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

Key words: TAP 20 • integrin • PK Cθ • endothelial cells • migration
formation in vitro, thus raising the possibility that regul-
a- lily, PKC selective inhibition of a specific member of the PKC fam-

31995). Our previous study (Tang et al., 1997) revealed that
incells revealed that

1996). Binding of RA CK 1, the receptor
for activated protein kinase C (PKC), to the β1, β2, and β5
cytosolic tails appears to depend upon stimulation with
phorbol esters (Liliental and Chang, 1998), indicating that
RA CK 1 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 cytosolic tails have been identi-
fied. β3-endonexin, a novel 111-residue polypeptide that
is capable of binding the cytosolic tail of the β3 integrin
subunit was identified by a yeast two-hybrid screening
strategy (Shattil et al., 1995; Eigenthaler et al., 1997; Kash-
iwagi et al., 1997). This polypeptide does not bind other
integrin tails, including those of β1, β2, and αβ1β2. Transient
coexpression of integrin receptor αβ1β2 and β3-endonexin in
CHOcells revealed that β3-endonexin can modulate the
affinity state of αβ1β2 in a manner that is structurally
specific and subject to metabolic regulation. A β2 integrin
cytosolic domain binding protein, cytohesin-1, has been
found to increase αLβ2-mediated cell adhesion (Kolanus
et al., 1996). Ca2+ and integrin-binding protein (CIB) inter-
acts with the integrin αβ1β2 tail (Näk et al., 1997). Finally,
ICA P-1 (integrin cytoplasmic domain–associated protein-1),
interacts specifically with the β1 integrin cytoplasmic
domain (Chang et al., 1997). The effects of CIB and
ICA P-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 angiogene-
sis induced by fibroblast growth factor 2 or by tumor nec-
rosis factor α; and a pathway initiated by vascular endo-
thelial 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 fam-
ily, PKCθ, prevents EC migration, proliferation, and tube
formation in vitro, thus raising the possibility that regula-
tion of EC function by PKCθ is accomplished by modula-
tion of αvβ5 function. One mechanism by which PKCθ
might regulate αvβ5 function is by controlling the expres-
sion of one or more integrin regulatory proteins.

Materials and Methods

Molecular Cloning

RNA display was performed using an RNA map kit (GenHunter) accord-
ing to the manufacturer’s instructions. The resulting PCR products were
cloned into plasmid pBluescript for sequencing analysis. To screen the rat
PC12 cDNA, β111 library (Clontech) for full-length theta-associated pro-
tein (TAP) 20, both the 5’ and 3’ insert screening amplimers of αβ111 as
the forward primers and a TAP 20 3’ end sequence ACC A T A G A A T
G C A G A C A G A 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 TAP 20 in mammalian cells, we constructed a
TAP 20 + green fluorescent protein (GFP) expression plasmid. The
EGFP (enhanced GFP) gene from plasmid pEGFP-C1 (Clontech) includ-
ing 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. TAP 20 cDNA was then cloned into
the HindIII-XbaI restriction sites in the plasmid pEGFP-C1. Thus, in this
vector the TAP 20 and EGFP genes are controlled by separate CMV
promoters. To localize TAP 20 protein in the cell, we cloned the TAP 20
cDNA into the BglII-Smal restriction sites in plasmid pEGFP-C1 for
expression of a GFP-TAP 20 fusion protein in mammalian cells.

ECV304 cells (a human bladder carcinoma cell line, distributed by
American Type Culture Collection) were cultured in M199 medium sup-
plemented with 10% FBS and antibiotics (GIBCO BRL). M V3 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% CO2
atmosphere. Monolayers of cells were transfected with either control vector or
TAP 20 construct using lipofectin (GIBCO BRL) or GenePorter (Gene
Therapy System) for H UVEC. 1 d after transfection of GFP-containing
plasmids, cells were harvested and suspended in Hank’s buffer (137 mM
NaCl, 5.4 mM KCl, 5.6 mM dextrose, 4.2 mM NaHCO3, 0.4 mM
Na2PO4, 0.44 mM KH2PO4, pH 7.4), and were sorted by GFP fluores-
cence using a FACScan flow cytometer (Becton Dickinson). The sorted
cells were recultured in the complete medium. Expression of TAP 20 was
confirmed by Western analysis using the anti-T A P 20 antibody. The sorted
cells were tested in the cell adhesion, migration, and tube formation ex-
periments.

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 T A P 20 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 deoxy-
cholate, 0.1% SDS, 10 mg/ml PMSF, 30 ml/ml aprotinin) per 107 cells. Sam-
ples were run on a 15 or 10% SDS polyacrylamide gel and electrophoreti-
cally transferred to Immobilon-P membranes (Millipore). The membranes
were then hybridized with indicated antibodies (anti-αv polyclonal anti-
body, Santa Cruz Biotechnology), PK Cα, β1, and β3 mAb (Transduc-
tion Laboratories), and anti-β1 polyclonal antibody (Chemicon). In PBS
containing 5% dry milk and detected via ECL (a mersham Pharmacia Bio-
tech). The affinity-purified rabbit polyclonal anti-T A P 20 antibody was
produced by QC B Inc.

Adhesion Assay

Cell adhesion was performed as described previously (G Iancotti and R
u slahti, 1990; L eavesley et al., 1992) with minor modifications. In brief, 96-
well polystyrene plates (Costar) were coated with 10 μg/ml ICAM-1
monoclonal antibody (Transduction Laboratories), and anti-β1 polyclonal
antibody (Chemicon). To localize TAP 20 protein in the cell, we cloned the TAP 20
cDNA into the BglII-Smal restriction sites in plasmid pEGFP-C1 for
expression of a GFP-TAP 20 fusion protein in mammalian cells.

Cell Migration Assay

To explore the function of TAP 20 in mammalian cells, we constructed a
TAP 20 + green fluorescent protein (GFP) expression plasmid. The
EGFP (enhanced GFP) gene from plasmid pEGFP-C1 (Clontech) includ-
ing its 5’ end cytomegalovirus (CMV) promoter to 3’ end polyA signal
region, was inserted into the EcoRI-KpnI restriction sites (non-multicloning
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The Journal of Cell Biology, Volume 147, 1999 1074
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 Dickenson) plates were prepared in 12-well plates following the manufacturer’s instructions. Atrial transfecting and sorting, HUVEC (~80% confluent) were treated with trypsin, and 5 × 10^5 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 M193 (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; Transduction Laboratories), or 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), 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: KLLTHDRKEFAKFEEERARAKWFTANPNYLKEATSTFTNITYRG) or β5 (amino acid sequence: KLLTHDRKEFARFKDFQFSRYTMYEASNPLYRKPSTHTVDFTFKNFKNSYNTVD) 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. A filter with 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 monoclonal antibody (Amerham Pharmacia Biotech), ββ monoclonal 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 PKCβ with the mRNA display method (Liang and Pardee, 1992) using mRNA from clonal populations of rat capillary endothelial cells (RCE), in which either the kinase-negative PKCβ (PKCβ-kn), a dominant negative inhibitor or a constitutively active form of PKCβ (PKCβ-ca) were overexpressed (Tang et al., 1997). An mRNA 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) Differential expression of the peptide TAP20 in RCE with varying PKC activity levels. Total RNA (B) and RCE lysate (C) were prepared from wt RCE (wt) or cells stably expressing control plasmid (v), PKCα (ca1 and ca2), or PKCβ-kn (kn1 and kn2). The full-length TAP20 cDNA was used as probe on Northern blot (B), and the TAP20 antibody was used as probe on Northern blot (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 PKCα was identified. Northern transfer analysis showed that this 0.8-kb mRNA is highly expressed in PKCα-ca RCE but is dramatically suppressed in PKCα-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 PKCα, as suggested by the Northern transfer analysis. In the PKCα-kn 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 PKCα-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 PKCα.

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 cDNA s 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 was used to detect protein expression. Lane 1 and 5, wt untransfected cells (wt); lanes 2 and 4, GFP/pRc transfected cells (GFP); lanes 3 and 5, TAP20 + GFP/pRc transfected cells (TAP20 + GFP); lanes 3 and 7, TAP20 + GFP/pRc transfected cells (TAP20 + GFP); and lane 4, GFP-TAP20/pRc-transfected cells (GFP-TAP20).

Expression of TAP20 in human cell lines. (A) Plasmid constructs. The EGF 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 GFP cDNA s 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. A anti-TAP20, anti-PKCα, 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); lanes 3 and 7, TAP20 + GFP/pRc transfected cells (TAP20 + GFP); and lane 4, GFP-TAP20/pRc-transfected cells (GFP-TAP20).
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, antiantigens 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 antibody, BHA 2.1 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 BHA 2.1 did not significantly affect cell adhesion on VN (comparing the BHA 2.1 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 BHA 2.1 (31.6 ± 7.7% of added cells) to an extent similar to that with cells on VN plates without BHA 2.1 antibody (Fig. 3 A; VN group of bars). In the experiments with the anti-VN receptor antibodies, the effects of the anti-αvβ3 antibody LM 609 were compared with those of the control antibody BHA 2.1. The degree of adhesion in the BHA 2.1 group was used as basal (100%) for comparison with that of the LM 609 and P1F6 groups in Fig. 3 B. LM 609 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 BHA 2.1 and LM 609 was not statistically significant among the three groups (P > 0.5). In contrast, the anti-αvβ5 antibody P1F6 compared with the control antibody BHA 2.1 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 BHA 2.1 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 LM 609. On the other hand, the VN receptor function of αvβ5 integrin was attenuated by TAP20 overexpression. Thus, blockage of αvβ5 by 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 MV 3 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 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 antiantigens antibodies (10 µg/ml) as indicated: the anti-αvβ3 antibody, LM 609; anti-αvβ5, P1F6, and as a control
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
cells, the number of focal adhesions was significantly reduced by 60–70% (Fig. 4D), 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. 5A). 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-
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. A asterisk indicates P < 0.01, TAP20 versus the controls. This enhancement (TAP20 versus the controls) could not be blocked by the control anti-α2β1 antibody, 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

Since TAP20 modulated cell adhesion and migration, we asked whether overexpressing cell 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,
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 of 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 PKCζ 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). A 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 L M 609, indicating that TAP20 may specifically interact with the αvβ5 integrin. A GST-TA20 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 αβ5β3 receptor affinity when overexpressed in CHO cells (Kashiwagi et al., 1997), TAP20 interacts with the β5 integrin and negatively regulates αvβ3-based adhesion and focal adhesion formation. Therefore, the effect of TAP20 on αvβ5 integrin 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 outside-in 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 PKC ζ 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 PKC ζ 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 PK Cs 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 Apr 1999
Revised: 19 October 1999
Acepted: 2 October 1999

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