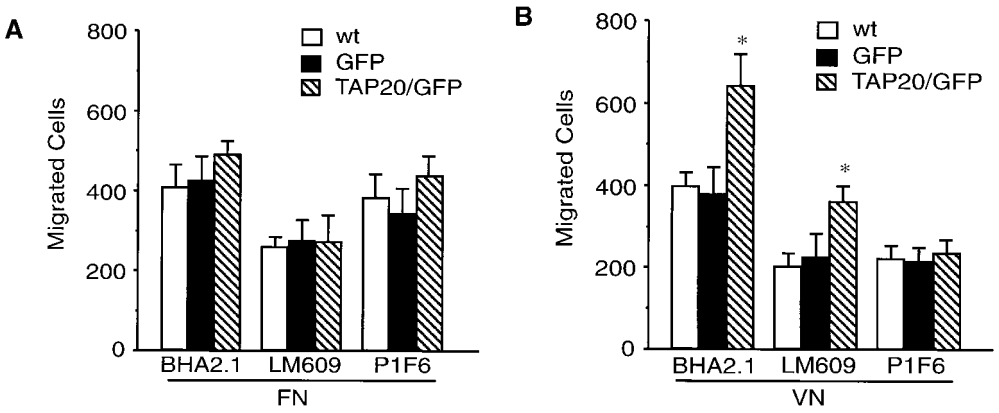


Figure 5. Modulation of cell migration by TAP20. Transfected HUVEC cells were sorted with GFP fluorescence and recultured for 1–2 d. Cells were briefly treated with trypsin and suspended in medium containing 0.1% BSA, with addition of antibodies as indicated: anti-α2β1, BHA2.1; anti-αvβ3, LM609; and anti-αvβ3, P1F6. Cells (50,000/0.1 ml) were seeded in coated FluoroBlok Insert precoated with either FN (A) or VN (B). Cells were incubated for 4 h at 37°C. After fixation, migrated cells were visualized under a fluorescence microscope. Cells that had migrated through the membrane of FluoroBlok Inserts were quantitated by counting the fluorescent cells in two random nonoverlapped 100× view fields. (A) Data are expressed as the mean ± S.D. (error bars) of four separate experiments. Asterisk indicates $P < 0.01$, TAP20 transfectants (TAP + GFP) versus GFP alone transfected cells by *t* test.

hanced by expression of TAP20 (640.4 ± 78.8 [TAP20] versus 395.2 ± 36.2 [wt] and 377.2 ± 65.6 [GFP]). This enhancement (TAP20 versus the controls) could not be blocked by the control anti-α2β1 antibody, BHA2.1, or by the anti-αvβ3 antibody, LM609, but it could be blocked by the anti-αvβ3 antibody, P1F6 (Fig. 5 B).

Since TAP20 modulated cell adhesion and migration, we asked whether overexpressing TAP20 would alter the ability of cells to form tubes on matrix gel. When the GFP-sorted HUVEC cells were cultured in matrix gel, a three-dimensional matrix, tube formation by TAP20 + GFP transfectants was significantly enhanced (Fig. 6). By 6 h,

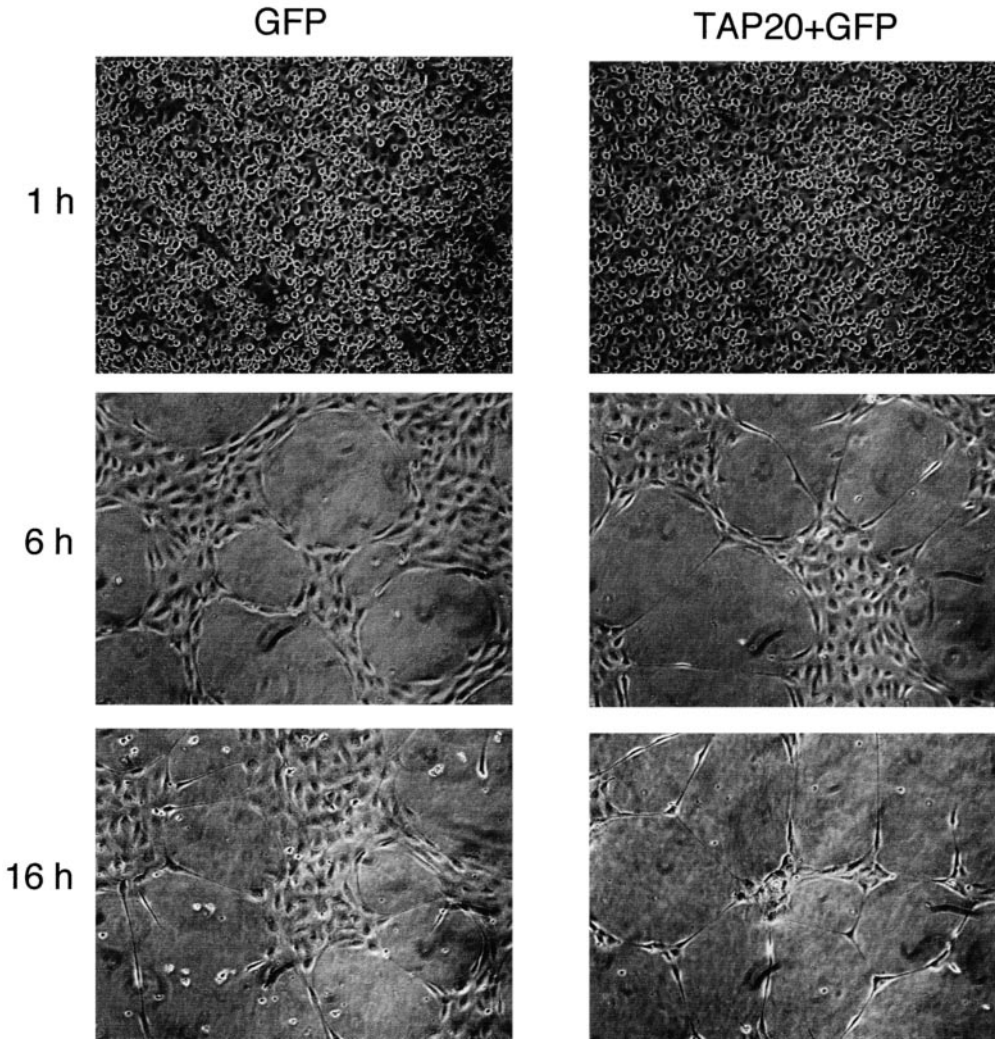


Figure 6. Enhancement of HUVEC tube formation on matrix gel by TAP20. Transfected cells were sorted with GFP fluorescence and were seeded on top of the matrix gel with complete M199 medium. The photographs were taken using light microscopy (100× view field) with a video TV camera system, at the timepoints indicated.

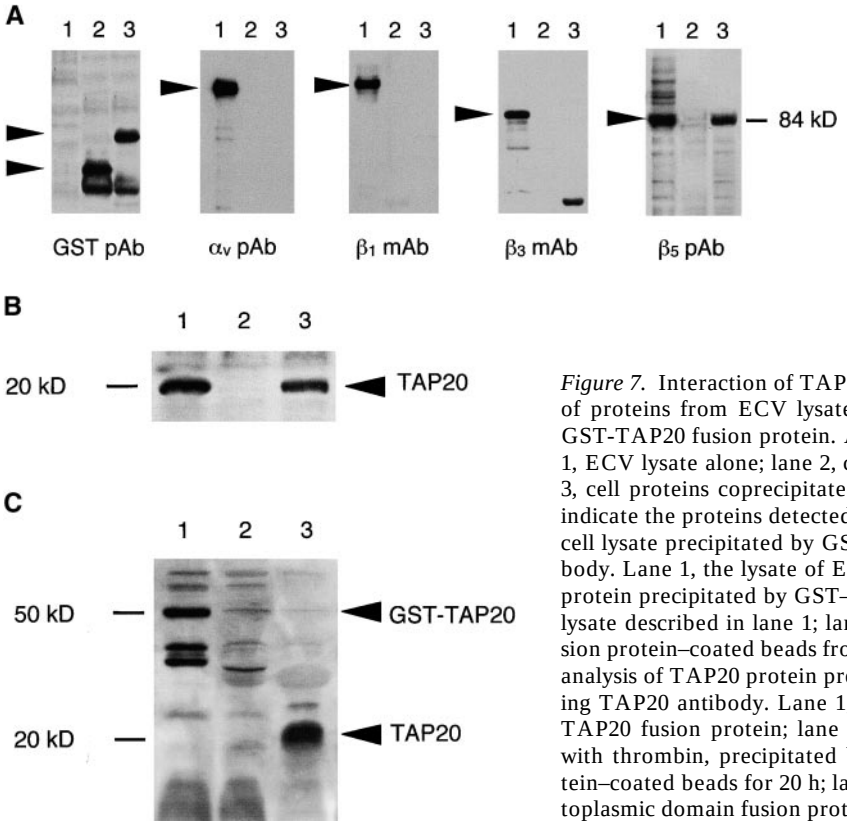


Figure 7. Interaction of TAP20 with β5 integrin subunit. (A) Western analysis of proteins from ECV lysate precipitated by immobilized GST (control) or GST-TAP20 fusion protein. Antibodies used for the blots are indicated. Lane 1, ECV lysate alone; lane 2, cell proteins coprecipitated with GST alone; lane 3, cell proteins coprecipitated with GST-TAP20 fusion protein. Arrowheads indicate the proteins detected. (B) Western analysis of TAP20 protein in ECV cell lysate precipitated by GST-integrin tail fusion protein, using TAP20 antibody. Lane 1, the lysate of ECV cells transfected with TAP20 + GFP; lane 2, protein precipitated by GST–β3 tail fusion protein–coated beads from the cell lysate described in lane 1; lane 3, protein precipitated by the GST–β5 tail fusion protein–coated beads from the cell lysate described in lane 1. (C) Western analysis of TAP20 protein precipitated by GST-integrin tail fusion protein, using TAP20 antibody. Lane 1, lysate from *E. coli* BL21 cells expressing GST-TAP20 fusion protein; lane 2, purified GST-TAP20 fusion protein digested with thrombin, precipitated by the GST–β3 cytoplasmic domain fusion protein–coated beads for 20 h; lane 3, TAP20 protein precipitated by GST–β5 cytoplasmic domain fusion protein–coated beads at 4°C for 20 h.

tube structures were observed in the TAP20 + GFP transfectants, but not in the control GFP cells. The number of tubes formed by TAP20 + GFP transfectants appeared to be dramatically increased at all timepoints compared with that by the control cells.

TAP20 Interacts with the Cytoplasmic Domain of β5 Integrin

The experimental evidence above suggested that the effects of TAP20 on cells were mediated by the αvβ5 integrin VN receptor. Immunoblotting with anti–β5 integrin antibody showed that the expression of the β5 integrin in TAP20 transfectants was similar to that in the control cells (Fig. 2 B). Therefore, we asked whether the effects of TAP20 could result from direct interaction with the αvβ5 integrin. We used a GST-TAP20 fusion protein purified with glutathione Sepharose beads to precipitate proteins from cell lysate, and probed proteins bound to the complexes with the antibodies against αv, β1, β3, and β5 integrin subunits. GST alone did not precipitate any protein recognized by these antibodies. Only the β5 integrin subunit was precipitated by the GST-TAP20 fusion protein (Fig. 7 A). To further confirm the TAP20–β5 integrin interaction, we used GST fusion proteins containing the cytoplasmic tail of either the β3 or β5 integrin subunit to precipitate TAP20 protein from lysate of ECV cells transfected with TAP20 + GFP. Immunoblotting with anti-TAP20 antibody demonstrated that TAP20 protein could coprecipitate with the GST–β5 tail fusion protein (Fig. 7 B). To rule out an indirect interaction between the GST-TAP20 protein and the β5 integrin complex, or between the GST–β5 tail and the TAP20 protein complex, we then used the GST-integrin tail fusion proteins to precipitate purified TAP20 protein. The GST-TAP20 fusion protein was first purified with glutathione Sepharose beads, and then was digested with thrombin. After incubation of the GST–integrin tail–coated glutathione Sepharose beads with TAP20 released by thrombin, GST–β5 tail beads were able to precipitate TAP20, as shown in immunoblotting with TAP20 antibody (Fig. 7 C).

Discussion

In this study, we identified a cDNA that encodes a novel 20-kD, 176–amino acid protein in RCE. Several observations in this study suggest that TAP20 is involved in integrin function in intact cells: (a) expression of TAP20 is dependent on PKCθ activity, which is required by RCE for migration and proliferation (Tang et al., 1997); (b) overexpression of TAP20 in human ECV cells resulted in an attenuation of cell attachment to VN and a decrease of focal adhesion formation on VN-coated plates; (c) expression of TAP20 in HUVEC cells resulted in an increase of cell migration on VN-coated plates, which can be blocked by an antiintegrin β5 antibody, P1F6; (d) expression of TAP20 in HUVEC cells enhances tube formation on matrix gel; and (e) the GST fusion protein coprecipitation study demonstrated that TAP20 binds to the cytoplasmic domain of the β5 integrin subunit.

β3-endonexin (Shattil et al., 1995) is the only gene closely related to TAP20. The overall amino acid sequence of TAP20 and the long form of β3-endonexin share 56%

homology, and thus, these two proteins are encoded by two different genes. Because of different mRNA splicing, there are two forms of β3-endonexin–related messages in cells, but only the short form peptide has β3 integrin binding activity (Shattil et al., 1995). An anti-TAP20 polyclonal antibody raised against a portion of the COOH terminus sequence of TAP20 recognizes a 20-kD band that is identical to calculated molecular weight of TAP20, indicating that a full-length form of TAP20 exists in cells. Overexpression of TAP20 in cells and the TAP20 binding experiments provide evidence that the full-length TAP20 protein can bind to the β5 integrin.

Our data show that overexpression of TAP20 in ECV cells affects cell adhesion and migration on VN, but not on other ECM proteins such as FN and LN, indicating that TAP20 regulates the function of the αv integrin family. Furthermore, TAP20 effects were inhibited by a specific αvβ5 integrin–blocking antibody P1F6, not by an anti-αvβ3 antibody LM609, indicating that TAP20 may specifically interact with the αvβ5 integrin. A GST-TAP20 fusion protein coprecipitated specifically with detergent-solubilized β5 integrin from ECV cells, and thus the binding of TAP20 to β5 integrin does not depend on the existence of αv. A GST–β5 tail fusion protein coprecipitated specifically with TAP20 protein, indicating that the TAP20 binding site is in the cytoplasmic domain of β5 integrin. Unlike β3-endonexin, which binds the β3 integrin and increases the $\alpha_{IIb}\beta3$ receptor affinity when overexpressed in CHO cells (Kashiwagi et al., 1997), TAP20 interacts with the β5 integrin and negatively regulates αvβ5-based adhesion and focal adhesion formation. Therefore, the effect of TAP20 is to decrease the affinity of its associated integrin receptor αvβ5 for its ligand VN. These effects also differ from those of the αvβ5 blocking antibody P1F6, which can prevent cell migration and angiogenesis initiated by VEGF or PKC activation (Friedlander et al., 1995), presumably by blocking the outside-in component of the integrin bidirectional signaling pathway (Klemke et al., 1994; Shattil and Ginsberg, 1997). This discrepancy suggests that TAP20 regulates EC functions by changing the inside-out component of αvβ5 signaling and thus modulating the functions of the αvβ5 integrin, indicating a different integrin regulating mechanism. Formation of focal adhesions requires ECM-integrin ligation and integrin clustering. The decrease in focal adhesions in the TAP20-overexpressing cells could result from the attenuation of ECM–β5 integrin ligation by TAP20. On the other hand, TAP20 may interfere with the interaction between β5 integrin and the cytoskeleton, which is required for focal adhesion formation. Perhaps as a result of decreased binding to VN, TAP20 enhances EC migration and in vitro tube formation. It is also possible that, despite negative modulation of β5 integrin function, TAP20 may upregulate recruitment of other molecules needed for migration, for instance.

This study demonstrates that TAP20 requires enzymatically active PKCθ for its transcription. It is very likely that TAP20 expression is PKC isoenzyme specific. Direct interaction of TAP20 with the cytoplasmic tail of the β5 integrin subunit is at least one of the pathways by which PKCθ might modulate EC migration and tube formation. Therefore, controlling TAP20 expression presents a mechanism, alternative to direct protein phosphorylation (Lewis et al., 1996), by which PKCs can regulate integrin function, and thus angiogenesis.

We thank S. Zhou for technical assistance.

This work was supported by grants from the National Heart, Lung, and Blood Institute to J.A. Ware.

Submitted: 6 April 1999
Revised: 19 October 1999
Accepted: 22 October 1999

References

Chang, D.D., C. Wong, H. Smith, and J. Liu. 1997. ICAP-1, a novel β_1 integrin cytoplasmic domain-associated protein, binds to a conserved and functionally important NPXY sequence motif of β_1 integrin. *J. Cell Biol.* 138:1149–1157.

Chen, Y.P., I. Djaffar, D. Pidard, B. Steiner, A.M. Cieutat, J.P. Caen, and J.P. Rosa. 1992. Ser-752→Pro mutation in the cytoplasmic domain of integrin beta 3 subunit and defective activation of platelet integrin alpha IIb beta 3 (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. *Proc. Natl. Acad. Sci. USA*. 89:10169–10173.

Clark, E.A., and J.S. Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science.* 268:233–239.

Clark, E.A., and R.O. Hynes. 1997. 1997 Keystone symposium on signal transduction by cell adhesion receptors. *Biochim. Biophys. Acta.* 1333:R9–R16.

Crowe, D.T., H. Chiu, S. Fong, and I.L. Weissman. 1994. Regulation of the avidity of integrin alpha 4 beta 7 by the beta 7 cytoplasmic domain. *J. Biol. Chem*. 269:14411–14418.

Eigenthaler, M., L. Hofferer, S.J. Shattil, and M.H. Ginsberg. 1997. A conserved sequence motif in the integrin beta3 cytoplasmic domain is required for its specific interaction with beta3-endonexin. *J. Biol. Chem*. 272:7693–7698.

Faull, R.J., N.L. Kovach, J.M. Harlan, and M.H. Ginsberg. 1993. Affinity modulation of integrin alpha 5 beta 1: regulation of the functional response by soluble fibronectin. *J. Cell Biol.* 121:155–162.

Folkman, J., and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem*. 267:10931–10934.

Friedlander, M., P.C. Brooks, R.W. Shaffer, C.M. Kincaid, J.A. Varner, and D.A. Cheresh. 1995. Definition of two angiogenic pathways by distinct alpha v integrins. *Science.* 270:1500–1502.

Giancotti, F.G., and E. Ruoslahti. 1990. Elevated levels of the alpha 5 beta 1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849–859.

Gumbiner, B.M. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell.* 84:345–357.

Hannigan, G.E., C. Leung-Hagesteijn, L. Fitz-Gibbon, M.G. Coppolino, G. Radeva, J. Filmus, J.C. Bell, and S. Dedhar. 1996. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. *Nature.* 379:91–96.

Hughes, P.E., and M. Pfaff. 1998. Integrin affinity modulation. *Trends Cell Biol.* 8:359–364.

Hughes, P.E., M.W. Renshaw, M. Pfaff, J. Forsyth, V.M. Keivens, M.A. Schwartz, and M.H. Ginsberg. 1997. Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell.* 88:521–530.

Hynes, R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.

Kashiwagi, H., M.A. Schwartz, M. Eigenthaler, K.A. Davis, M.H. Ginsberg, and S.J. Shattil. 1997. Affinity modulation of platelet integrin $\alpha_{IIb}\beta_3$ by β_3-endonexin, a selective binding partner of the β_3 integrin cytoplasmic tail. *J. Cell Biol.* 137:1433–1443.

Kerr, S.M., S. Vambrie, S.J. McKay, and H.J. Cooke. 1994. Analysis of cDNA sequences from mouse testis. *Mamm. Genome.* 5:557–565.

Klemke, R.L., M. Yebra, E.M. Bayna, and D.A. Cheresh. 1994. Receptor tyrosine kinase signaling required for integrin alpha v beta 5-directed cell motility but not adhesion on vitronectin. *J. Cell Biol.* 127:859–866.

Kolanus, W., W. Nagel, B. Schiller, L. Zeitlmann, S. Godar, H. Stockinger, and B. Seed. 1996. Alpha L beta 2 integrin/LFA-1 binding to ICAM-1 induced by cytohesin-1, a cytoplasmic regulatory molecule. *Cell.* 86:233–242.

Kucik, D.F., M.L. Dustin, J.M. Miller, and E.J. Brown. 1996. Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J. Clin. Invest.* 97:2139–2144.

Laudanna, C., J.J. Campbell, and E.C. Butcher. 1996. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science.* 271:981–983.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell.* 84:359–369.

Leavesley, D.I., G.D. Ferguson, E.A. Wayner, and D.A. Cheresh. 1992. Requirement of the integrin beta 3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. *J. Cell Biol.* 117:1101–1107.

Lewis, J.M., D.A. Cheresh, and M.A. Schwartz. 1996. Protein kinase C regulates alpha v beta 5-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. *J. Cell Biol.* 134:1323–1332.

Liang, P., and A.B. Pardee. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science.* 257:967–971.

Liliental, J., and D.D. Chang. 1998. Rack1, a receptor for activated protein kinase C, interacts with integrin beta subunit. *J. Biol. Chem*. 273:2379–2383.
Marte, B.M., and J. Downward. 1997. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem. Sci.* 22:355–358.
Naik, U.P., P.M. Patel, and L.V. Parise. 1997. Identification of a novel calcium-binding protein that interacts with the integrin alphaIIb cytoplasmic domain. *J. Biol. Chem*. 272:4651–4654.
O'Toole, T.E., Y. Katagiri, R.J. Faull, K. Peter, R. Tamura, V. Quaranta, J.C. Loftus, S.J. Shattil, and M.H. Ginsberg. 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* 124:1047–1059.
O'Toole, T.E., J. Ylanne, and B.M. Culley. 1995. Regulation of integrin affinity states through an NPXY motif in the beta subunit cytoplasmic domain. *J. Biol. Chem*. 270:8553–8558.
Schwartz, M.A., M.D. Schaller, and M.H. Ginsberg. 1995. Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* 11:549–599.
Shattil, S.J., and M.H. Ginsberg. 1997. Integrin signaling in vascular biology. *J. Clin. Invest.* 100:S91–S95.
Shattil, S.J., T. O'Toole, M. Eigenthaler, V. Thon, M. Williams, B.M. Babior, and M.H. Ginsberg. 1995. Beta 3-endonexin, a novel polypeptide that interacts specifically with the cytoplasmic tail of the integrin beta 3 subunit. *J. Cell Biol.* 131:807–816.
Shattil, S.J., H. Kashiwagi, and N. Pampori. 1998. Integrin signaling: the platelet paradigm. *Blood*. 91:2645–2657.
Stewart, M.P., A. McDowall, and N. Hogg. 1998. LFA-1–mediated adhesion is regulated by cytoskeletal restraint and by a Ca2+-dependent protease, calpain. *J. Cell Biol.* 140:699–707.
Tang, S., K.G. Morgan, C. Parker, and J.A. Ware. 1997. Requirement for protein kinase C theta for cell cycle progression and formation of actin stress fibers and filopodia in vascular endothelial cells. *J. Biol. Chem*. 272:28704–28711.
van Muijen, G.N., K.F. Jansen, I.M. Cornelissen, D.F. Smeets, J.L. Beck, and D.J. Ruiter. 1991. Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. *Int. J. Cancer.* 48:85–91.
Wang, R., S.J. Shattil, D.R. Ambruso, and P.J. Newman. 1997. Truncation of the cytoplasmic domain of beta3 in a variant form of Glanzmann thrombasthenia abrogates signaling through the integrin alpha(IIb)beta3 complex. *J. Clin. Invest.* 100:2393–2403.
Zhang, Z., K. Vuori, H. Wang, J.C. Reed, and E. Ruoslahti. 1996. Integrin activation by R-ras. *Cell.* 85:61–69.