PETA-3/CD151, a member of the transmembrane 4 superfamily, is localised to the plasma membrane and endocytic system of endothelial cells, associates with multiple integrins and modulates cell function

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SUMMARY

The Transmembrane 4 Superfamily member, PETA-3/CD151, is ubiquitously expressed by endothelial cells in vivo. In cultured human umbilical vein endothelial cells PETA-3 is present on the plasma membrane and predominantly localises to regions of cell-cell contact. Additionally, this protein is abundant within an intracellular compartment which accounts for up to 66% of the total PETA-3 expressed. Intracellular PETA-3 showed colocalisation with transferrin receptor and CD63 suggesting an endosomal/lysosomal localisation which was supported by immuno-electronmicroscopy studies. Co-immunoprecipitation experiments investigating possible interactions of PETA-3 with other molecules demonstrated associations with several integrin chains including $\beta1$, $\beta3$, $\beta4$, $\alpha2$, $\alpha3$, $\alpha5$, $\alpha6$ and provide the first report of Transmembrane 4 Superfamily association with the $\alpha6\beta4$ integrin. Using 2-colour confocal microscopy, we demonstrated similar localisation of PETA-3 and integrin chains within cytoplasmic vesicles and endothelial cell junctions. In order to assess the functional implications of PETA-3/integrin associations, the effect of anti-PETA-3 antibodies on endothelial function was examined. Anti-PETA-3 mAb inhibited endothelial cell migration and modulated in vitro angiogenesis, but had no detectable effect on neutrophil transendothelial migration. The broad range of integrin associations and the presence of PETA-3 with integrins both on the plasma membrane and within intracellular vesicles, suggests a primary role for PETA-3 in regulating integrin trafficking and/or function.

Key words: TM4SF, Integrin, Cell migration, Multi-protein complex, Protein trafficking, Angiogenesis

INTRODUCTION

The vascular endothelium provides a dynamic non-thrombogenic barrier between the bloodstream and extravascular tissues. The cellular interactions required for the maintenance and function of the vascular endothelium are tightly regulated and are mediated through soluble factors and adhesion molecules. Members of one such family of adhesion molecules, the integrins, are involved in cell-matrix and cell-cell adhesive events through their interaction with the cytoskeleton. Integrins are comprised of heterodimers of an α and β chain and it is the association of these chains which defines ligand specificity (reviewed by Schwartz et al., 1995). Cultured endothelial cells (EC) express many integrins including several of the $\beta1$ subfamily, $\alpha v \beta3$, $\alpha6\beta4$, $\alpha v \beta5$ and these molecules are involved in many aspects of endothelial

function including vasculogenesis, angiogenesis, wound healing, recruitment of leukocytes to sites of inflammation and regulation of thrombosis (reviewed by Shattil and Ginsberg, 1997).

Previously, we described the cloning of PETA-3 (Fitter et al., 1995), a member of the transmembrane 4 superfamily (TM4SF) (reviewed by Wright and Tomlinson, 1994; Maecker et al., 1997), which was assigned CD151 at the VIth International Leucocyte Typing workshop (Ashman et al., 1997). Subsequently, PETA-3 was independently cloned from the adult T cell leukaemia cell line, SF-HT, and termed SFA-1 (SF-HT-activated gene 1) (Hasegawa et al., 1996). Immunohistochemical studies have demonstrated expression of PETA-3 by epithelium, muscle, Schwann cells and ubiquitous expression by endothelium in vivo. Within haemopoietic cells, PETA-3 is mostly restricted to platelets and

megakaryocytes (Sincock et al., 1997; Ashman et al., 1991) where it may be involved in recognition of subendothelial matrix during thrombus formation and in thrombopoiesis, respectively.

Recent studies have demonstrated that PETA-3 is localised to endothelial cell junctions and have suggested that complexes of PETA-3 and α3β1 integrin modulate endothelial cell motility (Yanez-Mo et al., 1998). Several other members of the TM4SF, notably CD9, CD63, CD81 and CD82 have also been demonstrated to associate with integrins of the β1 subfamily including α3β1 (Berditchevski et al., 1995, 1996; Nakamura et al., 1995; Hadjiargyrou et al., 1996), α4β1 (Rubinstein et al., 1994; Mannion et al., 1996) and α6β1 (Berditchevski et al., 1995, 1996; Hadjiargyrou et al., 1996; reviewed by Porter and Hogg, 1998; Hemler et al., 1996). Furthermore, monoclonal antibodies (mAbs) against CD9 and CD81 have been shown to modulate cellular motility and adhesion (reviewed by Maecker et al., 1997).

The abundant expression of PETA-3 in EC, its association with β1 integrins, the induction of homotypic adhesion in haemopoietic cell lines by PETA-3 mAbs (Fitter et al., 1999) and the recent finding that phosphatidylinositol-4 kinase (PI4-K) associates with this molecule (Yauch et al., 1998) suggest a possible role for PETA-3 in the regulation of integrin function in EC. This study examines the subcellular localisation of PETA-3, further characterises the association of a broader range of integrin molecules, and examines functional aspects of PETA-3/integrin complexes in cultured human umbilical vein endothelial cells (HUVEC). Additionally we report the novel finding that the majority of PETA-3 resides within an intracellular endosomal/lysosomal compartment and propose that this molecule may be involved in integrin transport and/or turnover from the plasma membrane.

MATERIALS AND METHODS

Cell culture

HUVEC were isolated by collagenase treatment of umbilical veins, as previously described (Wall et al., 1978). Cells were maintained in M199 medium supplemented with Earle's salts (Cytosystems, Sydney, Australia), 20% foetal bovine serum, 20 mM Hepes, 2 mM glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate (Cytosystems), 0.225% sodium bicarbonate, antibiotics (penicillin and gentamycin), 20 μg/ml heparin, (Sigma, St Louis, MO), and 20 μg/ml endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA). EC were cultured in gelatin-coated tissue culture flasks and passaged when confluent by detachment with trypsin/EDTA (Cytosystems). Passage 1 to 4 cells were used for experiments.

Antibodies

The anti-PETA-3 mAbs 11B1.G4 and 14A2.H1, anti-CD9 mAb, 1AA2.H9, anti-CD63 mAb, 12F12, anti-β1 integrin mAb 61-2C4 and anti-PECAM-1 mAb B2B1 have been described previously (Sincock et al., 1997; Ashman et al., 1997; Cole et al., 1989). Other mAbs were obtained as follows; anti-β1 integrin mAb 8A2 (Kovach et al., 1992) was kindly provided by Dr N. Kovak (University of Washington, WA), anti-α2 16B4 (Serotec, Oxford, UK); anti-α3, P1B5 (Gibco BRL, Gaithersburg MD); anti-α5, PHM2 (Becker et al., 1981), provided courtesy of Dr R. Faull (Royal Adelaide Hospital, Australia); anti-α6, G₀H3 (PharMingen, San Diego, CA); anti-β3, AP-3 (ATCC, Rockville, MD) and anti-β4 439-9B (PharMingen). The anti-transferrin receptor mAb OKT-9 was obtained from the ATCC, anti-γ-

adaptin mAb 100/3 was purchased from Sigma and anti-p180 (Savitz and Meyer, 1990) rabbit polyclonal antiserum was the generous gift of Dr P. Meyer (University of California and Los Angeles, CA). The anti-CD44, mAb H9H11 (unpublished), was provided by Dr P. Simmons (Hanson Centre for Cancer Research), anti-CD34 mAb, 561 (unpublished) provided courtesy of Dr G. Gaudinack (The National Hospital, Oslo, Norway), anti-von Willebrand factor mAb, 49-5C3 (J. Gamble, unpublished) and anti-VE-cadherin mAb, 55-7H1 (Martin-Padura et al., 1997) provided by Dr L. Wayner (University of Minnesota, Minneapolis, MI). Rabbit polyclonal anti-PETA-3 antibody was raised against PETA-3 purified from platelets as described (Fitter et al., 1995). The specificity of this reagent was verified by analysis of binding to PETA-3, CD63 and CD9 transfectants, and by western blotting studies. Isotype matched non-binding mAbs 1B5 (IgG1) and 1D4.5 (IgG2a) (Sincock et al., 1997) or normal rabbit serum (NRS) were included in all experiments as specificity controls. For isotype matched binding controls, 3D2 (IgG1) anti-PECAM-1 and 61-2A1 (IgG2a) anti-E-selectin were used.

Indirect immunofluorescence labelling

For confocal microscopy, cells were plated at 5×10^4 per well in chamber slides (Lab Tek, Nunc, Roskilde, Denmark) precoated with 50 μg fibronectin (Boehringer Mannheim, Mannheim, Germany) and grown to various degrees of confluence over 2-4 days. Cells were fixed (47.5% acetone, 47.5% methanol, 5% formaldehyde) for 30 seconds on ice. For non-permeabilising fixation, cells were fixed with 2% formaldehyde for 5 minutes on ice. After washing, cells were incubated with primary mAb supplemented with 10% NRS and 0.1% azide for 45 minutes on ice. Cells were then washed and incubated with rabbit anti-mouse Ig FITC (AMRAD, Melbourne, Australia) for 45 minutes on ice. For 2-colour labelling, cells were incubated with both primary mAbs, washed then incubated with goat anti-mouse IgG1 Tri-Color (Caltag, San Francisco, CA) and anti-IgG2a FITC, (Caltag), supplemented as above. For wheatgerm agglutinin (WGA) staining, cells were incubated with 5 μg/ml biotinylated WGA, washed and incubated with streptavidin-Tri-Color (Caltag). After labelling, cells were washed, fixed with 1% paraformaldehyde and mounted in 86% glycerol containing 1% propyl-gallate (Sigma). Confocal microscopy was performed using a MRC 600 confocal microscope (Bio-Rad, Hercules, CA). Isotype matched negative control mAbs or normal rabbit serum were included in all indirect immunofluorescence labelling experiments as specificity controls.

Quantitation of intracellular antigens was performed using flow cytometry and saponin permeabilisation as previously described (Fitter et al., 1999). Briefly, cells were harvested with 1 mM EDTA, washed in cold wash buffer (PBS, pH 7.4, 0.1% BSA, 0.1% azide) and fixed with 2% formaldehyde for 10 minutes at room temperature. Cells were labelled by indirect immunofluorescence and bound mAb was detected by incubation with r-phycoerythrin-conjugated goat anti-mouse immunoglobulin (Caltag). mAb binding was quantitated by a Coulter (Hialeah, FL) EPICS XL flow cytometer. Percentage intracellular antigen was calculated by comparison of the mean fluorescence intensity of labelled formaldehyde fixed cells with formaldehyde fixed saponin permeabilised cells after correction for isotype match negative control binding.

Electron microscopy

EC monolayers were fixed with 8% paraformaldehyde in 0.1 M phosphate buffer, pH 7.35, for 1 hour at room temperature. Cells were then washed with 0.2 M phosphate buffer, scraped from the culture dish and pelleted at 8,000 g. The cells were then resuspended in warm gelatin (10% in phosphate buffer) and repelleted. After cooling, gelatin-embedded cells were infiltrated with PVP-sucrose overnight at 4°C and processed for frozen sectioning as described previously (Parton et al., 1997). Ultrathin frozen sections (60-80 nm) were labelled, stained, and viewed (Jeol 1010, Centre for Microscopy and

Microanalysis, University of Queensland) according to published techniques (Parton et al., 1997).

Immunoprecipitation and western blotting

EC were lysed with CHAPS lysis buffer (1% CHAPS; Sigma), 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and containing 40 µg/ml each of PMSF, TPCK, TLCK and p-nitrophenyl-p'-guanidino-benzoate-HCl, all from Sigma) for 30 minutes on ice. Alternatively 1% NP-40 buffer (1% NP-40, Fluka (Buchs, Switzerland), 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, supplemented as above) was used for some experiments as indicated. Specific molecules were immunoprecipitated by incubation of lysate from 10^7 cells with 2-5 µg of purified antibody and goat anti-mouse immunoglobulin-Sepharose for 2 hours at 4°C. A cocktail of isotype matched negative control mAbs 1D4.5 and 3D3.3 was included in all immunoprecipitation experiments as specificity control. Immunoprecipitates were washed four times in 1% lysis buffer followed by one wash in 0.1% lysis buffer, eluted with SDS-PAGE loading buffer at 100°C for 4 minutes and separated on 12% PAGE gels under non-reduced conditions. Proteins were electroblotted to polyvinyl difluoride (PVDF) membrane (Micron Separations Inc., Westborough, MA), then blots were washed in Tris buffered saline supplemented with 0.1% Tween-20 (TBST). Detection of PETA-3 was performed by incubation with biotinylated 11B1.G4 in TBST for 2 hours. After washing, bound primary antibody was detected using streptavidin-alkaline phosphatase (Molecular Probes, Eugene, OR) and visualised using the VISTRA ECF substrate (Amersham, Buckinghamshire, UK) and a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA).

For re-precipitation experiments EC were surface labelled with 0.5 mg/ml Sulfo-NHS-Biotin (Pierce, Rockford, IL) for 30 minutes at room temperature. Unreacted biotin was washed from the cells before addition of CHAPS lysis buffer. Immunoprecipitation with the anti-PETA-3 mAb 11B1.G4 was then carried out as described. 11B1.G4 immunoprecipitates were washed as above then eluted in 1% NP-40 supplemented with 0.2% SDS for 2 hours at 4°C. Eluted proteins were diluted 1:2 in NP-40 buffer, then reprecipitated as described above. Reprecipitated proteins were washed twice in NP-40 buffer before separation on 12% SDS-PAGE gels under reduced conditions. After transfer to PVDF membrane, labelled proteins were detected with streptavidin-alkaline phosphatase and visualised as described.

Motility assay

Third passage ECs were aliquotted into gelatin coated 6 well plates at 5×10^5 per well. After culture for 3 days, confluent monolayers were 'wounded' with a 4 mm latex scraper and the edge of the wounds marked. Monolayers were immediately washed to remove debris and complete EC medium with or without mAbs (1-10 µg/ml), as indicated, was added. Five randomly selected points along each wound were marked and the horizontal distance of migrating cells from the initial wound was measured at the times indicated using ×200 magnification with a 50 µm grid in the eyepiece. For each timepoint, measurements were taken from duplicate wells with three wounds per well. In order to determine whether mAb-induced proliferation contributed to the movement of cells into the wound, total protein content of all wells was measured. At the conclusion of the experiment, wells were washed twice with cold PBS and the cells lysed with 1% NP-40 for 2 hours on ice. Total protein content of all lysates was then measured, using a Bradford Protein Determination Kit (Bio-Rad) in accordance with the manufacturer's instructions.

In vitro angiogenesis assay

Matrigel (Collaborative Biomedical Products) was diluted 1:1.5 in cold serum free M199 medium supplemented as described above, with the omission of EC growth factors and heparin. Aliquots (100 µl) were plated into flat bottomed 96-well microtitre plates and allowed to gel for 1-2 hours at 37°C. EC were then plated into each well at a concentration of 2.5×10^4/well in 150 µl volume of complete EC medium supplemented with 1-10 µg/ml mAb as indicated. Capillary tubes were defined as cellular extensions linking cell masses or branch points and were quantitated from photographs of duplicate wells at 10 and 20 hours time points. Both tube length and number of tubes were quantitated. Statistical analysis of pooled data from three experiments was performed by 2-tailed t-tests for both tube length and number. mAb treatments were compared to both untreated and isotype matched non-binding controls for each time point ($P<0.05$ was considered statistically significant).

RESULTS

## PETA-3 is localised to perinuclear vesicles and endothelial cell junctions

Preliminary flow cytometric studies to investigate the expression of TM4SF members and integrins by cultured EC were carried out. Antibodies against TM4SF proteins varied in their level of surface binding with very strong staining of CD9, strong PETA-3, and low staining of CD63. Binding of the anti-$\beta 1$ mAb, 2C4 was the highest of the β chains examined, with lower levels of $\beta 3$ and $\beta 4$ detected. Expression of $\alpha 2$ and $\alpha 6$ were also high, with lower $\alpha 3$ and $\alpha 5$ levels detected. The $\alpha 4$ chain was not detected (data not shown).

The cellular distribution of PETA-3 in EC was investigated by indirect immunofluorescence and confocal microscopy. In formaldehyde-fixed confluent EC monolayers, anti-PETA-3 mAb strongly stained regions of cell-cell contact (Fig. 1A) as previously described (Yanez-Mo et al., 1998). However, upon permeabilisation, PETA-3 stained the perinuclear region in addition to cellular margins (Fig. 1B). At low culture density, PETA-3 showed stronger, more diffuse staining of the perinuclear region (Fig. 1C). Although staining of the plasma membrane at low culture density was diffuse, concentration of PETA-3 in regions of cell-cell contact was still apparent. Staining for CD9 showed a similar junctional distribution to PETA-3, however, no intracellular staining was observed in permeabilised cells (Fig. 1D). The distribution of intracellular PETA-3 differed from that of the endoplasmic reticulum marker, gp180 (Savitz and Meyer, 1990), which appeared as a diffuse network extending to the periphery of the cell (Fig. 1E).

Since PETA-3 staining did not resemble the endoplasmic reticulum, the possibility of an endosomal/Golgi localisation was examined. Consistent with this, staining of low density ECs with WGA, which binds the Golgi-apparatus, endosomes and plasma membrane (Tartakoff and Vassalli 1983), displayed a perinuclear morphology strongly resembling PETA-3 (Fig. 1F). Furthermore 2-colour staining for PETA-3 and WGA showed strong intracellular colocalisation (data not shown).

In order to quantitate the level of intracellular PETA-3, ECs were labelled by indirect immunofluorescence in the presence and absence of the permeabilising agent saponin, and analysed by flow cytometry (Fig. 2). PETA-3 staining was enhanced approximately 3-fold in saponin treated cells, indicating that the intracellular pool comprised approximately 66% of the total PETA-3 expressed (Fig. 2A). In contrast, no intracellular CD9 was detected (Fig. 2B), whilst approximately 85% of CD63 was found to be intracellular (Fig. 2C). The detection of intracellular antigens by saponin permeabilisation was consistent with that observed by confocal microscopy (see Fig. 1C,D, and below).

Fig. 1. Characterisation of the subcellular localisation of PETA-3 in cultured EC. (A) PETA-3 staining of formaldehyde fixed non-permeabilised cells. (B) Staining of PETA-3 in permeabilised EC. (C) Subconfluent ECs showed strong perinuclear staining for PETA-3. (D) Expression of CD9 was restricted to the plasma membrane, primarily at cell junctions in permeabilised cells. (E) Staining for the endoplasmic reticulum marker gp180. (F) Wheatgerm agglutinin. Bar, 55 μm.
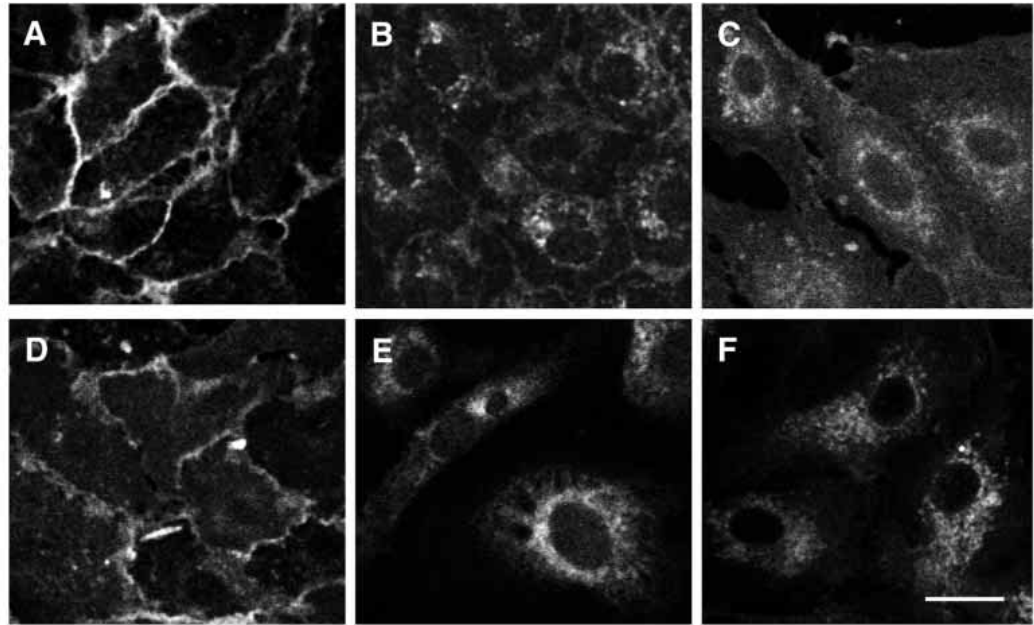
The level of surface expression or subcellular distribution of PETA-3 were not modulated by treatment of EC with tumour necrosis factor-α, interferon-γ, vascular endothelial growth factor and basic-fibroblast growth factor despite upregulation of E-selectin and ICAM-1 after 6 and 48 hours post-treatment, respectively (data not shown).

Intracellular PETA-3 resides within an endosomal compartment

To further characterise the intracellular compartment in which PETA-3 resides, 2-colour colocalisation studies were carried out. The morphology of the Golgi apparatus, as determined by staining with mAb against γ-adaptin (Robinson and Pearse, 1986), did not correspond with PETA-3 suggesting that the majority of PETA-3 does not reside within this compartment (Fig. 3A,B,C). Furthermore treatment with brefeldin A (Fujiwara et al., 1988) for 10 minutes failed to alter the localisation of PETA-3, although redistribution of γ-adaptin from the Golgi was observed (data not shown). Staining of Weibel-Palade bodies for von Willebrand factor (Ewenstein et al., 1987) failed to significantly colocalise with PETA-3 (Fig. 3D,E,F). In contrast, intracellular transferrin receptor, which is present in the early sorting endosome and the perinuclear early recycling endosome (Gruenberg and Maxfield, 1995), showed some colocalisation with PETA-3, especially within the perinuclear region (Fig. 3G,H,I). CD63, which is present in lysosomes and Weibel-Palade bodies (Metzelaar et al., 1991), showed strong colocalisation with PETA-3 in the perinuclear region (Fig. 3J,K,L).

PETA-3 is localised to endosomal structures by immuno-electron microscopy

Due to the limited resolution of confocal analysis, the distribution of PETA-3 was further examined at the ultrastructural level using anti-PETA-3 antibodies on ultrathin frozen sections of EC. Similar labelling was observed with four different antibody preparations; affinity purified anti-PETA-3 rabbit polyclonal (100 μg/ml), rabbit polyclonal whole serum

(diluted 1:50), purified 11B1.G4 (100 μg/ml) and 11B1.G4 culture supernatant. Low but specific labelling was observed on the plasma membrane (Fig. 4a,d) and in endosomal structures (Fig. 4b-d). From the morphology of the labelled structures these were identified as tubulo-vesicular early endosomes (Fig. 4d, and inset) and late endosomes (Fig. 4b and c). The majority of labelling was associated with the internal vesicles of the late endosomes, however some was also associated with the limiting membrane. Consistent with the endocytosis of PETA-3, low levels of labelling were also seen associated with coated pits. Negligible labelling was associated with other cellular organelles such as the endoplasmic reticulum, nucleus, and Golgi complex (see Fig. 4b) although some Weibel-Palade bodies showed a low level of membrane associated labelling (not shown).

PETA-3 associates with multiple integrins in endothelial cells

To examine possible association of PETA-3 with other proteins, confluent fourth passage EC monolayers were lysed in 1% CHAPS lysis buffer and immunoprecipitated with antibodies as indicated (Fig. 5A,B). Western blotting with anti-PETA-3 mAb 11B1.G4 showed co-immunoprecipitation of PETA-3 with the integrin β3, β4, α2 and α6 chains (Fig. 5A) in addition to the previously described β1 (Ashman et al., 1997), α3 (Yanez-Mo et al., 1998; Yauch et al., 1998) and α5 (Hasegawa et al., 1998) associations. PETA-3 has a single N-linked glycosylation site (Fitter et al., 1995) and the doublet represents the mature, fully glycosylated form (32 kDa) and the immature form (27 kDa) as verified by treatment with Endoglycosidase F (data not shown). In order to assess the specificity of PETA-3/integrin interactions, immuno-precipitations with several other endothelial adhesion molecules and transferrin receptor were carried out (Fig. 5A). PETA-3 was not detected in PECAM-1, transferrin receptor, CD34, CD44, or VE-cadherin precipitates but did co-immunoprecipitate with the other TM4SF members CD9 and CD63 as observed in haemopoietic cells (Fitter et al., 1999).

Given the hydrophobic nature of TM4SF proteins, the use of mild detergents has been suggested to lead to artifactual associations (Hemler et al., 1996). Therefore, co-immunoprecipitation experiments using more stringent conditions (1% NP-40), were carried out in parallel to the s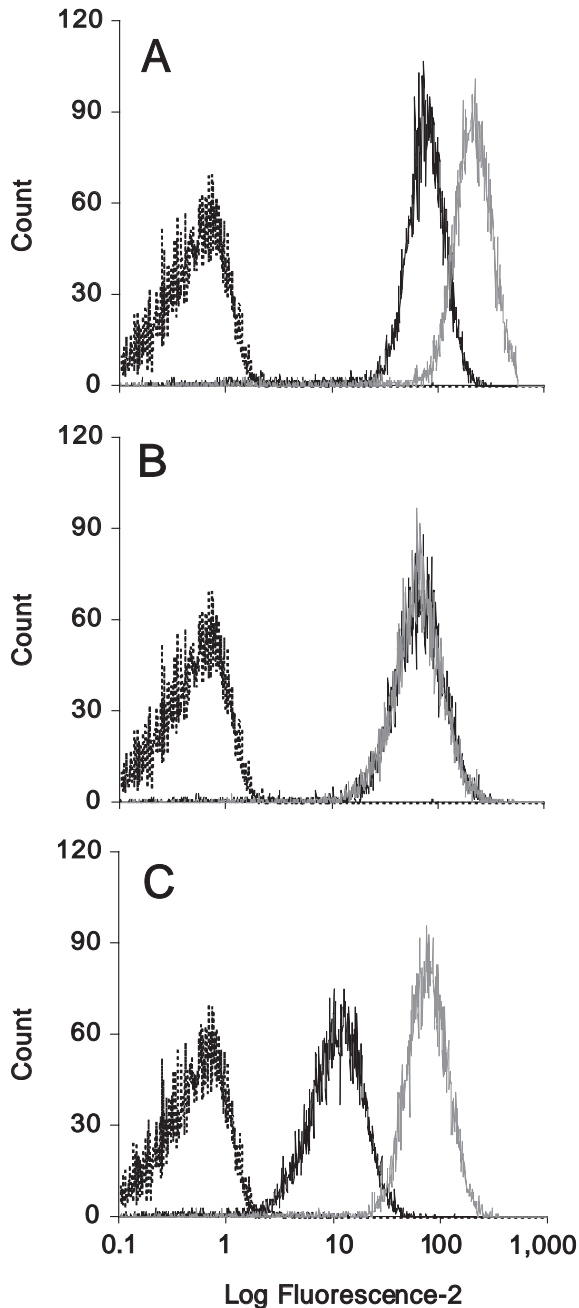tudies performed with CHAPS (Fig. 5B). PETA-3 co-immunoprecipitated with several integrin chains as was observed with CHAPS lysates, however, the associations with $\beta 3$, $\alpha 2$, $\alpha 5$ and the TM4SF members CD9 and CD63 were not observed under these conditions.

Since the association of PETA-3 with the $\beta 3$, $\alpha 2$ and $\alpha 5$ chains was not stable in NP-40, we further investigated their interaction with PETA-3 by re-precipitations from re-solubilised PETA-3 immunoprecititates derived from CHAPS lysates. Using this methodology, $\beta 3$, $\alpha 2$ and $\alpha 5$ integrin chains were readily re-precipitated from PETA-3 immunoprecipitates (Fig. 5C). Similarly, $\alpha 3$ and $\alpha 6$ were also detected. The lower levels of $\alpha 3$ and $\alpha 6$ detected by re-precipitation may reflect the inability of NP-40 to disrupt their interaction with PETA-3 (see Fig. 5B). Conversely, the stronger $\alpha 2$ and $\alpha 5$ signals obtained might be due to the lability of these interactions in NP-40 used in resolubilisation. $\beta 1$ and $\beta 4$ associations were not examined by this methodology. Re-precipitation experiments were also carried out with mAbs to transferrin receptor, CD34, CD44, VE-cadherin and PECAM-1, all of which failed to re-precipitate from NP-40 solubilised 11B1.G4 immunoprecipitates (Fig. 5C). The ability of these control mAbs to immunoprecipitate was verified from surface biotinylated CHAPS lysates (Fig. 5D). All mAbs immunoprecipitated substanital amounts of protein of predicted molecular weight, with the exception of transferrin receptor which was weak due to the majority of this antigen residing within intracellular endosomes.

Comparison of the subcellular distribution of integrins and PETA-3

To examine whether integrin associations with intracellular PETA-3 might exist, the colocalisation of integrins in HUVECs was examined by confocal microscopy. Staining of the integrin $\beta 1$ chain was essentially identical to the localisation of PETA-3, as both cell junctions and intracellular vesicles were labelled by 2-colour confocal microscopy (Fig. 6A,B). Staining for $\beta 3$ (Fig. 6C) also displayed a similar morphology to that of PETA-3. The $\alpha 2$ chain showed predominantly junctional staining, as previously reported (Fig. 6D; Lampugnani et al., 1991), whilst $\alpha 5$ (Fig. 6E) and $\alpha 6$ (Fig. 6F) were similar to that of $\beta 1$, $\beta 3$ and PETA-3. Both anti-$\beta 4$ and $\alpha 3$ mAbs labelled fixed EC poorly and were not suitable for immunohistology (data not shown).

Anti-PETA-3 mAbs inhibit EC motility

Previously, it has been demonstrated that $\beta 1$ and $\beta 3$ integrins mediate endothelial cell migration (Leavesley et al., 1993). Since PETA-3 associated with $\beta 1$ and $\beta 3$, and TM4SF proteins have been proposed to modulate integrin function, we examined the effect of anti-PETA-3 mAbs on EC migration. Confluent EC monolayers were wounded, treated with 2-10 µg/ml mAb and the degree of cell migration assessed over 30 hours (Fig. 7). Perturbation of EC migration was analysed by statistical analysis using 2-way analysis of variance (ANOVA). Incubation of cells with 10 µg/ml each of isotype matched non-binding control mAbs, or isotype matched binding control mAbs (3D2, anti-PECAM-1 and 61-2A1, anti-E-selectin) did not alter the rate of migration when compared to untreated ECs ($P>0.05$). In contrast, anti-PETA-3 mAbs 14A2.H1 and 11B1.G4 (10 µg/ml) inhibited the migration of cells into the wound by 32% and 41%, respectively, at 30 hours post-

Fig. 2. Quantitation of intracellular PETA-3 by immunofluorescent labelling of ECs in the absence and presence of the permeabilising agent saponin and flow cytometry. Histograms represent (A) PETA-3, (B) CD9 and (C) CD63 binding to non-permeabilised (black) or saponin permeabilised (grey) EC. Negative control mAb binding (dotted) is shown for comparison. Data presented are representative histograms from quadruplicate tests. Numbers in parenthesis represent mean fluorescent intensity of respective peaks, boldface = saponin treatment.
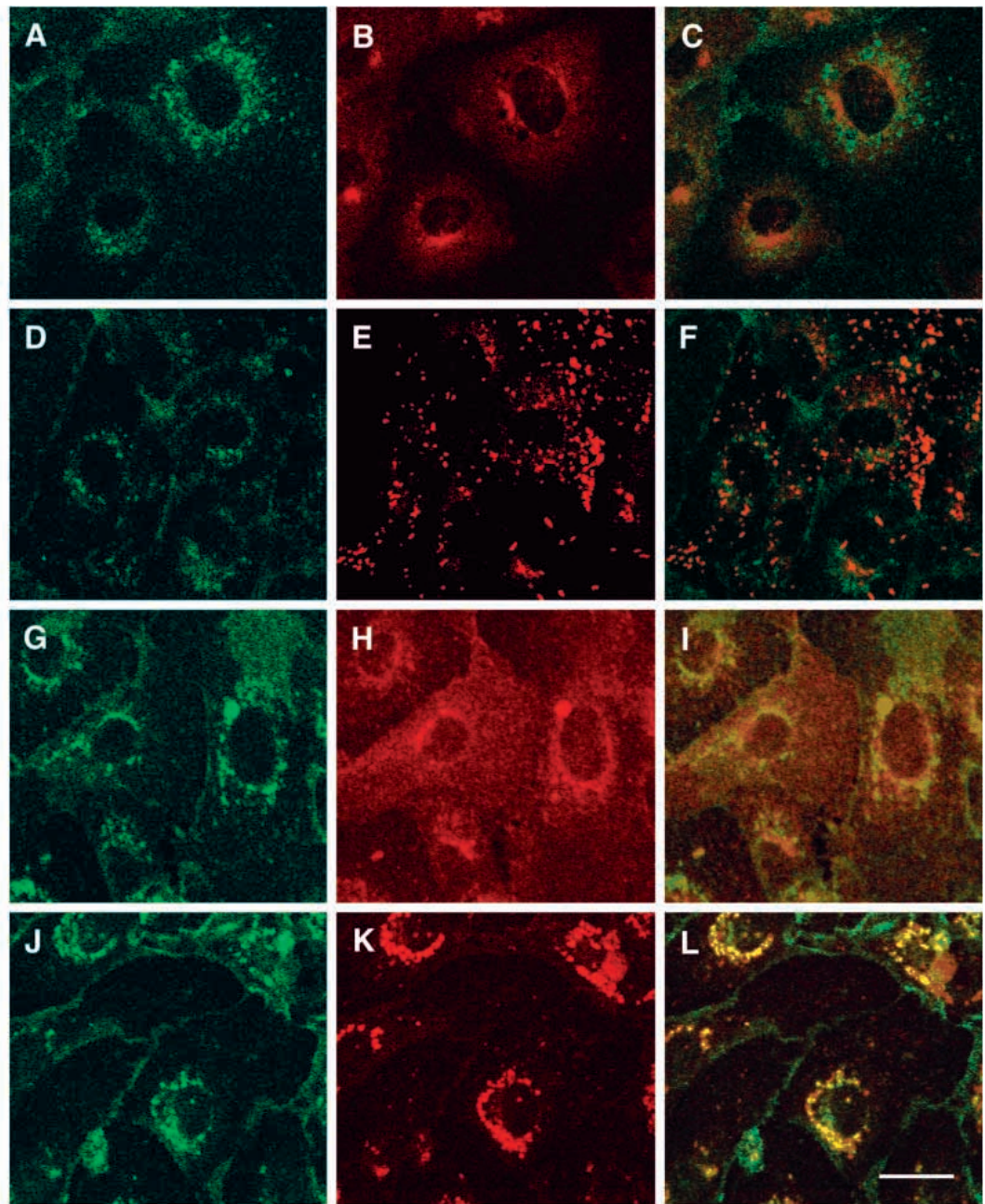
Fig. 3. Comparison of PETA-3 labelling with markers of different subcellular compartments. Staining for PETA-3 shown in green with γ-adaptin, von Willebrand factor, transferrin receptor or CD63 labelling of the same fields shown in red. Merges of red and green images were generated to demonstrate colocalisation (yellow). (A-C) Staining of the trans-Golgi network for γ-adaptin or (D-F) Weibel-Palade bodies with von Willebrand factor did not colocalise with PETA-3. (G-I) PETA-3 partially colocalised with transferrin receptor within the perinuclear region. (J-L) The perinuclear staining of PETA-3 colocalised strongly with CD63. Bar, 55 μm.

wounding when compared to negative control mAb treatments and untreated EC ($P<0.01$). Treatment of ECs with 2 μg/ml of the β1-activating mAb 8A2, or 10 μg/ml anti-CD9 mAb 1AA2.H9 also resulted in reduced migration, 52% and 33% inhibition at 30 hours, respectively ($P<0.01$). These findings were reproduced in two separate experiments performed and

for concentrations of anti-PETA-3 mAb 11B1.G4 ranging from 1-10 μg/ml. Inhibition in response to 11B1.G4 was effective over a range of concentrations (1-10 μg/ml), whereas lower doses of 14A2.H1 were less effective (data not shown). To determine whether mAb induced proliferation contributed to the movement of cells into the wound, total protein content

Fig. 4. Ultrastructural localisation of PETA-3. Ultrathin frozen sections of EC were labelled with affinity purified PETA-3 polyclonal antibody followed by 10 nm Protein A-gold. (a and b) Low power overviews showing the general distribution of PETA-3 labelling. (a) The plasma membranes of two closely apposed cells whereas panel b shows the juxtanuclear area of a cell. The plasma membrane (P) and a putative late endosomal structure (L, panel b) show significant labelling (labelling on plasma membrane

indicated by arrows in all panels; labelling in putative endosomal structures by arrowheads). The ER surrounding the nucleus (N) as well as the Golgi complex (G) are unlabelled. (c) A representative image of a putative late endosome (L). (d) Labelling on the plasma membrane (P), a coated pit (double arrowheads) and tubular elements, typical of early endosomal or recycling endosome tubules; inset shows a representative early endosome (E). Bars, 100 nm.
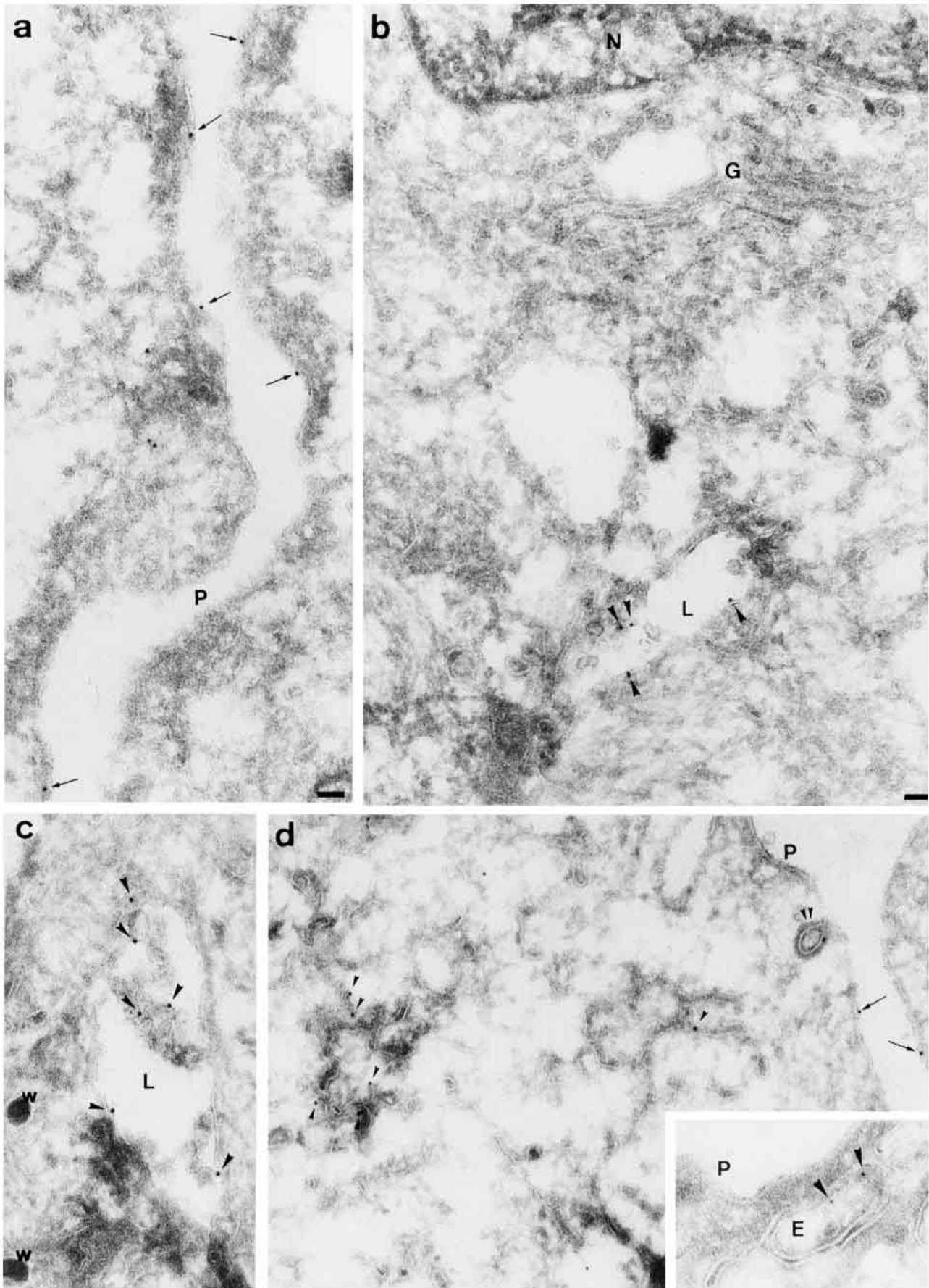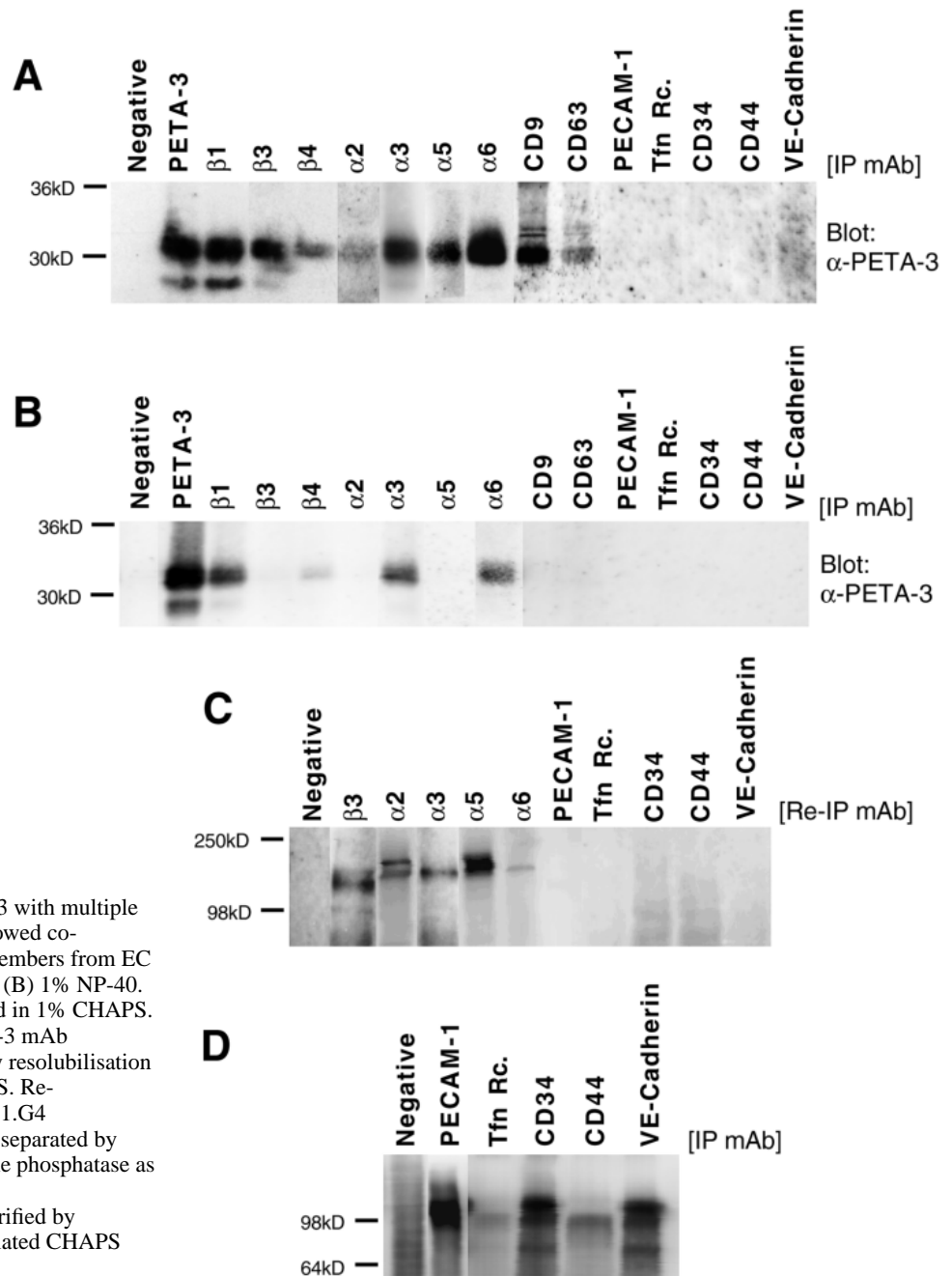
Fig. 5. Co-immunoprecipitation of PETA-3 with multiple integrins. Western blotting for PETA-3 showed co-precipitation with integrins and TM4SF members from EC lysates prepared with (A) 1% CHAPS and (B) 1% NP-40. (C) EC were surface biotinylated and lysed in 1% CHAPS. Immunoprecipitations with the anti-PETA-3 mAb 11B1.G4 were carried out and followed by resolubilisation of immunoprecipitates in NP-40/0.2% SDS. Re-precipitations from NP-40 solubilised 11B1.G4 immunoprecipitates were then performed, separated by PAGE and detected by straptavidin-alkaline phosphatase as described. (D) Control mAbs used for co-immunoprecipitation experiments were verified by immunoprecipitation from surface biotinylated CHAPS lysates.

within each well was determined at the cessation of the experiment. No significant change in total protein was observed for any of the treatments (data not shown).

Anti-PETA-3 mAbs inhibit in vitro capillary formation

Treatment of EC with the activating $\beta1$ mAb 8A2 has been previously shown to inhibit in vitro angiogenesis as assessed by capillary tube formation in Matrigel (Gamble et al., 1993). To investigate whether PETA-3 modulates this process (possibly through its association with $\beta1$ or other integrins), the effect of anti-PETA-3 mAb on in vitro tube formation was investigated. EC cultured on Matrigel in the presence of 1-10 µg/ml anti-PETA-3 mAbs showed retarded tube formation (Fig. 8) in three separate experiments performed. Although not

as effective as mAb 8A2, anti-PETA-3 mAbs significantly altered tube formation when compared to untreated, binding or non-binding negative control mAbs. Table 1 shows the pooled data from three experiments and statistical analysis was performed on these pooled data. At the 10 hour time point, treatment with 8A2 showed a statistically significant decrease ($P<0.05$) in tube length and increase in tube number when compared to controls. Although the anti-PETA-3 mAbs 11B1.G4 and 14A2.H1 (1-10 µg/ml) showed similar results at 10 hours, only the increase in the number of tubes in 11B1.G4 treated EC was statistically significant ($P<0.05$) at this time point when compared to controls. However, at 20 hours both the decrease in tube length and the number of tubes formed were significantly altered by both anti-PETA-3 mAbs as well
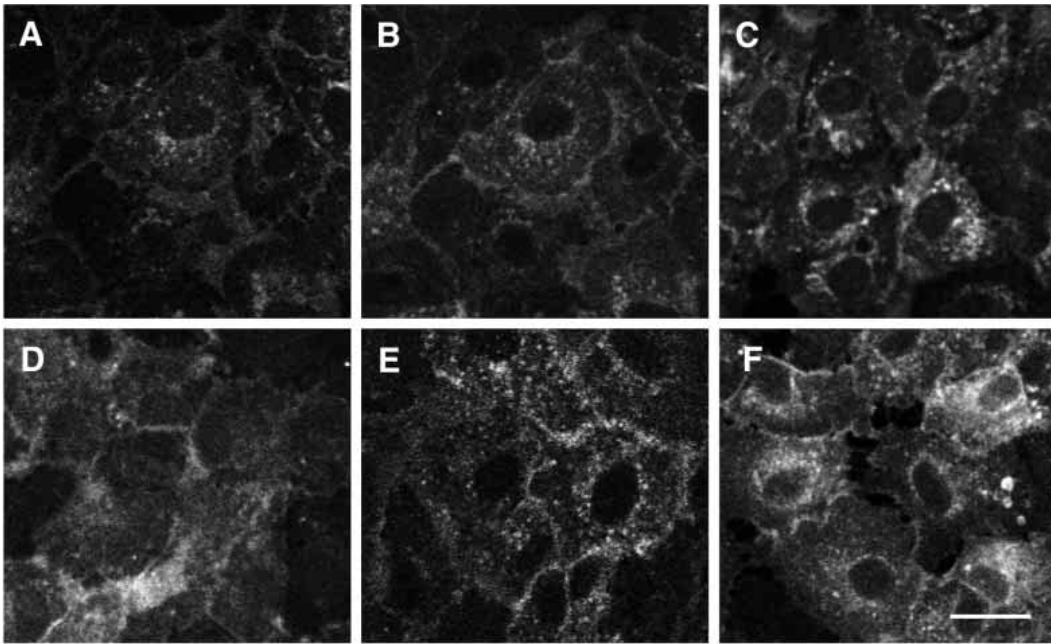
Fig. 6. The subcellular localisation of integrins resembles PETA-3 in EC. EC were labelled by indirect immunofluorescence and examined by 2-colour confocal microscopy. (A) PETA-3 and (B) β1 integrin labelling of the same field. For comparison, labelling of (C) β3, (D) α2, (E) α5 and (F) α6 are also shown. Bar, 55 μm.

as mAb 8A2 when compared to controls. The anti-CD9 mAb 1AA2.H9 failed to modulate tube formation.

DISCUSSION

PETA-3 is localised to the endocytic pathway

Cell surface PETA-3 is predominately localised to EC junctions in a similar manner to CD9, as has been described (Yanez-Mo et al., 1998). However, the data presented here show that the majority of this protein is intracellular (like CD63). Thus unlike CD9 and CD63, PETA-3 does not appear to be restricted to a single subcellular compartment. Furthermore, while the carboxyl-terminal cytoplasmic tail of CD63 contains a putative lysosomal targeting motif (Metzelaar et al., 1991), the domains of PETA-3 responsible for intracellular and plasma membrane targeting remain undefined. The morphology of PETA-3 staining did not resemble the endoplasmic reticulum marker gp180 or the Golgi marker, γ-adaptin (Robinson and Pearse, 1986). Furthermore, the localisation of PETA-3 was not sensitive to treatment with brefeldin A which disrupts the Golgi. These data, together with the observation that the majority of PETA-3 is fully glycosylated and showed co-localisation with WGA, imply that this molecule predominantly resides within a post-Golgi compartment. Based on colocalisation studies using transferrin receptor and CD63 mAbs and labelling of PETA-3 by immuno-electron microscopy, we conclude that the majority of intracellular PETA-3 is associated with endosomes, late endosomes and/or lysosomes. Also, like the recycling mannose 6-phosphate receptor in late endosomes (Griffiths et al., 1988), PETA-3 was mostly present on internal membranes of these structures.

The labelling of clathrin coated pits and vesicles suggests that PETA-3 is internalised from the plasma membrane. Additionally, localisation in tubular vesicular elements of early endosomes, and the colocalisation with perinuclear transferrin

receptor, which is predominantly associated with the perinuclear recycling vesicle, imply recycling of cell surface PETA-3 via the endocytic pathway. Perhaps PETA-3 is involved in the fusion of cellular membranes, in particular vesicles within the endocytic pathway or it might be involved in protein-protein interactions mediating internalisation and/or sorting events.

PETA-3 associates with multiple integrins

Association of PETA-3 with integrins and the two TM4SF members CD9 and CD63 was demonstrated by co-immunoprecipitation under mild lysis conditions. Precipitation from CHAPS lysates using mAbs to the various α chains and blotting for PETA-3 showed association with β1, β3, β4, α2,
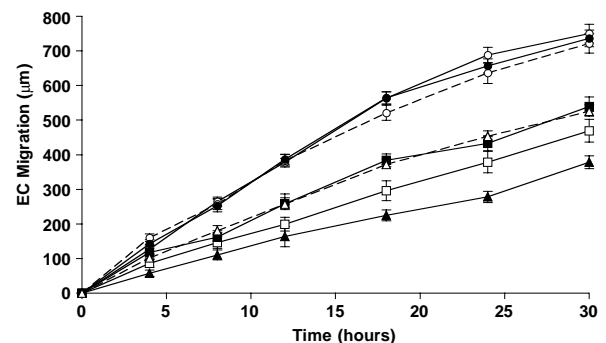

Fig. 7. Anti-PETA-3 mAbs inhibit EC motility. EC monolayers were scraped and the rate of cell migration into the 'wound' was assessed over 30 hours. Data points represent the mean ± s.e.m. of 30 measurements. (□) 11B1.G4 (10 μg/ml), (■) 14A2.H1 (10 μg/ml), (△) 1AA2.H9 (10 μg/ml), (▲) 8A2 (2 μg/ml). (○, solid line) Untreated, (●) isotype matched non-binding control mAbs (10 μg/ml), (○, broken line) isotype matched binding mAb 3D2 (anti-PECAM-1; 10 μg/ml), EC migration in the presence of 10 μg/ml mAb was analysed by 2-way ANOVA. *$P<0.01$ when compared to untreated, isotype matched binding and non-binding controls.
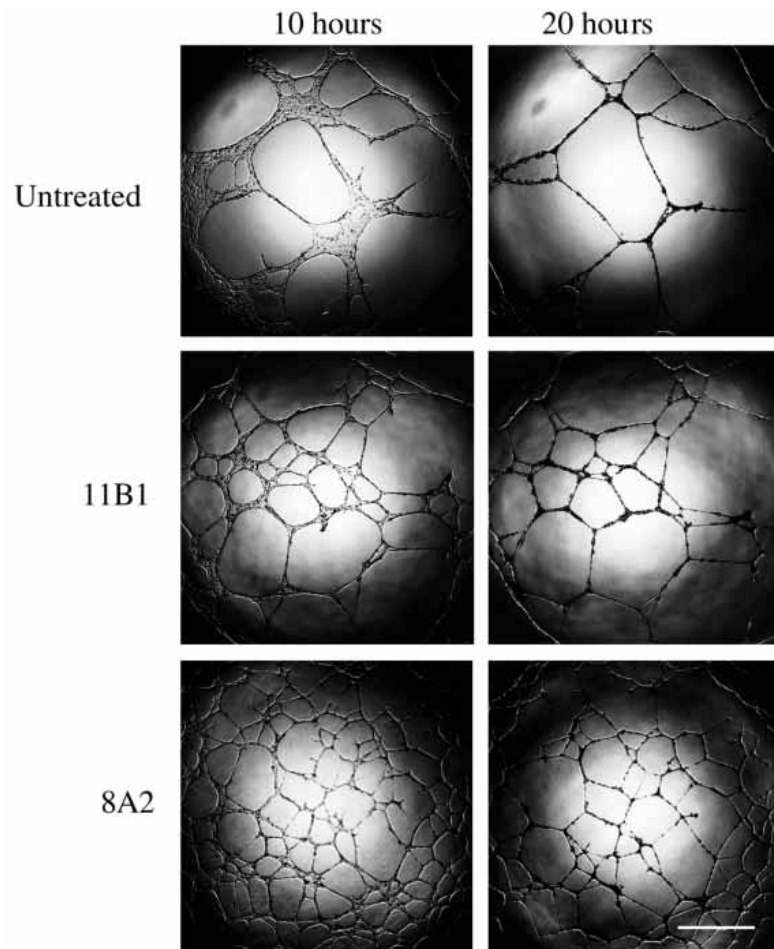
Fig. 8. Anti-PETA-3 mAbs modulate the formation of capillaries in vitro. EC were plated onto Matrigel at 2.5×10^4/well in the presence or absence of mAbs as indicated. Treatment with anti-PETA-3 mAb 11B1.G4 (10 µg/ml) or β1 mAb 8A2 (2 µg/ml) resulted in more tubes of shorter length. Data shown are representative photographs of one of three separate experiments. Bar, 800 µm.

α3, α5 and α6 integrin chains. In addition, when surface-biotinylated cells were subjected to mild detergent lysis, immunoprecipitation with PETA-3 mAb, solubilisation of the precipitates in stronger detergent and re-precipitation with mAbs to integrin chains, we were able to specifically examine PETA-3/integrin complexes at the cell surface. Using this protocol, surface association with all integrins examined could be demonstrated. The PETA-3/integrin and PETA-3/CD63 associations reported in this study add to the initial characterisation of PETA-3 associations with β1 integrins and CD81 (Ashman et al., 1997; Yanez-Mo et al., 1998; Yauch et al., 1998; Hasegawa et al., 1998).

Apart from the 'classical' complexes of TM4SF members with α3β1, α4β1 and α6β1 (reviewed by Hemler et al., 1996; Maecker et al., 1997), associations with the β3, β4, α2 and α5 integrin chains observed in this study have been less widely reported. As β4 only associates with α6, we conclude that PETA-3 associates with the α6β4 complex. Similarly β3 in EC is paired with αv, and although co-immunoprecipitation with αv was not investigated due to the lack of a suitable antibody, the data suggest that PETA-3 complexes with αvβ3. Other TM4SF proteins have also been shown to associate with non-β1 integrin sub-families, including CD9 with αIIbβ3 and α4β7 and CD63 with αLβ2 (Slupsky et al., 1989; Mannion et al., 1996; Skubitz et al., 1996). Despite the promiscuous association of PETA-3 with integrins observed in this study, the lack of co-immunoprecipitation with other EC adhesion

molecules and transferrin receptor demonstrates the specificity of the reported interactions. Furthermore, several of the PETA-3/integrin associations were NP-40 stable. Therefore we conclude that PETA-3 associates with a wide range of integrins including members of the β1, β3 and β4 sub-families, thus extending the range of known TM4SF/integrin associations. Additionally, we have observed association of PETA-3 with CD9 and CD63 in EC. In contrast to the results with certain

Table 1. Anti-PETA-3 mAbs 11B1.G4 and 14A2.H1 modulate in vitro capillary formation

Treatment	10 hours		20 hours	
	Length (µm)	Number	Length (µm)	Number
Untreated	408±46	11.0±0.5	537±68	22.5±1.4
3D3.3+1D4.5 (Control)	374±16	12.6±2.7	473±46	26.0±1.6
3D2 (PECAM-1)	441±57	12.1±3.1	560±45	22.4±2.4
61-2A1 (E-selectin)	430±63	9.3±1.5	561±56	21.8±2.4
1AA2.H9 (CD9)	423±23	9.7±1.6	466±65	24.6±4.7
11B1.G4 (PETA-3)	344±33	24.3±1.3*	364±24*	33.9±3.0*
14A2.H1 (PETA-3)	374±14	10.7±1.6	386±29*	31.3±2.2*
8A2 (β1)	221±7*	175.8±40.1*	260±23*	126.1±17.7*

Anti-PETA-3 mAbs modulate in vitro capillary formation resulting in more tubes of shorter length. Results presented are the means ± s.e.m. of pooled data from three assays performed in duplicate (see Fig. 8). *t*-tests were performed to compare each mAb treatment with untreated and isotype matched non-binding controls, *$P<0.05$.

integrins, the association of PETA-3 with CD9 and CD63 was not detected when cells were lysed using 1% NP-40. Others have also reported loss of TM4SF/TM4SF and TM4SF/integrin associations in detergents which disrupt hydrophobic interactions (Fitter et al., 1999; Mannion et al., 1996).

Anti-PETA-3 antibodies modulate EC function

The data presented here demonstrate inhibition of EC migration in wounded monolayers by anti-PETA-3 mAbs. In some cases, anti-integrin mAbs reverse the effects of anti-TM4SF mAbs on adhesion and migration (Masellis-Smith and Shaw 1994; Shaw et al., 1995; Behr and Schriever 1995). Thus the functional responses induced by anti-TM4SF mAbs may be mediated indirectly. Since EC motility is inhibited by anti-β1 and β3 antibodies (Leavesley et al., 1993) and PETA-3 associates with these integrins, we postulate that the effects of anti-PETA-3 mAbs on EC migration are due to modulation of integrin function. The effect of anti-PETA-3 mAbs on EC motility has recently been reported by other investigators (Yanez-Mo et al., 1998) and was attributed to the association of PETA-3 with α3β1 integrin, since these mAbs caused a small but statistically significant increase in adhesion to fibronectin, type I collagen and laminin. Although modulation of integrin affinity has been demonstrated by the direct binding of anti-β1 mAbs such as 8A2 and QE2.E5 (Kovach et al., 1992; Faull et al., 1996), other studies to date have failed to demonstrate altered adhesion of cells to ECM after stimulation by anti-TM4SF mAbs (Mannion et al., 1996; Fitter et al., 1999).

We have also demonstrated modulation of in vitro capillary tube formation on the complex matrix Matrigel, by anti-PETA-3 mAbs. This process involves EC movement, realignment to form sprouts and subsequent fusion and extension to form mature capillary tubes (Gamble et al., 1993). mAbs against α6 and β1 integrins block endothelial tube formation on Matrigel, thus demonstrating a major role for α6β1 in this process (Davis and Camarillo, 1995). Although the mechanism of altered tube formation by anti-PETA-3 mAbs remains undefined, it does not solely correlate with decreased cellular motility, as treatment of EC with anti-CD9 mAb which also retarded EC migration, did not influence tube formation. In contrast to cell migration and tube formation, no detectable effect on neutrophil trans-endothelial migration was observed when EC were treated with F(ab′)$_2$ fragments of anti-PETA-3 mAbs under previously established conditions (Cooper et al., 1995; and data not shown). Therefore PETA-3 does not appear to be involved in leukocyte recognition of endothelial cells or the transendothelial migratory process.

PETA-3, integrins and endocytosis: a functional link?

Endocytosis and subsequent recycling of integrins to the leading edge promotes cell migration (reviewed by Lauffenberger and Horwitz, 1996). Recycling of α5β1, αvβ3 and α6β4, which all associate with PETA-3, has been described (Bretscher, 1992; Sczekan and Juliano, 1990; Panetti and McKeown-Longo, 1993; Bretscher, 1989; Raub and Kuentzel, 1989). However, the mechanism by which integrins are internalised, sorted and recycled to the leading edge of migrating cells remains unclear. The similar intracellular localisation of PETA-3 and integrins suggests that PETA-3 may enter the endocytic pathway complexed with these molecules. Interestingly, internalisation of PETA-3/integrin complexes appears to be selective as CD9, which is also present in integrin complexes on the plasma membrane, was not detected within the endocytic pathway. Therefore we propose that PETA-3 may play a specific role in the internalisation and recycling of integrin complexes through the endocytic pathway. Furthermore, the functional effects of anti-PETA-3 antibodies may be due to disruption of integrin turnover.

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