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The association between CD36 and Lyn protein tyrosine kinase is mediated by lipid

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Abstract

CD36 is a transmembrane glycoprotein receptor that engages in signal transduction implicated in important physiological and pathophysiological events. CD36 in platelets has been shown physically and functionally to associate with members of the Src family of protein tyrosine kinases, Fyn, Lyn, and Yes, but the nature of this important association has never been rigorously examined. Here, we show that CD36 does not associate with Lyn through a protein-mediated interaction. In COS cells transfected with both CD36 and Lyn these molecules did not co-precipitate, suggesting a requirement for an intermediary molecule absent from the COS cells. Yeast two-hybrid analysis confirmed that the carboxylterminal cytoplasmic tail of CD36 did not bind Lyn directly, and no Lyn binding protein bound to CD36 in a cDNA library screen. Conversely, when the CD36–Lyn association seen in platelets was analysed by biophysical parameters, dissociation occurred at 37 °C and also by solubilisation in octylglucoside, indicative of a lipid-mediated association. Since both CD36 and Lyn are enriched in Triton X-100-insoluble rafts at the plasma membrane, these findings point to the importance of raft-associated lipids in CD36-mediated signal transduction.

Keywords: CD36; Diabetic nephropathy; Glycosynapse; Lyn protein tyrosine kinase; Plasma membrane rafts; Platelets; Signal transduction; Yeast two-hybrid assay

Few proteins are as functionally versatile as CD36, the inaugural member of an evolutionary conserved small gene family (reviewed in [1]). CD36 is classified as a Class B scavenger receptor and in this capacity it has been shown to bind advanced glycation end products (AGE) and anionic phospholipids. It also functions as a receptor for erythrocytes parasitized by *Plasmodium falciparum* (IRBC), and IRBC binding to monocytes induces the production of reactive oxygen species that may play a role in the pathophysiology of *falciparum* malaria [2]. CD36 also engages in the binding and uptake of oxidised low-density lipoprotein (OxLDL), and this receptor function accounts for some 50% of OxLDL uptake by human and mouse macrophages [3,4] considered to constitute an early event in the development of atherosclerotic lesions.

Signalling through CD36 binding to apolipoproteins present in the atheroma may further exacerbate the developing plaque by stimulating the production of inflammatory mediators [5]. CD36-mediated signalling is also strongly implicated in the regulation of angiogenesis mediated by thrombospondin-1 (TSP-1), a large secreted glycoprotein identified as a ligand of CD36. TSP-1 is a natural inhibitor of angiogenesis that limits vessel density, and studies *in vitro* and *in vivo* have established that this inhibitory function of TSP-1 requires binding to CD36 [6,7]. Following TSP-1 binding it is considered that CD36 signals to induce apoptosis of activated microvascular endothelial cells [7].

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However, the most striking phenotype of CD36 null mice is defects in lipid metabolism manifested by increased levels of plasma cholesterol, triacylglycerol, and fatty acids, and fasting hypoglycemia [8], and CD36 expression and function may be modulated in obesity and type 2 diabetes [9,10]. Recently, it was shown that under the influence of glucose—which promotes CD36 translation [11]—CD36 is expressed by human proximal tubule epithelial cells (PTEC) [12]. Such CD36 expression was found to be necessary and sufficient to induce PTEC apoptosis upon binding its ligands, AGE-albumin and free fatty acid [12]. The findings suggest that such CD36-mediated apoptosis of PTEC may trigger tubular epithelial degeneration and interstitial fibrosis, resulting in diabetic nephropathy, known to be a common complication of types 1 and 2 diabetes and the most common cause of kidney failure [12].

The molecular mechanism by which CD36-mediated signalling induced PTEC apoptosis was shown to involve the Src family protein tyrosine kinase (SFK) Fyn and possibly others leading to activation of p38 MAPK and increased activity of effector caspase 3 [12]. A similar pathway was shown for the CD36-induced apoptosis of endothelial cells caused by TSP-1 [7], and anti-CD36 monoclonal antibody (mAb) crosslinking studies with monocytes demonstrated direct initiation of SFK activation and downstream ERK 1/2 and p38 MAPK activation [13]. MAb to CD36 also induce platelet aggregation [14] and a possible biochemical mechanism for the participation of CD36 in signalling was suggested by the work of Huang et al. [15], who reported that immunoprecipitates from resting platelets lysed in Triton X-100 co-precipitated Fyn, Lyn, and Yes SFKs. Similar kinase profiles could be found associated with CD36 isolated from melanoma cells, erythroleukemia cells, and endothelial cells [15,16]. The nature of the association between CD36 and SFK is not known. Based upon a motif contained within the short C-terminal cytoplasmic domain of CD36 it has been suggested that there may be a direct protein–protein interaction with SFK [17], but this has not been tested experimentally. Here, we demonstrate the absence of such a direct protein interaction, and the biophysical properties of the co-precipitated Lyn and CD36 from platelets suggest that the association is mediated by lipid. Since CD36 and active Lyn are highly enriched within plasma membrane microdomains/rafts in platelets [18,19] it is suggested that CD36-mediated signalling is initiated by lipid associations within these domains.

Materials and methods

Antibodies. Monoclonal antibody (mAb), VM58, and polyclonal (pAb) against CD36 were as previously described [20]. FAK pAb [21] was a gift of Dr. S. Hanks. Nonimmune mouse and rabbit immunoglobulin (Ig), pAb to Lyn, and pAb to Lck were purchased from IMVS (Adelaide, Australia), Santa Cruz Biotechnology (Santa Cruz, CA), and Upstate Biotechnology (Lake Placid, NY), respectively.

Cell transfection. cDNA encoding human CD36, human Lyn (a gift of Dr. Y. Yaminashi, The University of Tokyo, Japan), and murine FAK [21] was subcloned to pEF.BOS [22]. COS-7 cells were transiently transfected by electroporation as previously described [23]. Co-expression in individual cells was validated using immunofluorescent microscopy with >50% expressing transfected protein.

Immunoprecipitation and in vitro kinase (IVK) assay. Isolated human platelets [18,19] or transfected COS-7 cells were lysed in RIPA [23] or 1% Triton X-100 buffer and precleared with species-matched Ig before immunoprecipitation with specific antibodies [20,23]. IVK assays were carried out as described previously [19] and samples resolved by SDS–PAGE, transferred to nitrocellulose (Sartorius, Germany), and the results evaluated by autoradiography followed by ECL-immunoblotting [20,23].

Yeast two-hybrid assay. Yeast two-hybrid assay was performed in *Saccharomyces cerevisiae* HFc7 strain by colony lift or quantitative liquid β-galactosidase assay as previously described [24]. The cytoplasmic domain of CD36 (*Hae*III fragment) was introduced into the *Sma*I site of the GAL4 DNA binding domain (BD) vector, pGBT9*lacZ*. Full-length Lyn was amplified by PCR using oligonucleotides 5′-CCCGAAGA ATTCACGCGAGCGG-3′ and 5′-TGAGGGATCCACCCCTGCCAA ATGGACGGG-3′, and subcloned into both pGBT9*lacZ* and the GAL4 activation domain (AD) vector pGAD424 (Clontech, Palo Alto, CA) using the introduced restriction sites. Similarly, full-length FAK was amplified using oligonucleotides 5′-TTCTGAGTCGACATGACAGA-TACC-3′ and 5′-GGTGGAATGCTGCAGAAGATCTCCC-3′, and subcloned into pGAD424. Control vectors used were pGBT9-CD4 (carboxylterminal 117 amino acids of human CD4), pGAD424*lacZ*-Lck (aminoterminal 213 amino acids of murine Lck), pVA3 (murine p53 BD vector), and pLAM5′ (amino acids 66–230 of human lamin-C in BD vector). A library screen using pGBT9*lacZ*-CD36 was conducted against a human leukemia library produced from unstimulated Jurkat T cells in the pGAD10 vector (Clontech).

Isolation of glycolipid-enriched microdomains (GEMs). Isolations were performed using cold 1% Triton X-100 lysis buffer as described previously [18,19,23]. In some experiments GEMs were solubilised with 1% octylglucoside (octyl-β-D-glucopyranoside; Boehringer–Mannheim, Germany).

Results and discussion

To investigate the nature of the CD36–SFK association we chose a transfection model using Lyn as a SFK representative together with CD36 to enable subsequent mutagenic analysis. For this, a control vector and the cDNAs for both CD36 and Lyn (singularly and in combination) were transfected into COS-7 cells. Following expression, cell lysates were immunoprecipitated with CD36, Lyn or control antibodies and the precipitates subjected to IVK assays. It was found that none of the antibodies precipitated phosphoproteins from COS-7 cells transfected with vector alone or with CD36 alone. Immunoprecipitations of Lyn from cells transfected with either Lyn alone, or with CD36 and Lyn, precipitated a phosphoprotein band corresponding to the M_r of $p56^{lyn}$. However, this band was absent from the CD36 immunoprecipitation from cells transfected with CD36 and Lyn (data not shown). Immunoblotting of the same nitrocellulose membrane confirmed that CD36 was present in the samples immunoprecipitated with anti-CD36 from cells transfected with CD36. Conversely no CD36 was observed to co-precipitate with Lyn from the cells transfected with CD36 and Lyn. Therefore, under these experimental conditions, CD36 did not associate with Lyn kinase, perhaps indicating that intermediary protein(s) absent from this system are required to permit their association.

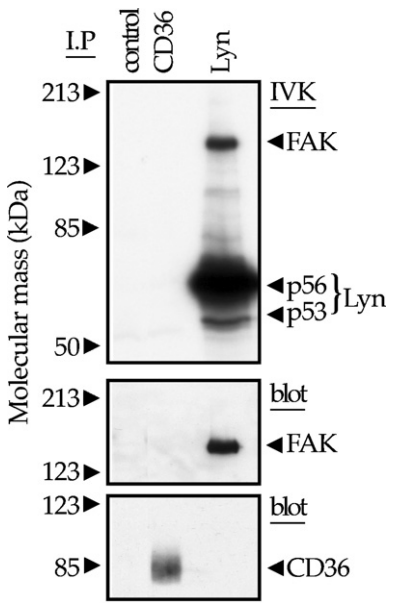

Fig. 1. CD36 does not directly associate with Lyn in transfected COS-7 cells. Cells transfected with CD36, Lyn, and FAK were lysed in RIPA and immunoprecipitated with CD36 mAb, Lyn pAb, and control Ig. Immunocomplexes were subjected to IVK assay and labelled phosphoproteins detected by autoradiography (top). The same membrane was then immunoblotted for FAK (middle) and CD36 (bottom) using pAbs.

Peptide affinity chromatography using a peptide corresponding to the carboxylterminal tail of CD36 identified focal adhesion kinase (FAK) as a putative CD36-binding protein (Drs. R. Andrews and M.C. Berndt, personal communication), and FAK is also known to directly bind Lyn [25]. To determine if CD36 associated as a complex with Lyn and FAK, COS-7 cells were co-transfected with CD36, Lyn, and FAK. The cell lysate was immunoprecipitated with control, CD36 and Lyn antibodies, and an IVK assay performed (Fig. 1). The control immunoprecipitant did not reveal any specific phosphoproteins, but the immunoprecipitation for Lyn precipitated three strong bands representative of transfected Lyn (56 kDa), the endogenous $p53^{Lyn}$ isoform, and FAK (125 kDa). Notably, no trace of FAK or Lyn could be observed in the CD36 immunoprecipitation in either the IVK assay or FAK immunoblot (Fig. 1). These results argue against the notion that CD36 can associate with Lyn through a complex with FAK and, in confirming the association between FAK and Lyn, a positive control is also provided for our immunoprecipitation results.

Next, we turned to the yeast two-hybrid system since this is a sensitive measure of protein–protein interactions. To test for a direct binding association between CD36 and Lyn in this system we inserted the cytoplasmic tail and partial transmembrane domain of CD36 in the 'bait' construct pGBT9*lacZ* (Fig. 2A) and the complete coding sequence of Lyn in the pGAD424 vector. That no association was detected is shown, together with controls, in Fig. 2B and Table 1. The cytoplasmic tail of CD36 contains the sequence CACRSKTIK, conforming to a consensus sequence (CXCX5K) known to form a tetrahedral metal complex with two cysteine residues (CXXC) within the aminoterminus of Lck kinase. Based on this, Shattil and Brugge [17] postulated a similar association between CD36 and SFKs. Clearly this does not occur with Lyn in the yeast two-hybrid system, and neither Lyn, Fyn nor Yes contains the CXXC motif. However, when we tested for CD36 binding to Lck in the yeast two-hybrid system, a positive reaction was obtained (Fig. 2B). Further, conformity with the prototypic interaction between CD4/CD8 and Lck was demonstrated by mutating the two cysteines within the cytoplasmic tail of CD36 to serines, when it was found that these mutations abrogated the interaction (SAS; Fig. 2B). This result substantiates the notion of Shattil and Brugge [17], but whether this binding association between CD36 and Lck identified in the yeast two-hybrid system is physiological requires more analysis; in

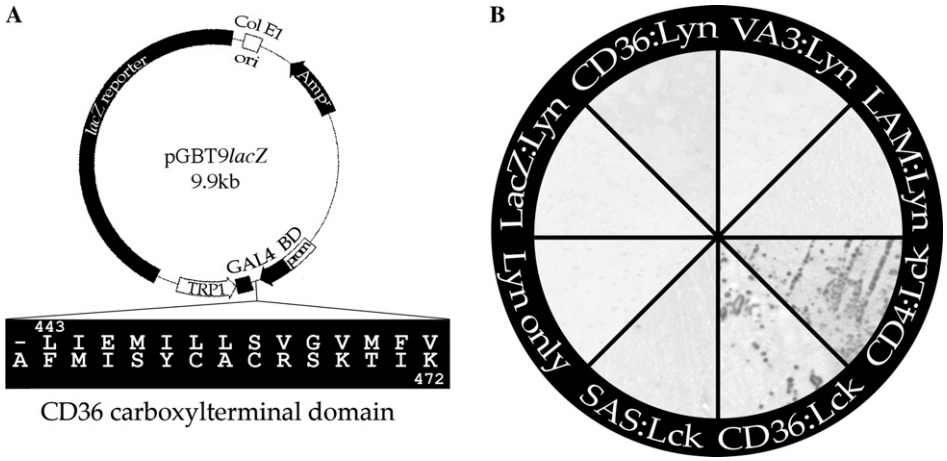

Fig. 2. Yeast two-hybrid analysis indicates CD36 does not interact with Lyn. (A) Schematic showing the carboxylterminal region of CD36 (Leu^{443}-Lys^{472}) cloned into the pGBT9*lacZ* vector to generate a GAL4 BD fusion. (B) Colony lift assays were performed with the indicated AD:BD plasmid combinations.

Table 1
CD36 interacts with Lck but not with Lyn or FAK in the yeast two-hybrid assay

Interaction (BD–AD)	Colony lift assay	Quantitative assay
CD4–Lck	Blue	1
CD36–Lck	Blue	0.33 ± 0.04
SAS–Lck	White	0.02 ± 0.02
pVA3–Lck	White	0.01 ± 0.01
CD36–Lyn	White	0.01 ± 0.01
CD4–Lyn	White	0.01 ± 0.01
pVA3–Lyn	White	0.02 ± 0.02
CD36–FAK	White	0.02 ± 0.01

Yeast were transformed with the indicated plasmids before performing colony lift and quantitative assays [24]. Values (means ± the standard error of the mean) were normalized against the CD4–Lck interaction.

preliminary experiments with cultured human monocytes we failed to demonstrate co-precipitation of the two molecules (not shown). We also found that FAK bound to Lyn in the yeast two-hybrid assay (not shown) thus substantiating reported co-precipitation results [25]; however, in this assay FAK did not bind to CD36 (Table 1).

Together with the immunoprecipitation data from transfected COS cells these results show that CD36 does not bind directly to Lyn, and also that FAK does not function to bridge these molecules. Therefore in an attempt to identify a possible intermediary we used the CD36 "bait" construct (Fig. 2A) to screen a cDNA library in the pGAD424 "prey" vector (see Materials and methods). The entire library of 2×10^6 independent clones was screened, and three clones were isolated and confirmed as true binding partners when tested against a range of controls. Sequencing identified each of the CD36-binding proteins and these will be reported elsewhere: however, none of these known proteins is able to bind Lyn and therefore cannot act as an intermediary protein for the Lyn–CD36 association.

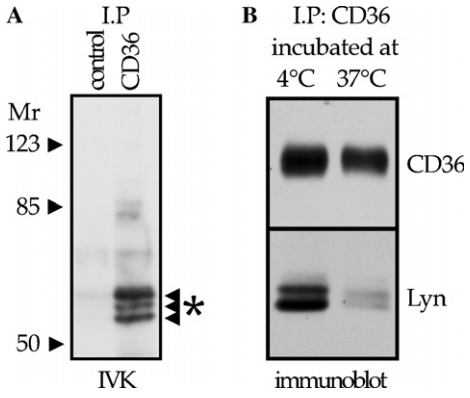

Fig. 3. The association of Lyn SFK with CD36 in platelets is temperature sensitive. (A) Human platelets were lysed with RIPA and immunoprecipitated (IP) with control Ig or CD36 mAb and the immunocomplexes subjected to IVK assay. Asterisk denotes three specific phosphoproteins detected by autoradiography. (B) Platelets were lysed in cold Triton X-100 buffer and CD36 immunoprecipitated as for (A). Precipitated complexes were held at 4 °C or incubated at 37 °C for 15 min before rewashing and immunoblotting for CD36 (top) and associated Lyn kinase (bottom).

The initial finding of the association between CD36 and Lyn (and also Fyn and Yes) was obtained from platelets [15], and this report has been cited over 150 times [ISI, September 2006], therefore we re-examined the association of these anucleate cells with a view to identifying an intermediary molecule using a proteomic approach. First we confirmed that CD36 co-precipitated Lyn from lysates of washed human platelets. The results of the IVK reaction demonstrated that CD36 was associated with three phosphoproteins that ranged in M_r from 53 to 62 kDa (Fig. 3A). Based upon the results presented by Huang et al. [15], these sizes are consistent with being $p53^{Lyn}$ and $p56^{Lyn}$ isoforms of Lyn and the upper band at ~62 kDa may represent unresolved $p60^{Fyn}$ and $p62^{Yes}$ SFK. The identity of the Lyn bands was confirmed by immunoblotting (data not shown). In platelets, both CD36 and active Lyn are highly enriched within membrane microdomains, or rafts, whether these are isolated as Triton X-100 detergent-insoluble material (GEMs) or by detergent-free methodology [18,19]. In order to isolate and identify CD36-associated molecules within rafts we isolated the cold Triton X-100-insoluble band obtained by centrifugation of the lysates through sucrose gradients. Attempts to immunoprecipitate CD36 from this fraction were unsuccessful. Immunoprecipitation of Lyn from this fraction, however, also was problematic since we obtained particulate material that appeared as nonspecific aggregates (not shown). Therefore to obviate this problem, the isolated GEM fraction was solubilised in 1% octylglucoside prior to immunoprecipitation and blotting. Under these conditions both CD36 and Lyn yielded satisfactory immunoprecipitates; however, unexpectedly, the molecules did not co-precipitate—indicating that they no longer formed an association in this detergent (not shown).

These results, apparent insolubility in ionic detergents such as Triton X-100, together with solubility in the same buffer supplemented with octylglucoside are highly reminiscent of GPI-anchored proteins [26]: in particular, solubilisation of GPI-anchored proteins in octylglucoside has been shown to remove their associated SFK [27], whereas the protein–protein mediated association between CD4 and Lck remains intact in this detergent [27]. The association between GPI-anchored proteins and SFK is considered to occur in membrane rafts where the predominantly unsaturated fatty acid residues in the GPI moiety penetrate into the inner cytoplasmic leaflet to directly interact with aliphatic chains of the SFK. This association is strictly dependent upon the raft lipids retaining their lipid ordered phase. Biochemically this correlates directly with raft protein solubility in detergent such that the association between GPI-anchored proteins and phosphoproteins correlates with the insolubility of the protein [28], and the same effect occurs with increasing the temperature when raft proteins become soluble in Triton X-100 at 37 °C [29]. Therefore, we tested the solubility of the CD36–Lyn

association at 37 °C. Isolated human platelets were lysed in 1% Triton X-100 and immunoprecipitated for CD36. Equal portions of the precipitated complexes were held at either 4 or 37 °C, rewashed, and subjected to immunoblotting for CD36 and Lyn. It was found that warming the complex caused dissociation between CD36 and Lyn (Fig. 3B); in time-course experiments dissociation began after 5 min and was complete by 60 min (data not shown).

Taken together, these data strongly indicate that the association between CD36 and SFK is mediated by a lipid interaction, likely lipid–lipid. This is the case with the GPI-anchored proteins but is very unusual for a transmembrane protein. However, CD36 has been shown to be palmitoylated [18,30], and there are precedents that such acylation of a transmembrane glycoprotein can function in signalling. Thus, palmitoylation of the integrin β-subunit of α6β4 has been shown to be essential for its association with SFK within rafts [31], and it is also required for the formation of the tetraspannin web at that site [32]. Further, the palmitoylation of CD36 has been shown to interact with Src previously [33] when it was found that a number of dually acylated proteins, including CD36, could interfere with the Src-mediated phosphorylation of caveolin in a process dependent upon the lipid modification.

Our data also point to the likelihood that the CD36–SFK association occurs within lipid rafts as represented by the biochemical GEM fraction. In platelets, both CD36 [18,34] and active Lyn [19] are enriched within this fraction with important physiological and practical implications and considerations. Finally another interesting question arises from this work. CD36 has been reported to co-precipitate SFK from melanoma cells and endothelial cells as well as platelets [15,16], yet in repeated attempts (with and without iodoacetamide in the lysis buffer, not shown) we were unable to co-precipitate transfected CD36 and Lyn from COS cells when we could do so readily from platelets. This failed co-precipitation was not a consequence of either CD36 or Lyn being unable to localise to membrane rafts in the transfected COS cells since we have shown previously that both molecules are enriched with the biochemically isolated GEM from these cells [19]. In this regard it is of interest that Gousset et al. [34] found that whereas CD36 selectively partitioned within the GEMs isolated from platelets, the GPI-linked protein, CD55, did not do so; a result that differs from studies with COS cell transfectants where it was shown that all GPI-linked proteins tested localised to such GEMs [35]. Hakomori [36] has pointed to the heterogeneity of so-called membrane rafts which he has redefined as the glycosynapse, the constituents of which, in part at least, are defined by their ganglioside content. The ganglioside content of COS cells differs from that of platelets or melanoma cells [37] therefore this could account for different protein–lipid and lipid–lipid interactions occurring within different glycosynapses, with important functional consequences, and this consideration is currently under study.

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