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Translocation of protein tyrosine phosphatase Pez/PTPD2/PTP36 to the nucleus is associated with induction of cell proliferation

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SUMMARY

Pez is a non-transmembrane tyrosine phosphatase with homology to the FERM (4.1, ezrin, radixin, moesin) family of proteins. The subcellular localisation of Pez in endothelial cells was found to be regulated by cell density and serum concentration. In confluent monolayers Pez was cytoplasmic, but in cells cultured at low density Pez was nuclear, suggesting that it is a nuclear protein in proliferating cells. This notion is supported by the loss of nuclear Pez when cells are serum-starved to induce quiescence, and the rapid return of Pez to the nucleus upon refeeding with serum to induce proliferation. Vascular endothelial cells normally exist as a quiescent confluent monolayer but become proliferative during angiogenesis or upon vascular injury. Using a 'wound' assay to mimic these events in vitro, Pez was found to be nuclear in the cells that had migrated and were proliferative at the 'wound' edge. TGFβ, which inhibits cell proliferation but not migration, inhibited the translocation of Pez to the nucleus in the cells at the 'wound' edge, further strengthening the argument that Pez plays a role in the nucleus during cell proliferation. Together, the data presented indicate that Pez is a nuclear tyrosine phosphatase that may play a role in cell proliferation.

Key words: Tyrosine phosphatase, Pez, Nuclear localisation, HUVEC, Proliferation, Quiescence

INTRODUCTION

Reversible tyrosine phosphorylation catalysed by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) is a major regulatory mechanism for a diverse number of cellular functions (for a review, see Neel and Tonks, 1997). Although there has been a surge in the numbers of PTPs identified in the last 5 years, relatively little is known about the specific functions and modes of regulation of the individual PTPs.

The PTP Pez (PTPD2/PTP36) is a member of the subfamily of PTPs with homology to the band 4.1 proteins, characterised by the presence of N-terminal FERM (4.1, ezrin, radixin, moesin) domains and C-terminal PTP catalytic domains. Other members of the FERM subfamily of PTPs include PTPD1, PTPH1 and PTPMeg1. Pez was first cloned in a screen for PTPs expressed in normal breast tissue (Smith et al., 1995). It is also expressed in varying amounts in other tissues including kidney, skeletal muscle, lung and placenta. In human umbilical vein endothelial cells (HUVEC), the Pez mRNA is one of the most highly expressed PTP mRNAs (C. Wadham and Y. Khew-Goodall, unpublished observation), suggesting it may be a critical PTP in regulating endothelial cell function.

The FERM superfamily of proteins include the ERM (ezrin, radixin and moesin) proteins and talin. A feature common to all these proteins is that they interact through their N termini with integral plasma membrane proteins and through their C termini with the cytoskeleton, thus forming a bridge between the plasma membrane and the cytoskeleton (Tsukita and Yonemura, 1997, 1999). It has been postulated that the family of FERM proteins, particularly the ERM proteins, play a role in organising the cytoskeleton, perhaps in response to external cues.

By analogy to the other members of the FERM family of proteins, it is reasonable to predict that the FERM subfamily of PTPs is similarly localised and perform similar functions. Some evidence exists to support this. Ogata et al. (1999a,b) recently demonstrated that ectopic overexpression of PTP36, the murine homologue of Pez, led to decreases in actin stress fibre and focal adhesions, resulting in loss of cell-matrix adhesion and cell proliferation. They showed that ectopically expressed PTP36 was associated with the plasma membrane and the cytoskeletal fraction, suggesting that it may play a role in the regulation of the cellular cytoskeleton. Studies on PTPH1 have also shown that the N-terminal portion can mediate associations of PTPH1 with plasma membrane structures (Zhang et al., 1999). In addition, the FERM domain of PTPH1 can inhibit the catalytic activity of PTPH1 in vitro (Zhang et al., 1995), suggesting that it has functions other than in tethering PTPH1 to the plasma membrane. Furthermore, PTPH1 has been shown to be complexed to the adaptor molecule 14-3-3 when it is phosphorylated (Zhang et al., 1997), and the major substrate of PTPH1 has been identified, using a substrate-trapping mutant of PTPH1, to be the valosin-

containing protein (VCP, also known as p97/CDC48) (Zhang et al., 1999), a protein with various potential functions including cell-cycle regulation (Madeo et al., 1998). The studies with PTPH1, a representative of the FERM subfamily of PTPs, demonstrate that members of this subfamily may have functions other than those related to cytoskeletal architecture.

In this report, we show that the subcellular localisation of Pez is regulated. Pez is cytoplasmic in confluent monolayers where the cells are quiescent, a state similar to the resting endothelium lining blood vessels. However, in actively proliferating cells growing at low cell density and in cells induced to proliferate from the resting state by serum addition or by the 'wounding' of a confluent monolayer, Pez is nuclear. Collectively, our data demonstrate that the presence of Pez in the nucleus correlates with cell proliferation and suggests that it has a nuclear role during cell proliferation. In blood vessels, the normally quiescent endothelium is induced to proliferate at sites of vascular injury and during angiogenesis. Therefore, Pez may play a role in regulating endothelial cell proliferation during vascular injury and angiogenesis.

MATERIALS AND METHODS

Antibodies

Anti-Flag epitope (M2) was obtained from AMRAD Biotech (Victoria, Australia) and anti-ERK1/ERK2 from Zymed Laboratories Inc. (San Francisco, CA, USA).

Generation of Pez-specific antibodies

Two peptides predicted to be immunogenic by the algorithm 'Antigenic' (GCG, University of Wisconsin, USA), located in the variable region of Pez, were synthesised. The peptides, PYTVPYGPQGVYSNKLVSPS corresponding to amino acids (aa) 495-510 ($Pez_{495-510}$) and SHEVPQLPQYHHKK corresponding to aa 683-696 ($Pez_{683-696}$), do not exhibit significant homology to other proteins in the protein databases, including other members of the FERM PTPs, suggesting that antibodies (Abs) raised against them would be specific to Pez. The peptides were conjugated to the carrier protein, ovalbumin (Sigma, St Louis, MO, USA) and used to immunise rabbits. Antisera obtained against each of the peptides were used in an ELISA to screen for binding to unconjugated peptide. Antisera generated to $Pez_{495-510}$ and $Pez_{683-696}$ were called PezR1 and PezR2, respectively. Whole antiserum to PezR1 and PezR2 were purified using peptide affinity columns generated by coupling the peptides $Pez_{495-510}$ and $Pez_{683-696}$ cross-linked to BSA to Affigel-15 (Bio-Rad Labs, Hercules, CA, USA) matrix, according to the manufacturer's instructions.

Western blotting, subcellular fractionation and immunoprecipitation

For western blotting, the lysates were electrophoresed on an 8% SDS-polyacrylamide gel (Laemmli, 1970), transferred onto Hybond-P PVDF membranes (Amersham, Buckinghamshire, England) and blocked with 5% milk, 0.1% Triton X-100 in phosphate-buffered saline (PBS). Crude antisera against Pez were used at 1:500 dilution, affinity-purified Ab were used at 1:50 or 1:100 dilution and the blots developed using an HRP-conjugated secondary Ab (Immunotech, Marseille, France) and enhanced chemiluminescence (Amersham). For quantitative western blotting analysis, the blots were developed using an alkaline phosphatase-conjugated secondary Ab, fluorescent Vistra ECF substrates (Amersham) and the resulting band intensities quantitated using a Fluorimager and the Imagequant software (Molecular Dynamics).

In studies where the Ab was preincubated with peptides prior to use in western blots, preincubation was carried out in 1 ml PBS containing 100 μg/ml peptide for 1 hour at room temperature or overnight at 4°C.

Total cell lysates were prepared by harvesting cells with trypsin, followed by washing in PBS prior to lysing in Laemmli sample buffer (Laemmli, 1970) containing 300 mM NaCl. Subcellular fractionation into nuclear and cytoplasmic fractions was carried out according to published protocols (Mui et al., 1995). Briefly, cells were harvested by trypsinisation, washed in PBS and resuspended in Buffer A (50 mM Hepes, pH 7.5, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM MgCl, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA, 1× protease inhibitor cocktail (Sigma)) containing 0.2% Nonidet P-40 (Sigma) for 1 minute on ice. The nuclear pellet was collected by a brief centrifugation for 15 seconds and washed twice with Buffer A. The nuclear pellet was then either lysed in Laemmli sample buffer to obtain a total nuclear fraction or resuspended in Buffer B (50 mM Hepes, pH 7.5, 100 mM NaF, 300 mM NaCl, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA, 1× protease inhibitor cocktail) and rocked vigorously to obtain nuclear extract (supernatant after centrifugation at 13000 rpm, 5 minutes at 4°C) or nuclear pellet fraction. Immunoprecipitations were carried out by lysing cells in RIPA buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1× protease inhibitor cocktail) followed by preclearing with protein-A sepharose beads. Precleared lysate was incubated with antiserum for 2 hours at 4°C, then protein-A sepharose beads were added and incubated a further 1 hour at 4°C. The protein-A sepharose complex was pelleted by centrifugation, washed twice with RIPA buffer and boiled in Laemmli sample buffer.

Confocal microscopy

HUVEC were plated onto fibronectin (50 μg/ml) coated glass Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA) at the indicated densities. Prior to staining, cells were fixed in 4% paraformaldehyde/PBS for 10 minutes, quenched with 10 mg/ml sodium borohydride for 15 minutes and then permeabilised by treatment with 0.1% Triton X-100. Affinity-purified Ab to Pez was used at 1:10 dilution (whole sera was used at 1:250) and indirect immunofluorescence was detected by incubation with biotinylated anti-rabbit secondary Ab (Rockland, Gilbertsville, PA, USA) followed by FITC-conjugated streptavidin (Dako A/S, Denmark). Confocal microscopy was carried out using a BioRad MRC-600 confocal microscope. For comparison of different treatments, the laser power, confocal aperture and contrast settings were kept constant.

Culture of primary HUVEC

HUVEC were extracted by collagenase treatment according to a modified version of Wall et al. (1978). Cells were grown in 25 cm^2 gelatin-coated tissue culture flasks (Costar, Cambridge, MA, USA) in endotoxin-free M 199 medium (Cytosystems, Sydney, Australia) supplemented with 20% FCS (PA Biological, Sydney, Australia), 20 mM Hepes, sodium pyruvate and non-essential amino acids at 37°C in a 5% CO_2 atmosphere. Cells were replated 2-5 days after establishment of culture by harvesting with 0.05% trypsin-0.02% EDTA. Endothelial cell growth supplement (Multicel, Trace Biosystems, Australia) at 25 mg/ml and heparin were added to cells that were passaged twice or more. In general, cells were used at first or second passage. All reagents used in the growth and passaging of HUVEC were made up under endotoxin-free conditions and contained between 10-100 pg/ml endotoxin determined by the *Limulus* amoebocyte assay.

Plasmids and transfection

Full-length Pez cDNA was tagged at the N terminus with the Flag-epitope and cloned into the eukaryotic expression vector pcDNA3

(Invitrogen, Groningen, The Netherlands). The Flag-tagged mutant, Δ4.1, was generated by deletion of amino acids 2-336, which essentially removes the entire FERM domain. HUVEC and 293 cells were transfected using Lipofectamine 2000 (Life Technologies Inc., Grand Island, NY). Cells were harvested or analysed 48 hours after transfection.

RESULTS

Endogenous Pez expression detected by Pez-specific antisera

Two specific antisera generated to two separate regions of Pez were used to determine the subcellular localisation of endogenous Pez in primary HUVEC. Each of the antisera detected the peptide that was used for immunisation but not the reciprocal peptide, indicating that they are each specific for the peptide used for immunisation (data not shown). The single open reading frame in the Pez cDNA sequence (Smith et al., 1995) predicted a protein of 1187 amino acids or approximately 130 kDa. By western blotting analysis of HUVEC lysate, each Ab (named PezR1 and PezR2) detected two major bands, but only one major band of approx. 130 kDa is common between the Abs and this was not detected by the preimmune sera (Fig. 1A,C). A Flag epitope-tagged Pez ectopically expressed in 293 cells comigrated with the 130 kDa band from HUVEC lysate and was recognised by PezR1, in both western blotting and immunoprecipitation (Fig. 1B). This suggests that the 130 kDa band from HUVEC detected by both Abs is most likely endogenous Pez. Further evidence that the 130 kDa band is indeed Pez is demonstrated in Fig. 1C, where preincubating the Abs with the peptides used for immunisation resulted in removal of the 130 kDa band but not the nonspecific bands. Preincubation with nonspecific peptides had no effect. Together these data suggest that we have generated two Pez-specific Abs that recognise endogenous Pez.

Both the antisera were affinity-purified using the appropriate peptide affinity columns. The resulting affinity-purified PezR1Ab recognised only the 130 kDa Pez band (Fig. 2A) and affinity-purified PezR2 detected predominantly the 130 kDa Pez band as well as a minor non-specific low molecular mass band (Fig. 2B). Both the affinity-purified Abs were subsequently used for detection of Pez by indirect immunofluorescence.

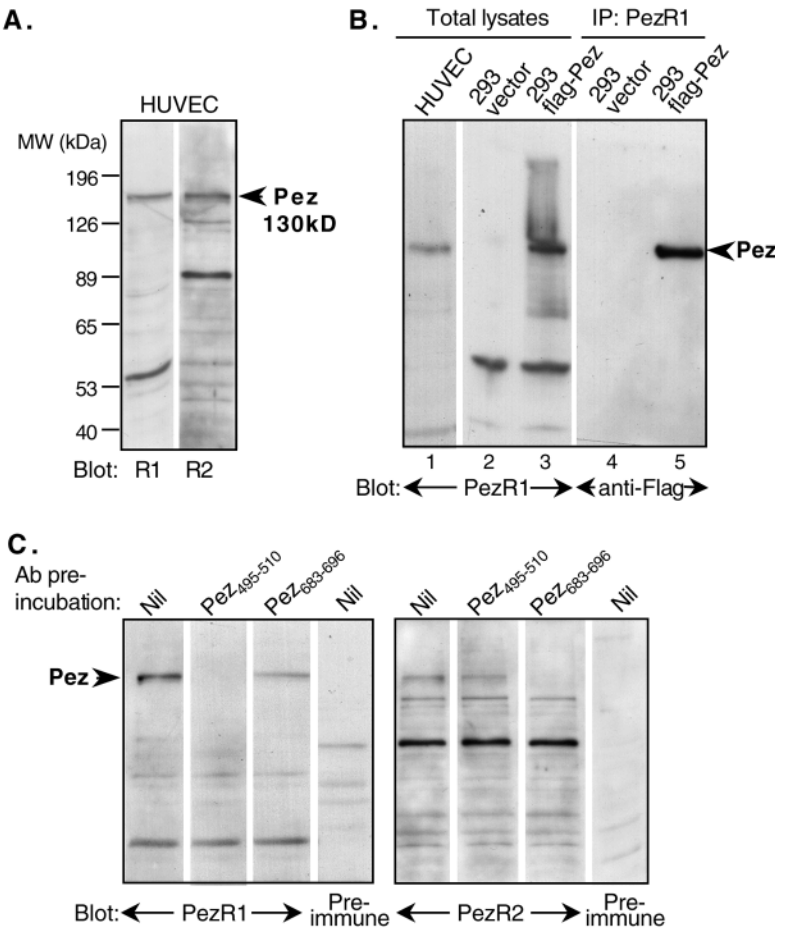

Fig. 1. Characterisation of anti-Pez antisera. (A) Western blot of HUVEC lysate with PezR1 and PezR2 antisera. The migration of prestained markers (MW) is shown. Each Ab recognises two major bands, of which only the 130 kDa Pez band is common. (B) Lanes 1-3: western blot of lysates from HUVEC or 293 cells transfected with empty vector or flag-tagged Pez using the PezR1 antiserum showing ectopically expressed Pez comigrating with endogenous Pez from HUVEC. Lanes 4 and 5: immunoprecipitation using the PezR1 antiserum was carried out using lysates of 293 cells transfected either with empty vector or flag-tagged Pez followed by western blotting with the anti-flag epitope Ab. (C) Western blot of HUVEC lysate with PezR1 (left) or PezR2 (right) antisera that was either untreated (Nil), preincubated with the specific immunising peptide ($Pez_{495-510}$ for PezR1, $Pez_{683-696}$ for PezR2) or the non-specific peptide ($Pez_{683-696}$ for PezR1, $Pez_{495-510}$ for PezR2), showing that the immunising peptide specifically blocks the Pez band. The right lane in each panel was blotted with the appropriate preimmune serum.

Endogenous Pez is nuclear or cytoplasmic depending on cell density

The subcellular localisation of Pez in primary HUVEC was examined by indirect immunofluorescence and confocal laser microscopy using both the affinity-purified PezR1 and PezR2 Abs. In confluent monolayers, as predicted by the presence of the FERM domain in the N terminus of Pez, both Abs stained predominantly the cytoplasmic compartment (Fig. 2A,B, confluent) and in some cells appeared to be concentrated at the plasma membrane at intercellular junctions (data not shown). When cells were plated sparsely, however, predominantly nuclear staining was observed using both Abs (Fig. 2A,B, sparse). An identical pattern of localisation was observed using the PezR1 whole serum whereas preimmune sera or sera preincubated with the corresponding immunising peptides showed either no staining or greatly reduced staining, respectively (data not shown). Because both affinity-purified Abs (one of which, PezR1, recognised only Pez by western blotting) showed an identical pattern of density-dependent staining, we concluded that Pez localises to the nucleus in sparsely plated cells but is cytoplasmic in confluent monolayers.

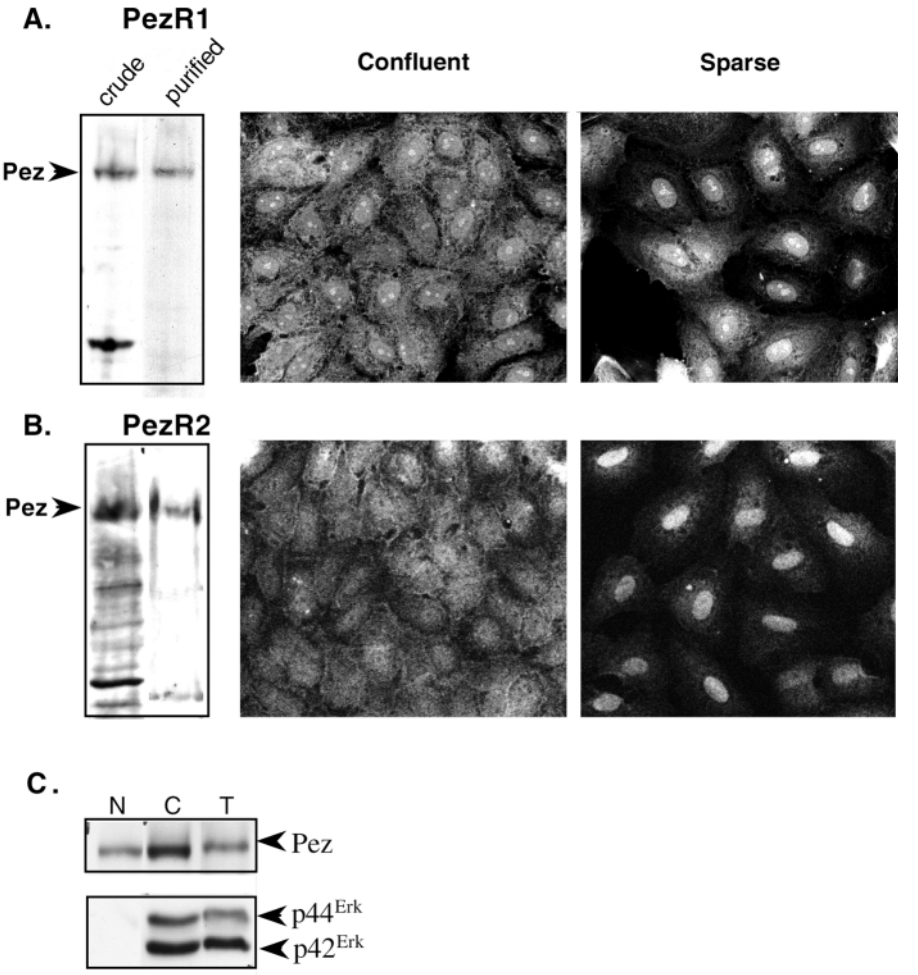

Fig. 2. Affinity purification of PezR1 and PezR2 Abs and their use in the subcellular localisation of endogenous Pez. (Left) Western blots of total HUVEC lysate to compare crude serum and affinity-purified PezR1 and PezR2 Abs. (Middle and right) Confocal micrographs showing indirect immunofluorescence of HUVEC plated as confluent (9×10^4 cells/well, middle) and sparse (2.5×10^4 cells/well, right) monolayers stained with either the affinity-purified PezR1 (A) or PezR2 (B) Abs. The staining with both Abs is predominantly diffuse cytoplasmic in the confluent monolayers and nuclear in sparse isolated cells. (C) Western blot of total HUVEC lysate (T) and after subcellular fractionation into crude nuclear (N) and cytoplasmic (C) extracts, using the PezR1 antiserum (top) and the anti-MAPK Ab (bottom). Pez was present in both nuclear and cytoplasmic fractions whereas the MAPK was detectable only in the cytoplasmic fraction.

To further verify that the nuclear staining observed by in situ immunofluorescence microscopy was due to the presence of Pez in the nuclear compartment, HUVEC were fractionated into nuclear and cytoplasmic extracts and western blotted with PezR1 Ab. The same 130 kDa band was detected in both the nuclear and cytoplasmic fractions (Fig. 2C). The nuclear fraction was shown to be free of cytoplasmic contamination by the complete absence of the cytoplasmic protein MAPK ($p44^{Erk}$ and $p42^{Erk}$) from the nuclear fraction. Thus Pez is indeed present in the nucleus.

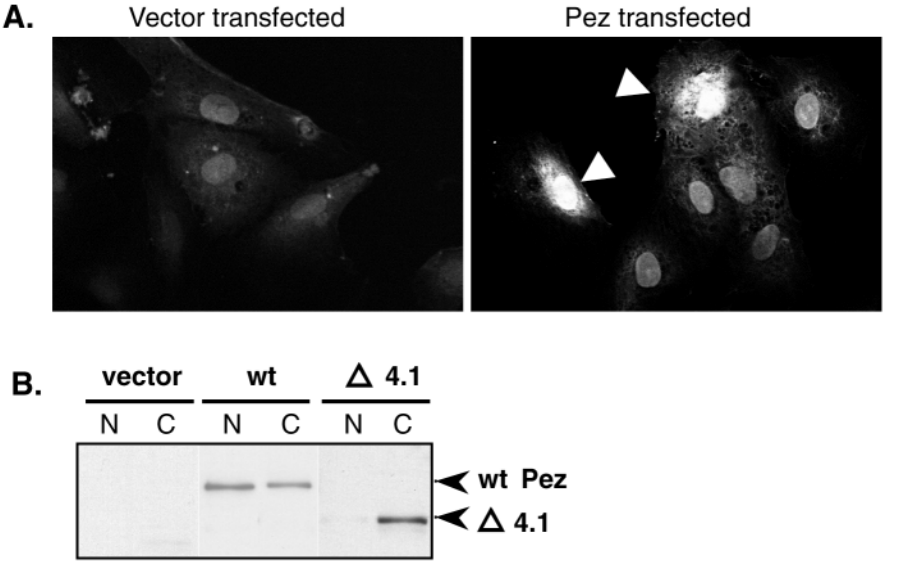

Fig. 3. Ectopically expressed Pez also localises to both the nucleus and cytoplasm. (A) Confocal microscopy of HUVEC transfected either with empty vector (left) or Flag-tagged Pez (right) and detected using the PezR1 antiserum. Pez-overexpressing cells (amongst a background of nontransfected cells showing endogenous Pez staining) are denoted by arrowheads. The figures shown are representative of at least three separate experiments, each using a different line of HUVEC. (B) Western blot of HEK293 nuclear (N) and cytoplasmic (C) lysates obtained after subcellular fractionation from cells transfected with either empty vector, wild-type (wt) Flag-tagged Pez or an N-terminal deletion mutant (Δ4.1). The ectopically expressed proteins were detected with an anti-Flag epitope Ab. Nothing was detected in the empty vector transfectants, wt Pez was present in both the nuclear and cytoplasmic fractions but the Δ4.1 mutant was only present in the cytoplasmic fraction.

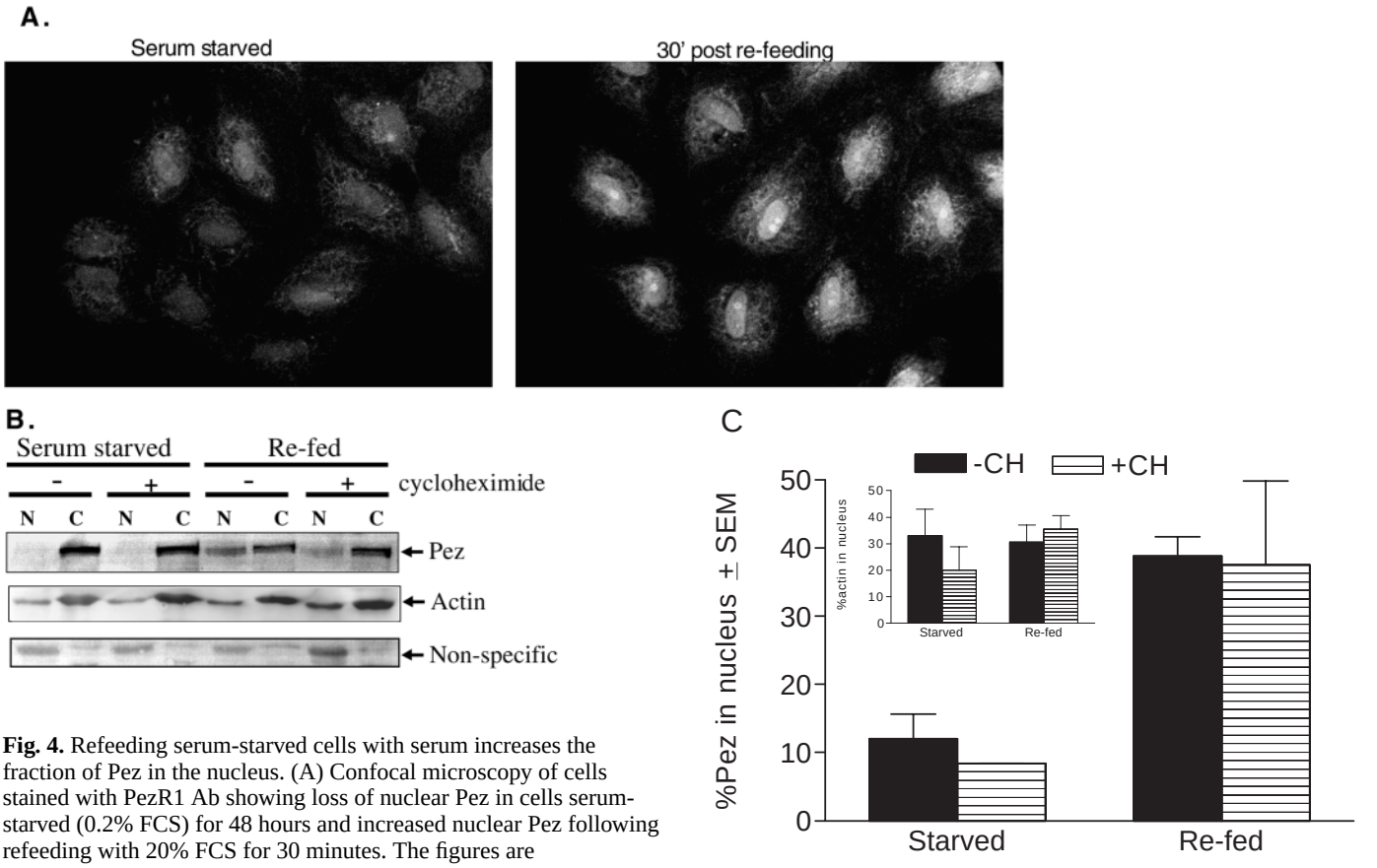

Fig. 4. Refeeding serum-starved cells with serum increases the fraction of Pez in the nucleus. (A) Confocal microscopy of cells stained with PezR1 Ab showing loss of nuclear Pez in cells serum-starved (0.2% FCS) for 48 hours and increased nuclear Pez following refeeding with 20% FCS for 30 minutes. The figures are representative of three separate experiments, each done in duplicate. (B) A representative western blot detected with the PezR1 Ab (top and bottom panels) used for quantitating the proportion of Pez in the nucleus. Serum-starved or refed cells (as in A) were fractionated into nuclear (N) and cytoplasmic (C) fractions. Lysates normalised for equal cell numbers were loaded onto each lane. Blots were also reblotted with an anti-actin Ab (middle panel) to further control for loading. The nonspecific band (bottom panel) is a low molecular mass nuclear protein also recognised by the PezR1 crude antiserum; it does not translocate into the cytoplasm when cells are serum-starved. In cells treated with cycloheximide, 10 μg/ml cycloheximide was added 15 minutes prior to refeeding with serum and left in for the entire duration of refeeding (30 minutes). (C) Quantitation of western blots carried out as described in B using a fluorimager and the Imagequant software. Following background subtraction, the percentage of Pez in the nucleus relative to total cellular Pez was plotted. No significant difference was observed in the percentage of nuclear Pez between refeeding in the presence or absence of cycloheximide (CH) (P=0.44, Student's t-test, n=2) but the percentage of nuclear Pez in refed cells compared to serum-starved cells was significantly different (P<0.01, Student's t-test, n=2). Inset shows quantitation of actin in the nuclear fraction relative to total cellular actin after the blots were reprobed with an anti-actin Ab, showing that there is no significant change in the compartmentalisation of actin upon refeeding with serum or treatment with cycloheximide.

To determine the subcellular localisation of ectopically expressed Pez, a Flag epitope-tagged Pez construct was transfected into HUVEC and the cells stained with the PezR1 Ab. By indirect immunofluorescence, cells transfected with Pez had greatly increased staining in both the nucleus and cytoplasm compared to endogenous Pez staining in empty vector transfected or untransfected cells (Fig. 3A). The presence of ectopic Pez in both compartments can also be demonstrated by western blotting following transfection into HEK293 cells using an Ab against the Flag epitope (Fig. 3B, wt). The presence of ectopic Pez in the nucleus cannot be attributed solely to overexpression because a truncated form with the N-terminal FERM domain deleted was not detected in the nucleus (Fig. 3B, Δ4.1). This further confirms that both endogenous and ectopically expressed Pez can localise to the nucleus and that nuclear localisation of Pez is likely to be dependent on sequences in the FERM domain.

Similar patterns of localisation were also observed using the PezR1 Ab in HeLa cells (data not shown), suggesting that this pattern of compartmentalisation was not confined to HUVEC.

Nuclear localisation of Pez is serum dependent

The above observation raised the possibility that Pez may be nuclear in proliferating cells but cytoplasmic in cells that are quiescent. HUVEC plated at low density were starved of serum (0.2% FCS, 48 hours) to induce quiescence and the localisation of Pez examined thereafter. By indirect immunofluorescence, nuclear Pez was significantly reduced in cells made quiescent by serum starvation but was increased following refeeding with 20% FCS for 30 minutes (Fig. 4A). To quantitate the amount of Pez in the nucleus following serum starvation and refeeding, nuclear and cytoplasmic fractions of cell lysates were examined for Pez content by western blotting and fluorimaging. From this analysis, only about 10% of total Pez

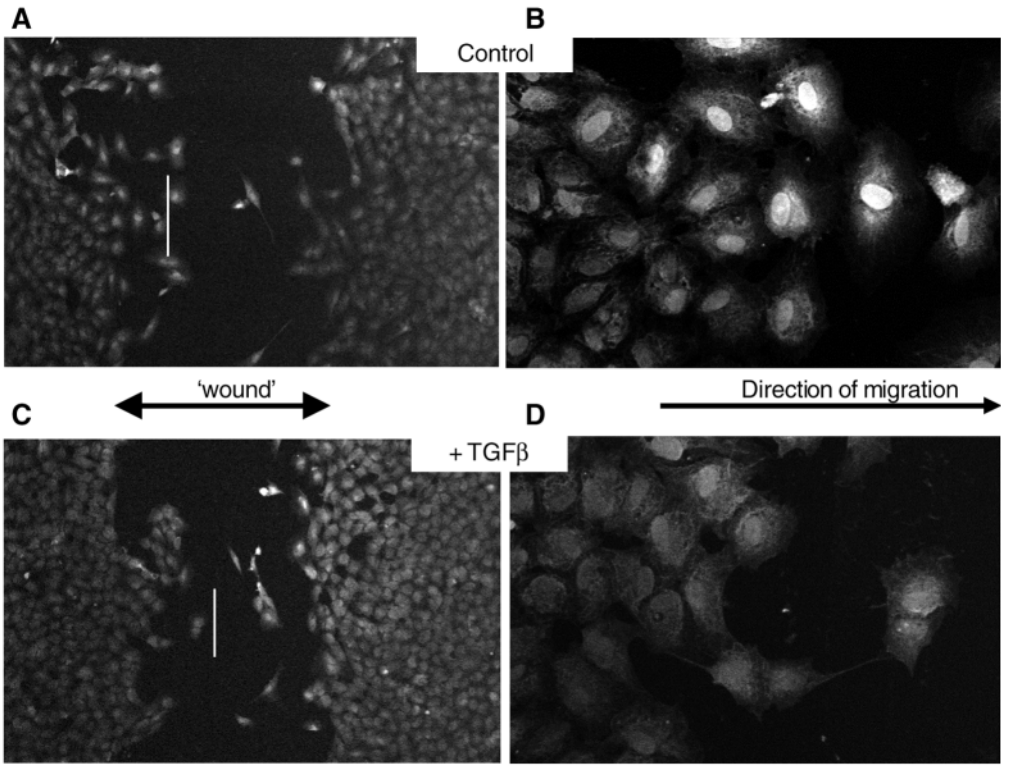

Fig. 5. TGFβ inhibits translocation of Pez to the nucleus in a 'wound' assay. Confocal microscopy of a confluent monolayer of HUVEC through which a 'wound' was made by denuding a linear region on the monolayer followed by staining with PezR1 antiserum 17-24 hours after 'wounding'. (A,B) Controls, no addition. (C,D) TGFβ (0.4 ng/ml) was added at the time the wound was made. (A and C) show the 'wound' into which a similar degree of cellular migration has occurred in both control and TGFβ cells. (B and D) are higher magnifications of the regions marked by a vertical line in A and C, respectively, showing translocation of Pez into the nucleus in the control but not TGFβ treated cells that have migrated into the 'wound'. The figures are representative of three separate experiments each done in duplicate.

remained in the nucleus after serum starvation but about 40-50% of total Pez was back in the nucleus as rapidly as 30 minutes after refeeding with serum (Fig. 4B,C), indicating that Pez is translocated back into the nucleus when quiscent cells (at G_0) are stimulated to proliferate upon the addition of serum. The increase in nuclear Pez in this short period of refeeding was unaffected by the presence of the protein synthesis inhibitor, cycloheximide, during refeeding, suggesting that the increase in nuclear Pez can occur in the absence of de novo protein synthesis. In contrast to Pez, there was no significant alteration in the proportion of actin in the nucleus when serum was added back to the cells and this was similarly unaffected by cycloheximide treatment (Fig. 4B,C, inset). Interestingly, the nuclear localisation of the non-specific band also recognised by the crude PezR1 antisera does not alter with serum starvation (Fig. 4B, non-specific) indicating that not all proteins translocate from the nucleus to the cytoplasm when cells become quiescent. The presence of this nonspecific band in the nucleus even after serum-starvation could account for some of the residual nuclear staining detected by immunofluorescence in Fig. 1A. We have also found that there is no significant alteration in the amount of total Pez in the cells after 30 minutes refeeding with serum (data not shown).

Pez translocates into the nucleus in cells at a 'wound' edge

At sites of vascular injury and during angiogenesis, endothelial cells migrate out of the intact confluent endothelium and proliferate. To mimic these events in vitro, a confluent monolayer of HUVEC was 'wounded' to generate a linear region denuded of cells (Fig. 5A,B). The cells from the confluent area subsequently migrate into the denuded area and proliferate. In cells at the 'wound' edge that have recently migrated into the 'wound', Pez is localised almost exclusively to the nucleus whereas cells in the confluent monolayer distal from the edge that have not migrated clearly showed Pez remaining in the cytoplasm (similar to Fig. 2A).

Translocation of Pez to the nucleus is inhibited by TGFβ

Because the cells at the 'wound' edge are both migratory and proliferative, the role of Pez in the nucleus of these cells could be related to either migration, proliferation or both. To differentiate between these possibilities, cells were treated at the time of generating the 'wound' with TGFβ, which inhibits proliferation by arresting cells at G_1 phase (Iavarone and Massague, 1997) but has no effect on cell migration. Following TGFβ treatment, cells at the 'wound'edge showed little or no increase in nuclear Pez compared to the cells in the confluent region distal from the 'wound', suggesting that TGFβ inhibited the translocation of Pez into the nucleus (Fig. 5C,D). There was no difference in the distance migrated or the number of cells migrating into the 'wound' between untreated and TGFβ-treated cells, confirming that TGFβ does not inhibit cell migration (Fig. 5A,C). These data further support our earlier observations that the translocation of Pez into the nucleus correlates with the transition from quiescence to proliferation.

DISCUSSION

Intracellular compartmentalisation is a powerful means of ensuring specificity of signal transduction pathways by limiting the access of activated, promiscuous enzymes to other downstream partners, while increasing the efficiency of reactions by grouping proteins involved in a particular cascade

together. Hence, the subcellular localisation of a PTP can be an indicator of the physiological role of the PTP.

Because a number of members of the FERM family of proteins are localised through their FERM domain to the plasma membrane-actin cytoskeleton interface (Tsukita and Yonemura, 1997, 1999), it was assumed that Pez would also be similarly localised. However, this had not been demonstrated for the endogenous protein. Using specific Abs to Pez, we demonstrated that the nuclear or cytoplasmic localisation of Pez was regulated by a number of factors including cell density and the presence or absence of serum. The presence of Pez in the nucleus of cells at low but not high density, its absence from the nucleus of serum-starved cells and its return to the nucleus when the cells are replenished with serum, all suggest that Pez is a nuclear protein during cell proliferation.

The resting endothelium that lines blood vessels is normally nonproliferative. At sites of vascular injury and during angiogenesis, however, endothelial cells can become migratory and proliferative. To address whether there is an alteration in the subcellular localisation of Pez when endothelial cells are induced from a quiescent state to migrate and proliferate, we used an in vitro 'wounding' assay to induce a small population of endothelial cells at the 'wound' edge to migrate and proliferate. Pez was indeed nuclear in the cells at the 'wound' edge that had migrated into the 'wound' but remained cytoplasmic in the confluent cells distal from the edge. TGFβ, which inhibits cell proliferation but not migration, inhibited the translocation of Pez to the nucleus of cells at the 'wound' edge, further strengthening the argument that Pez may play a nuclear role during cell proliferation. Although Pez is expressed in many different cell types to varying levels, its abundance in HUVEC suggests that it may be an important regulator of nuclear events in endothelial cells under situations where endothelial cell proliferation occurs.

Studies on the murine homologue of Pez, PTP36, showed that the epitope-tagged, ectopically expressed protein used in the studies was cytoplasmic in both HeLa cells and fibroblasts, with no report of its presence in the nucleus (Ogata et al., 1999a,b). We have observed, however, that Flag-epitope tagged Pez ectopically expressed in both HUVEC (Fig. 3A) and 293 cells (Fig. 3B) was predominantly present in the nucleus in cells at low density. The reason for the difference in subcellular localisation between Pez and PTP36 is not immediately obvious and requires further investigation. One reason may be that Pez and PTP36 are isoforms rather than orthologues. A precedence for differential localisation of isoforms of a member of the FERM family of proteins exists: Band 4.1 protein, commonly known for its role in cross-linking the plasma membrane of red blood cells to the spectrin cytoskeleton, also has a nuclear isoform (Krauss et al., 1997).

The presence of Pez in the nucleus of almost all of the cells in a low density asynchronous culture suggests that Pez is nuclear in proliferating cells through all the stages of the cell cycle. This remains to be confirmed by following the subcellular localisation of Pez through the stages of the cell cycle in synchronised cells. The data presented, taken together, suggest that Pez translocates into the nucleus at the G_0/G_1 transition and moves out of the nucleus when cells enter G_0. Cell proliferation is a complex, highly coordinated process that requires regulation of both the structural and biochemical changes that occur. Since changes occur in the activity of many nuclear proteins, understanding the function of Pez in the nucleus during cell proliferation will require identification of its nuclear substrates.

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