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src-related protein tyrosine kinases are physically associated with the surface antigen CD36 in human dermal microvascular endothelial cells**

Helen A. Bull[a], Paul M. Brickell[b], Pauline M. Dowd[a,*]

[a]*Division of Dermatology, Department of Medicine University College London Medical School, The Rayne Institute, 5 University Street, London WC1E 6JJ, UK*

[b]*Medical Molecular Biology Unit, Department of Molecular Pathology, University College London Medical School, London, UK*

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Abstract

src-related cytoplasmic PTKs are physically and functionally associated with cell surface receptors and are involved in signal transduction. In this paper we report the identification of *src*-related proteins $p59^{fyn}$, $pp60^{c\text{-}src}$ and $p62^{yes}$ in human microvascular endothelial cells cultured from normal human skin and their physical association with the thrombospondin receptor CD36. Such an association represents a potential signalling pathway by which thrombospondin may regulate angiogenesis.

Key words: Microvascular endothelium; CD36; *src*-related protein tyrosine kinase

1. Introduction

The microvascular endothelium is normally quiescent but is nevertheless able to undergo rapid differentiation and proliferation during angiogenesis and wound healing. The signalling pathways responsible for endothelial cell activation are not clear. However studies in other cell types have indicated that cell phenotype is influenced by its extra-cellular matrix (ECM) [1] and that cell surface ECM protein receptors are involved in relaying signals between the ECM and the cell [2]. CD36 is an integral membrane protein that is thought to have a role in signal transduction [3]. It is an 88 kDa antigen originally identified on platelets (and named GPIV) and subsequently shown to be expressed on a variety of cells including microvascular endothelium [4]. However, expression of CD36 on endothelium is not universal, even within the same vascular bed [5]. CD36 is a receptor for thrombospondin [6] and its expression on the microvascular endothelial cell surface may well be influenced by the presence of thrombospondin, as has been suggested by in vitro experiments using embryonic fibroblasts [7].

Antibody cross-linking of the CD36 receptor activates both platelets [8] and monocytes [9] and, in platelets, immunoprecipitation with anti-CD36 antibodies co-precipitates the *src*-related protein tyrosine kinases (PTKs) (EC 2.7.1.112) $p56/p58^{lyn}$, $p59^{fyn}$ and $p62^{c\text{-}yes}$ [10]. Phosphorylation of proteins on tyrosine residues by PTKs is an important mechanism regulating cellular responses to exogenous stimuli and there is increasing evidence that the *src*-related cytoplasmic PTKs are physically and functionally associated both with receptor PTKs and with cell surface membrane receptors that do not possess PTK activity [11]. For example, in T- cells, $p56^{lck}$ is associated with the CD4 and CD8α proteins [12] and $p59^{fyn}$ is associated with the T-cell antigen receptor complex [13].

src-related proteins have been identified in large vessel endothelium. The *fyn* gene has been cloned from a cDNA library prepared from human umbilical vein endothelial cells (HUVEC) [14] and c-*src* mRNA and protein have been identified in bovine aortic endothelial cells [15]. However, as already indicated, blood vessel endothelium is not homogeneous.

We have therefore sought evidence for the presence of *src*-related PTKs in human dermal microvascular endothelial cells (HDMEC). By analogy with platelets it may be proposed that CD36 is linked to cytoplasmic *src*-related PTKs in microvascular endothelium. Such an association would be a potentially important mechanism for regulating endothelial cell responses to changes in its ECM at sites of injury. We have therefore sought evidence for a physical association between CD36 and members of the *src*-related PTK family in HDMEC.

*Corresponding author. Fax: (44) (71) 380 9837.

Abbreviations: ECM, extracellular matrix; PTK, protein tyrosine kinase, HUVEC, human umbilical vein endothelial cells; HDMEC, human dermal microvascular endothelial cells; PHS, pooled human serum; Iscove's DMEM, Iscove's modification of Dulbecco's modified Eagle's medium; DTT, dithiotreitol; PMSF, phenyl methyl sulphonyl fluoride; LB, lysis buffer; BSA, bovine serum albumin; NRS, normal rabbit serum; SDS, sodium dodecyl sulphate; RIPA, radioimmunoprecipitation assay; TBS, Tris-buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; BCIP, bromo-4-chloro-3-indoyl phosphate; NBT, nitroblue tetrazolium;

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2. Materials and methods

2.1. Human dermal microvascular endothelial cell cultures

HDMEC were isolated from neonatal foreskin obtained after routine circumcision and established in culture as previously described [16]. The HDMEC were cultured on fibronectin-coated culture wells in Iscove's modification of Dulbecco's modified Eagles culture medium (Iscove's DMEM; Gibco, UK) containing 1-methyl-3-isobutylxanthine, dibutyryl cyclic-AMP and 30% (v/v) pooled human serum (PHS).

Confluent monolayers of cells between passages 2 and 4 were used for these experiments.

2.2. Immunoprecipitation

HDMEC were washed twice with serum-free culture medium containing 100 μM sodium orthovanadate (Na_3VO_4). Lysates were prepared by adding 0.5 ml lysis buffer (LB; 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) Nonidet P40, 1 mM EDTA, 1 mM dithiothreitol (DTT), 100 μM Na_3VO_4, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 20 μM leupeptin) to each 25 cm^2 culture flask. Lysates were then transferred to microfuge tubes, vortexed, incubated on ice for 30 min and centrifuged (10,000 × *g*, 10 min) to remove nuclei and cell debris. The lysates were divided into two aliquots and stored at −20°C. Protein concentrations were estimated using the Bradford assay with bovine serum albumin (BSA) prepared in 1/10 dilution of LB as the standard [17].

Immunoprecipitations were carried out using the method described by Courtneidge [18,19]. Fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem, UK) was first pre-incubated with normal rabbit serum (NRS). 500 μl of 10% (w/v) Pansorbin was incubated with 50 μl NRS on ice for 30 min, washed three times with immunoprecipitation buffer (RIPA; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1 mM EDTA, 1 mM DTT, 100 μM Na_3VO_4, 1 mM PMSF, 20 μM leupeptin) and resuspended in LB at a concentration of 5% (w/v). Cell lysates were then pre-adsorbed with NRS-treated Pansorbin. Lysate containing 100 μg of protein was added to 1/10 volume of NRS-treated Pansorbin and incubated on ice for 30 min. The Pansorbin was pelleted by centrifugation (10,000 × *g*, 5 min) and the supernatant transferred to a clean microfuge tube. 0.5 μg antibody protein was added to the pre-adsorbed lysate, mixed and incubated at 4°C overnight (18 h). The samples were then centrifuged (10,000 × *g*, 5 min) and the supernatant transferred to a clean microfuge tube. Fresh Pansorbin was prepared by washing three times in RIPA buffer and 5 μl of washed Pansorbin per μl antiserum were added to each tube. After 45 min incubation on ice, the Pansorbin-bound immune complexes were pelleted by centrifugation (10,000 × *g*, 10 min) and washed three times with LB buffer and once with Tris buffered saline (TBS; 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). The immune complexes were then eluted in 25 μl single-strength SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1% (w/v) SDS, 4% (w/v) sucrose, 4 mM EDTA, 90 mM Tris-HCl, pH 6.8).

2.3. Electrophoresis and immunoblotting

Cell lysates diluted in an equal volume of double-strength SDS-PAGE sample buffer and immune complexes suspended in single-strength SDS-PAGE sample buffer were heated at 100°C for 3 min and the insoluble material precipitated by centrifugation (10,000 × *g*, 5 min). Proteins were size-fractionated by SDS-PAGE using 3% (w/v) polyacrylamide in the stacking gel and 10% (w/v) polyacrylamide in the resolving gel and then electroblotted onto 0.45 μm nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) [20]. Residual protein binding sites were blocked by soaking the blots for 2 h in Tris/Tween buffer (150 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20) containing 3% (w/v) BSA. After washing three times for 5 min in Tris/Tween buffer the blots were incubated overnight at 4°C with different antibodies diluted in Tris/Tween buffer containing 3% (w/v) BSA. The next morning the blots were washed three times for 5 min with Tris/Tween buffer and then incubated with alkaline phosphatase conjugated goat anti-mouse or goat anti-rabbit IgG (Southern Biotechnologies, Birmingham, Alabama) diluted in Tris/Tween buffer with 3% (w/v) BSA for 2–3 h at room temperature. The blots were then washed twice for 5 min with Tris/Tween buffer and developed with bromo-4-chloro-3-indoyl phosphate/Nitro blue tetrazolium (330 μg/ml BCIP, 150 μg/ml NBT prepared in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 10–30 min at room temperature. The colour development was quenched by washing the blots under running water.

2.4. Antibodies and reagents

The antibodies used for the immune precipitations and Western blot analysis included an affinity purified polyclonal rabbit antiserum that recognizes a homologous region of the C-terminal of $p60^{c\text{-}src}$, $p59^{fyn}$ and $p62^{c\text{-}yes}$ (Santa Cruz Biotechnology, Santa Cruz, California), affinity purified polyclonal and monoclonal antibodies directed against the platelet receptor GP88 (CD36, GPIV)(generous gifts from Dr Michael Berndt, Baker Research Institute, Melbourne, Australia) and polyclonal antiserum (whole serum) directed against $p59^{fyn}$ (a generous gift from Professor Mike Waterfield, Ludwig Institute, UCL Branch).

3. Results

3.1. src-*related PTKs in human dermal microvascular endothelial cells*

In Western blots of protein extracted from HDMEC, the c-*src/fyn/yes* antiserum detected two strong bands with molecular weights of approximately 60 kDa (Fig. 1A) and the *fyn* antiserum detected a broad band with a molecular weight of approximately 60 kDa (Fig. 1B). These results demonstrate that microvascular endothelial cells express the *fyn* PTK and possibly the c-*src* and c-*yes* PTKs.

3.2. *CD36 in human dermal microvascular endothelial cells*

We next investigated the expression of immunoreactive CD36 in HDMEC. Anti-CD36 antibodies detected a broad band with an approximate molecular weight of between 80 kDa and 90 kDa in Western blots of protein

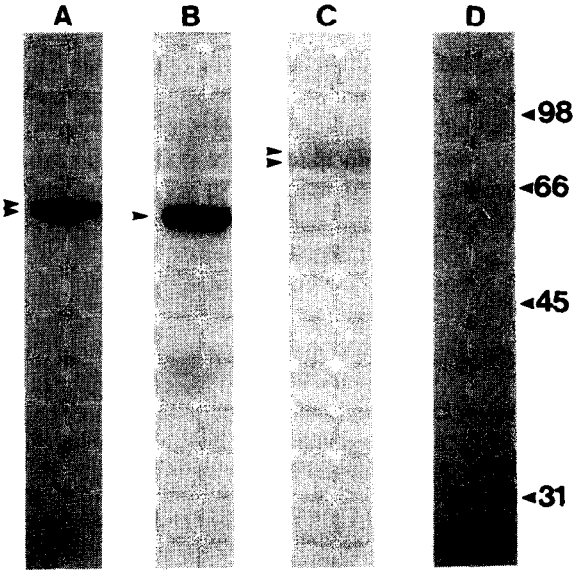

Fig. 1. Western blots of whole cell extracts of unstimulated HDMEC were incubated with anti-c-*src/fyn/yes*, antiserum (A), anti-*fyn* antiserum (B), anti-CD36 antiserum (C) followed by alkaline phosphatase conjugated second layer antiserum, or with alkaline phosphatase conjugated second layer antiserum alone (D). Molecular weight markers are indicated on the right-hand side of the blot.

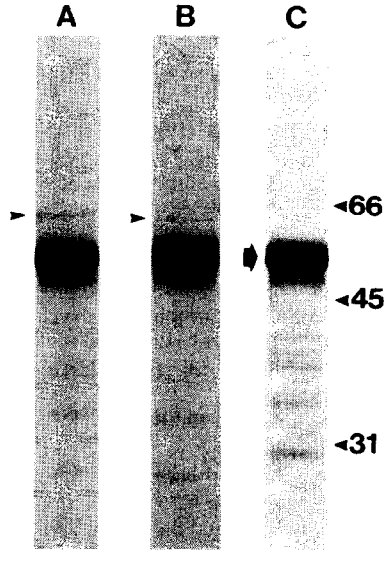

Fig. 2. Western blots of immune complexes precipitated with anti-CD36 antiserum were incubated with anti-c-*src/fyn/yes* antiserum (A) or anti-*fyn* antiserum (B) followed by alkaline phosphatase conjugated second antiserum layer, or with alkaline phosphatase conjugated second antiserum layer alone (C). Molecular weight markers are indicated on the right-hand side of the blot.

extracted from HDMEC (Fig. 1C). Two bands with molecular weights of approximately 80 kDa and 88 kDa were visible within the broad band.

3.3. *Association of CD36 with* src*-related proteins*

We next investigated whether *src*-related PTKs were physically associated with CD36 in HDMEC.

Protein extracts of HDMEC were immunoprecipitated with the polyclonal anti-CD36 antiserum and the resultant Western blots were incubated with the anti-c-*src/fyn/yes* antiserum or the anti-*fyn* antiserum (Fig. 2). Both the anti-c-*src/fyn/yes* antiserum (Fig. 2A) and the anti-*fyn* antiserum (Fig. 2B) detected a band with a molecular weight of approximately 60 kDa in CD36 immunoprecipitates. The broad intensely staining band of approximately 50–55 kDa results from binding of the second antibody to the immunoglobulin heavy chain in the Pansorbin immunoprecipitates (Fig. 2C).

Finally, protein extracts of HDMEC were immunoprecipitated with the anti-c-*src/fyn/yes* antiserum or the anti-*fyn* antiserum and the resultant immunoblots were incubated with the anti-CD36 antiserum (Fig. 3). This antiserum detected two bands with molecular weights of approximately 80 kDa and 88 kDa (Fig. 3A,C). A band with a molecular weight of approximately 70 kDa was also detected. This band was only a minor component in the c-*src/fyn/yes* immunoprecipitates but in the *fyn* immunoprecipitates, the bands with molecular weights of approximately 70 kDa and 80 kDa were stained with equal intensity whilst the 88 kDa band was hardly visible. As before, the second antibody also bound to the immunoglobulin heavy chain in the immunoprecipitates (Fig. 3B).

4. Discussion

These data indicate that *src*-related PTKs are present in human microvascular endothelium and that the membrane receptor CD36 is physically associated with $p59^{fyn}$ and possibly with $pp60^{c\text{-}src}$ and $p62^{c\text{-}yes}$.

The antibody raised against human platelet CD36 (GP88, GPIV) detected a broad band with molecular weight ranging from approximately 80–90 kDa in Western blots of whole cell extracts of HDMEC. Within the broad band it was possible to detect two bands with molecular weights of approximately 80 kDa and 88 kDa. These two bands were clearly defined on Western blots of immune complexes precipitated with the anti-c-*src/fyn/yes* antiserum and incubated with the anti-CD36 antiserum and an additional feint band with a molecular weight of approximately 70 kDa was also detected. In Western blots of anti-*fyn* immune complexes incubated with anti CD36 antiserum, the 70 kDa and 80 kDa bands were stained with equal intensity. CD36 is heavily glycosylated with both *N*- and *O*-linked carbohydrate moieties and the different bands present on Western blots may represent proteins with different degrees of glycosylation.

It is not possible to establish the nature of the interaction between CD36 and the *src*-related PTK from these data. The different staining intensities of the three CD36 bands in Western blots of anti-c-*src/fyn/yes* immune complexes and anti-*fyn* immune complexes incubated

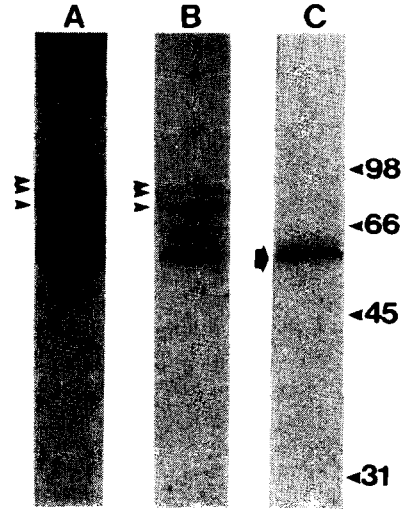

Fig. 3. Western blots of immune complexes precipitated with anti-c-*src/fyn/yes* antiserum (A,B) or anti-*fyn* antiserum (C) were incubated with the anti-CD36 antiserum followed by alkaline phosphatase conjugated second antiserum layer (A,C), or with alkaline phosphatase conjugated second antiserum layer alone (B). Molecular weight markers are indicated on the right-hand side of the blot.

with anti-CD36 antiserum may be due steric hinderance since the anti-c-*src*/*fyn*/*yes* antiserum is directed against the COOH-terminal common to all three proteins whilst the anti-*fyn* antiserum is directed against the unique NH_2-terminal. Alternatively, the $p59^{fyn}$ binding site on CD36 may be different to those of $pp60^{c\text{-}src}$ and $p62^{yes}$.

The function of $p59^{fyn}$ and *src*-related PTK in HDMEC is not known. Phosphotyrosine has been localised at focal contacts and intercellular junctions in a variety of cell types [21] and in early passage NIH 3T3 cells transfected with normal c-*src*, *fyn* or c-*fgr* cDNAs there was increased tyrosine phosphorylation of the focal adhesion kinase $p125^{fak}$ [22]. The increased tyrosine phosphorylation of $p125^{fak}$ was most evident in cells transfected with normal *fyn* cDNA suggesting that *src*-related PTK and $p59^{fyn}$ in particular are localized in focal adhesion plaques at least in 3T3 cells. Overexpression of normal $p59^{fyn}$ in NIH 3T3 cells contributes to the formation of non-contact inhibited clusters (transformed foci) [23] which is preceded by disruption of the focal adhesion plaques. These data indicate that $p59^{fyn}$ may play a role in the modulation of cell adhesion.

Our data suggest the possibility that $p59^{fyn}$ is involved in mediating changes in microvascular endothelial cell adhesion in response to binding of thrombospondin to CD36 on the cell surface. This represents a potentially important signal transduction pathway in angiogenesis since loss of contact between the endothelial cell and its ECM is an initial event in the generation of new blood vessels.

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