Outside-in Signaling Pathway Linked to CD146 Engagement in Human Endothelial Cells*

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CD146 (S-Endo 1 Ag or MUC18) is a transmembrane glycoprotein expressed on endothelial cells on the whole vascular tree. CD146 is located at the intercellular junction where it plays a role in the cohesion of the endothelial monolayer. CD146 engagement initiates an outside-in signaling pathway involving the protein tyrosine kinases FYN and FAK as well as paxillin. Here we report that CD146 engagement by its specific monoclonal antibody in human umbilical vein endothelial cells induces a Ca2+ influx that is sensitive to thapsigargin and EGTA treatment, indicating that CD146 engagement initiates a store-operated calcium mobilization. In addition, biochemical and pharmacological analysis revealed that CD146 engagement initiates the tyrosine phosphorylation of phospholipase C-γ, Pyk2, and p130Cas. Pharmacological inhibition of Ca2+ flux with 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acetoxymethyl ester and EGTA indicated that an increase in Ca2+ is required for Pyk2 and p130Cas tyrosine phosphorylation. Moreover, a complex association was observed between Pyk2, p130Cas, and paxillin. These results indicate that CD146 is coupled to a FYN-dependent pathway that triggers Ca2+ flux via phospholipase C-γ activation leading subsequently to the tyrosine phosphorylation of downstream targets such as Pyk2, p130Cas, FAK, and paxillin. In addition to its role in cell-cell adhesion, CD146 is a signaling molecule involved in the dynamics of actin cytoskeleton rearrangement.

CD146 is involved in cell-cell adhesion through a heterophilic ligand that still remains unknown (8). In melanoma cells, the homotypic interaction of CD146 and its ligand contributes to cohesive interactions among these cells (9). Similarly, the binding of CD146 to its putative receptor on trophoblasts confers a stationary phenotype so preventing trophoblastic migration/invasion within the myometrium (10). Immunohistochemical studies have revealed that CD146 is localized at the intercellular junction in endothelial cells (3). This localization is consistent with a role of CD146 in the control of cohesive cell-cell interactions.

It is well known that cell adhesion molecules located at the intercellular junction control the integrity of the endothelial monolayer (11). They promote adhesion through their extracellular domain, whereas the intracytoplasmic tail is implicated in the outside-in signaling pathway that is derived from their engagement (12–15). In endothelial cells, CD146 acts as a signal transduction molecule. CD146 initiates an outside-in signal cascade upon monoclonal antibody engagement. Whereas CD146 is not phosphorylated on tyrosine residues, its engagement promotes the recruitment of the Src family kinase p59Fyn as well as the tyrosine phosphorylation of a large panel of intracellular proteins including p125Fak (FAK) and paxillin, two proteins present in focal adhesion plaques (16).

Calcium is a central second messenger that mediates a large number of cellular processes such as cell division, gene transcription, and/or cell death (17, 18). Activation of multiple cell surface receptors linked to PTK activation leads to increases in intracellular calcium concentrations ([Ca2+]i) (19). A feature of PTK-induced increase in [Ca2+]i involves a two-step process characterized at first by a rapid, transient release of Ca2+ stored in the endoplasmic reticulum (20). This release of Ca2+ from intracellular stores occurs, at least in part, via activation of phospholipase C (21, 22). PLCγ activation results in increased production of inositol 1,4,5-triphosphate and emptying of inositol 1,4,5-triphosphate receptor-gated Ca2+ stores. Depletion of intracellular Ca2+ stores then induces sustained extracellular calcium influx via store-operated calcium entry (SOCE) (23). Numerous endothelial cell functions are regulated by elevation in [Ca2+]i, levels such as the secretion of endothelial-derived factor (EDF) (24). EDF is a potent vasoconstrictor that has been described as an immunomodulatory molecule (25). A role for EDF in the regulation of inflammatory processes has been suggested (26). Recent studies have shown that EDF controls the adhesion, migration, and functions of immune cells. The mechanism by which EDF exerts its effects is not fully understood. It has been proposed that EDF might stimulate lymphocyte adhesion and migration by inducing changes in the expression or activity of adhesion molecules such as CD146 (27).

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Mobilization of Ca\textsuperscript{2+} in Response to CD146 Engagement in HUVEC

We show here that engagement of CD146 in HUVEC triggers a store-dependent Ca\textsuperscript{2+} influx that requires the tyrosine phosphorylation (Tyr(P)) of FYN and PLC\gamma. In addition, CD146 engagement induces the Tyr(P) of the PTK Pyk2 (29–31), and the adaptor protein p130Cas\textsuperscript{Ca2+} (32, 33), by a process involving Ca\textsuperscript{2+} mobilization. Thus, these data confirm and expand the concept that besides its function as adhesive protein, CD146 is also a signaling molecule involved in the dynamics of actin cytoskeleton rearrangement.

EXPERIMENTAL PROCEDURES

Materials

Fluo-3-AM, Pluronic F-127, and BAPTA-AM were from Molecular Probes (Eugene OR). Thapsigargin, herbinmycin, and ionomycin were from Alexis Corp. (San Diego, CA); FP1 and U73122 were from Calbiochem. Culture medium and culture reagents were from Life Technologies, Inc. Chemical reagents were from Sigma, mAbs against CD146 were from Jackson Laboratories (Palo Alto, CA), and mAbs against phosphotyrosine (PY20), FYN, p130Cas\textsuperscript{Ca2+}, Pyk2, paxillin, and PLC\gamma were from Transduction Laboratories (Lexington, KY).

Methods

Cell Culture—HUVECs were isolated from umbilical cord veins according to the method of Jaffe et al. (34). They were used at subconfluent density after one passage. Cell monolayers were starved for 3 hr in serum-free RPMI containing 0.5% BSA. For drug treatment experiments, HUVECs were pretreated with the drug for the indicated time prior to CD146 engagement.

CD146 Engagement—CD146 clustering was performed as described previously (36). Briefly, quiescent HUVECs were incubated with 10 \mu g/ml anti-CD146 F(ab\textsuperscript{1})\textsubscript{2} mAb in HBSS 30 min at 4 °C; after washing, cross-linking was then performed with 20 \mu g/ml goat anti-mouse IgG F(ab\textsuperscript{1})\textsubscript{2} (GAM), and the cells were processed for calcium determinations. Control cells were incubated with isotype-matched IgG1 and cross-linked with GAM.

Inhibitor Treatments—Herbinmycin, FP1, and U73122 were dissolved in Me\textsubscript{2}SO and incubated with HUVECs in serum-free HBSS, at the required concentration, 30 min prior to engagement of CD146. When required, HUVECs were preincubated with BAPTA-AM for 30 min at 37 °C and washed twice in HBSS, and engagement of CD146 was performed as indicated above. To study the effect of EGTA on the tyrosine phosphorylation of signaling proteins, EGTA (5 mM) was added throughout the addition of GAM.

Intracellular Ca\textsuperscript{2+}—Serum-starved subconfluent HUVECs grown in 96-well plates were loaded with Fluo-3-AM (5 \mu M) and 0.2 mg/ml Pluronic F-127 by incubation in loading buffer (HBSS supplemented with 10 mM HEPES, pH 7.4, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 1% BSA) (35) for 30 min at 37 °C. Cells were then washed in loading buffer and incubated for at least 15 min at room temperature, washed, and incubated again in loading buffer. CD146 engagement was then performed using anti-CD146 mAb at 4 °C as described (16). Cells were then washed and incubated in Ca\textsuperscript{2+}-free loading buffer to which 2 mM CaCl\textsubscript{2} was added when required, and cross-linking with GAM was performed as described above. Intracellular Ca\textsuperscript{2+} levels were determined immediately upon addition of GAM to the Fluo-3-loaded cell monolayers at 37 °C using a plaque-reader spectrofluorimeter (Cytosensor series 4000 Perspective Biosystems). Levels of fluorescence from cells in individual wells were monitored at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The signal was calibrated by addition of 10 \mu M ionomycin containing Ca\textsuperscript{2+} (2 mM) to obtain maximal fluorescence (I\textsubscript{max}). After a stable fluorescence at 530 nm (I\textsubscript{max}), the medium was changed to containing one of 5 mM EGTA in calcium-free medium with 10 \mu M ionomycin (I\textsubscript{Rmax}). Ca\textsuperscript{2+} concentration was calculated using the method of Grynkiewicz et al. (36) using the equation: [Ca\textsuperscript{2+}] = K\textsubscript{d} (R - R\textsubscript{max})/R\textsubscript{max} - R with K\textsubscript{d} = 400 nm (37). In some experiments, HUVECs were pretreated at 37 °C before cross-linking for 5 min with 1 \mu M thapsigargin for measurement of calcium release from intracellular stores. The effect of extracellular calcium was analyzed in presence of 5 mM EGTA in the loading buffer. Calcium influx was then assayed as required. All pharmacological and monoclonal antibody treatments did not alter the cell viability assessed both by treatment with 10 \mu M ionomycin to trigger calcium influx and by trypan blue dye exclusion demonstrating that the cells were still viable.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as described previously (16). In brief, cell lysates (300–600 \mu g of total proteins/sample, i.e. 0.3 to 1 \times 10\textsuperscript{6} cells/sample) were immunoprecipitated with 4 \mu g of mAb for 3 hr at 4 °C under constant agitation, followed by a 2-h incubation with protein G-Sepharose beads. After washing, the recovered proteins were subjected to SDS-PAGE and transferred to nitrocellulose filters (PVDF membranes). After blocking, membranes were incubated in TBS-T 5% BSA containing indicated antibodies (1 \mu g/ml) for 1 hr at room temperature. Immunoreactive bands were visualized by chemiluminescence using horseradish peroxidase-conjugated anti-mouse IgG and ECL reagent. When required, membranes were stripped and rebotted with the indicated antibodies.

Statistical Analysis—Results are expressed as mean ± S.E. Calcium measurements were analyzed by the unpaired Student’s t test. Differences were considered significant with p < 0.05.

RESULTS

CD146 Engagement-induced [Ca\textsuperscript{2+}]\textsubscript{i} Increase in HUVECs—To determine whether the engagement of CD146 in HUVECs triggers an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, cells loaded with 5 \mu M Fluo-3-AM were pretreated with anti-CD146 mAb F(ab\textsuperscript{1})\textsubscript{2} fragment (10 \mu g/ml) at 4 °C for 20 min and equilibrated at 37 °C for 180 s, and [Ca\textsuperscript{2+}]\textsubscript{i} was measured after cross-linking with GAM F(ab\textsuperscript{1})\textsubscript{2} antibody (20 \mu g/ml). In the presence of 2 mM extracellular calcium, the engagement of CD146 resulted in an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, characterized by an initial peak followed by a phase showing a slow decrease (Fig. 1). The initial increase peaked from basal levels of 139.4 ± 20.6 to 912.9 ± 188.3 nm (mean values ± S.E., p < 0.001, n = 15) within 30 s upon GAM addition. This [Ca\textsuperscript{2+}]\textsubscript{i} rise was followed by a gradual decline in [Ca\textsuperscript{2+}]\textsubscript{i}, levels which at the end of the incubation had not yet returned to base-line levels. [Ca\textsuperscript{2+}]\textsubscript{i}, rise was dose-dependent, and 10 \mu g/ml anti-CD146 generated a maximal [Ca\textsuperscript{2+}]\textsubscript{i}, response (data not shown). No change in [Ca\textsuperscript{2+}]\textsubscript{i}, was observed when an isotype-matched antibody was used instead of anti-CD146 mAb indicating that intracytoplasmic calcium changes are specific for the engagement of CD146.

Role of Intracellular and Extracellular Sources of Calcium in Response to CD146 Engagement—To determine which Ca\textsuperscript{2+} sources contribute to the intracytoplasmic calcium flux in response to CD146 engagement, internal Ca\textsuperscript{2+} stores were depleted by 1 \mu M thapsigargin (TG), an inhibitor of endoplasmic Ca\textsuperscript{2+} pumps (38). Addition of 1 \mu M TG to HUVECs preincubated with anti-CD146 mAb induced a transient increase in [Ca\textsuperscript{2+}]\textsubscript{i}, upon 1565 nm (34) using the equation: 

\[ \text{[Ca}^{2+}]_i = K_d \frac{R - R_{\text{max}}}{R_{\text{max}} - R} \]
Effect of calcium chelators on calcium influx induced by CD146 engagement in HUVECs. HUVECs loaded as described in Fig. 1 were treated with anti-CD146 mAb F(ab')2 fragment and incubated with 1 μM TG in calcium-containing medium (A). Changes in [Ca\(^{2+}\)]\(_i\), were monitored for 180 s. After GAM cross-linking (arrow), intracellular calcium modifications were further assayed for 360 s. CD146 engagement without (●) and with (*) TG treatment is shown. B, 5 mM EGTA in calcium-containing medium. [Ca\(^{2+}\)]\(_i\), was assayed for 360 s after cross-linking with GAM (arrow) in the presence of EGTA. CD146 engagement without (●) and with (*) EGTA is shown. C, with GAM (20 μg/ml) in the absence of exogenous calcium for 300 s (GAM-Ca\(^{2+}\)). 2 mM extracellular calcium was then added (+Ca\(^{2+}\)), and calcium influx was monitored for an additional 200 s. The curves are representative of four experiments performed in triplicate.

nm in control cells, n = 4) which peaked in 20 s. After [Ca\(^{2+}\)]\(_i\), has returned down to base-line values within 180 s, GAM was added to the monolayer. Preincubation of HUVECs with TG (1 μM) completely blocked [Ca\(^{2+}\)]\(_i\), increase after addition of GAM as compared with [Ca\(^{2+}\)]\(_i\), increase obtained in cells without TG treatment (Fig. 2A, n = 4). These results indicated that the initial rise of [Ca\(^{2+}\)]\(_i\), in response to CD146 engagement was dependent on intracellular store depletion.

Cross-linking of CD146 was then performed in the presence or absence of EGTA (5 mM). EGTA had no effect on the initial transient increase but strikingly decreased the [Ca\(^{2+}\)]\(_i\), in the second phase from 522.5 ± 106.3 to 328.9 ± 45.8 nm (p < 0.002, n = 4) (Fig. 2B). The role of extracellular calcium was confirmed in a subsequent experiment where CD146 cross-linking was performed in the absence of extracellular calcium. As shown in Fig. 2C, only the first transient rise in [Ca\(^{2+}\)]\(_i\), was observed. After the peak has returned close to the basal level, subsequent addition of 2 mM Ca\(^{2+}\) to the extracellular medium restored the second slow phase of [Ca\(^{2+}\)]\(_i\), (Fig. 2C). Taken together, these data indicate that the second phase of slow decrease was on the dependence of extracellular calcium sources. These results suggest that CD146 engagement stimu-

lates Ca\(^{2+}\) influx into the cells that depends both on Ca\(^{2+}\) release from internal stores and Ca\(^{2+}\) entry from the extracellular milieu.

Involvement of Protein Tyrosine Kinases (PTK) in Calcium Flux Induced by CD146 Engagement—For receptors coupled to PTK, calcium mobilization requires the activation of PTK and the recruitment of PLC\(\gamma\) to the plasma membrane (20). Previous data have indicated that upon engagement, CD146 recruits PLC\(\gamma\), which in turn participates to the increase in Ca\(^{2+}\) entry through PLC\(\gamma\).

CD146 engagement also induced the Tyr(P) of PLC\(\gamma\) (Fig. 4, upper panel, lane b) which was not observed in control cells (lane a). PLC\(\gamma\) phosphorylation was abolished by preincubation of HUVECs with PP1 (10 μM) (lane c). Preincubation of cells with BAFTA-AM, a chelator of intracellular calcium, or EGTA did not inhibit the Tyr(P) of PLC\(\gamma\) (lanes d and e). These results suggest that the Tyr(P) of PLC\(\gamma\) induced by CD146 engagement is mediated by FYN activation. However, no direct association between CD146 and PLC\(\gamma\) was initiated by CD146 engagement (data not shown). The involvement of FYN and PLC\(\gamma\) in [Ca\(^{2+}\)]\(_i\), mediated by CD146 engagement was confirmed by using pharmacological inhibitors. Pretreatment of HUVECs for 30 min with 2 μM herbimycin (Fig. 5A) or 10 μM PP1 (Fig. 5B) prior to CD146 engagement extinguished [Ca\(^{2+}\)]\(_i\), increase induced by CD146 cross-linking. Similarly, preincubation of HUVECs 30 min with 10 μM U73122, an inhibitor of PLC\(\gamma\) (40), abolished the rise in [Ca\(^{2+}\)]\(_i\), in response to CD146 engagement (Fig. 5C). Taken together, these results are consistent with a scheme according to which CD146 engagement induces the recruitment and activation of FYN, leading to the Tyr(P) of PLC\(\gamma\), which in turn participates to the increase in [Ca\(^{2+}\)]\(_i\).
related to FAK, is activated by an increase in intracellular calcium concentration (reviewed in Ref. 41). To determine whether PYK2 was involved in the signaling pathway mediated by CD146 engagement, anti-Pyk2 immunoprecipitates were immunoblotted with anti-Tyr(P) PY20 and anti-Pyk2. GAM cross-linking performed for 15 min induced the Tyr(P) of a band of 100 kDa identified as Pyk2 (Fig. 6A, upper panel, lane b) that was not observed in the absence of CD146 engagement. The Tyr(P) of Pyk2 was abolished by PP1 (10 μM) (lane c), by the intracellular calcium chelator BAPTA-AM (25 μM, lane d), and by EGTA (5 mM, lane e). This phosphorylation was observed despite the absence of a molecular association between CD146 and Pyk2 (data not shown). These data indicate that Pyk2 is phosphorylated on tyrosine residues following CD146 engagement by a process requiring both the activation of an Src family PTK and calcium mobilization from intra- and extracellular stores.

The adapter protein p130Cas localizes to focal adhesion points and interacts with Pyk2 (32, 33). The role of Pyk2 as a transducing molecule toward proteins of the focal adhesion plaques was then investigated in response to CD146 engagement. Similar to experiments with Pyk2, CD146 cross-linking with GAM for 15 min induced the Tyr(P) of a band with a molecular mass of 130 kDa corresponding to p130Cas (Fig. 6B, upper panel, lane b). No phosphorylation was observed in HUVECs treated with a control isotype-matched (IgG1) mAb (lane a). The Tyr(P) of p130Cas was abrogated by pretreatment of HUVECs with PP1 (10 μM) (lane c), and was reduced by BAPTA-AM (25 μM) or EGTA (5 mM) (lanes d and e). Nevertheless, CD146 did not associate with p130Cas (data not shown). Pyk2 has been shown to associate with paxillin (42). Paxillin is tyrosine-phosphorylated upon CD146 engagement (16). Therefore, we investigated whether paxillin and also p130Cas associate with Pyk2 in HUVECs upon CD146 triggering. As shown in Fig. 7, anti-Pyk2 immunoprecipitates performed on anti-CD146-stimulated HUVEC lysates contained a 130-kDa band reactive with anti-p130Cas mAb as well as a 70-kDa band reactive with anti-paxillin mAb (lanes b). Of note, constitutive

**Fig. 4.** Effect of CD146 engagement on the tyrosine phosphorylation of PLCγ in HUVECs. After engagement of CD146, lysates (300 μg) were immunoprecipitated using anti-PLCγ mAb, separated on a 7.5% SDS-PAGE, and immunoblotted (IB) using PY20 anti-Tyr(P) mAb as indicated (upper panel). Lane a, IgG1 isotype-matched control; lane b, CD146 engagement; lane c, CD146 engagement in presence the of 10 μM PP1; lane d, 25 μM BAPTA-AM; lane e, 5 mM EGTA. Blots were then stripped and rebotted with anti-PLCγ (lower panel). The position of PLCγ is indicated by an arrow.

**Fig. 5.** Effect of kinase inhibitors on calcium influx induced by CD146 engagement in HUVEC. CD146 engagement was performed as described under “Experimental Procedures.” Calcium release in HUVECs loaded as described Fig. 1 was assayed for 360 s after GAM cross-linking (G). Prior to CD146 engagement, cells were incubated 30 min with inhibitors (*). A, herbimycin (Herb, 2 μM); B, 10 μM PP1; and C, 10 μM U73122. The curves are representative of four experiments performed in triplicate.

**Fig. 6.** Effect of CD146 engagement on the tyrosine phosphorylations of Pyk2 and p130Cas in HUVEC. Engagement of CD146 was performed as described under “Experimental Procedures.” Tyrosine phosphorylations of Pyk2 (A) or p130Cas (B) were studied by immunoprecipitation with anti-Pyk2 (600 μg of cell lysate) and anti-p130Cas (300 μg of cell lysate), respectively, and immunoblotting (IB) using PY20 anti-Tyr(P) mAb. Upper panel, lane a, IgG1 isotype-matched control; lane b, CD146 engagement; lane c, CD146 engagement in the presence of 10 μM PP1; lane d, 25 μM BAPTA; lane e, 5 mM EGTA. Blots were then stripped and rebotted with anti-Pyk2 mAb (A, lower panel) or anti-p130Cas (B, lower panel). The respective positions of Pyk2 and p130Cas are indicated by an arrow.
The time course of the Tyr(P) of FYN, PLCγ, Pyk2, and p130Cas upon CD146 engagement was then investigated. Cell lysates were immunoprecipitated with anti-Tyr(P) mAb and immunoblotted with anti-FYN, anti-PLCγ, anti-Pyk2, and anti-p130Cas. The position of the different proteins is indicated by an arrow.

**FIG. 7.** Molecular interactions between PYK2, p130Cas, and paxillin. CD146 engagement was performed as described under “Experimental Procedures.” Cell lysates (300 μg) were immunoprecipitated with anti-Pyk2 and immunoblotted (IB) with anti-p130Cas or anti-paxillin. Lanes a, IgG1 isotype-matched control; lane b, CD146 engagement. The position of the different proteins is indicated by an arrow.

The association of Pyk2 and paxillin but not of Pyk2 and p130Cas was observed in unstimulated HUVECs (lanes a).

**Time Course of Tyr(P) of FYN, PLCγ, Pyk2, and p130Cas—**The time course of the Tyr(P) of FYN, PLCγ, Pyk2, and p130Cas upon CD146 engagement was then investigated. Cell lysates were immunoprecipitated with anti-Tyr(P) mAb and immunoblotted with the indicated mAbs. CD146 engagement rapidly stimulates the Tyr(P) of FYN and PLCγ (Fig. 8) which reached their maximum 2 and 5 min, respectively, after GAM cross-linking. It should be noted that Tyr(P) of Fyn was detected as soon as 15 s upon CD146 engagement (data not shown). Maximal Tyr(P) of Pyk2 and p130Cas were observed after 20 min. The Tyr(P) of Pyk2 was transient, whereas that of p130Cas remained sustained after 30 min. These results indicate that CD146 engagement induces an outside-in signal pathway involving at first FYN and PLCγ followed by the phosphorylation of Pyk2 and p130Cas.

**DISCUSSION**

Previous data have indicated that CD146 initiates an outside-in signal transduction pathway that involves the recruitment of the Src PTK FYN and leads to the Tyr(P) of FAK and paxillin, two proteins present in the focal adhesion plaques (16). We demonstrate here that in endothelial cells, CD146 engagement promotes an increase in [Ca2+]i, both by Ca2⁺ release from TG-sensitive Ca2⁺ stores and entry of extracellular Ca2+. This process requires the activation of FYN and PLCγ. Moreover, Ca2⁺ appears to serve as a second messenger by coupling Ca2⁺ release to the Tyr(P) of the related FAK kinase, Pyk, and the adapter protein, p130Cas, and favoring their association.

The Ca2⁺ influx initiated by CD146 engagement in HUVECs is representative of the well known SOCE. Indeed, the initial rapid Ca2⁺ mobilization depends upon TG-sensitive stores, whereas the long lasting decrease depends on the extracellular Ca2+. This SOCE is mediated by activation of FYN and PLCγ (20), a key enzyme involved in calcium traffic (19). CD146 recruitment of FYN initiates the Tyr(P) of PLCγ, as evidenced by the inhibition of PLCγ Tyr(P) by PP1, and is consistent with the time course. These data of FYN and PLCγ phosphorylation are in agreement with previous reports indicating that FYN is involved in calcium mobilization by initiating the activation of PLCγ (44–46).

It should be noted that the mechanism leading to SOCE is not well elucidated. Some recent data suggest that Ca2⁺ influx results from a secretory pathway induced by a close interaction between ER calcium stores and the plasma membrane via an involvement of the actin cytoskeleton (47, 48). The secretion requires intracellular fusion events mediated by SNAP-25, a membrane protein belonging to the SNAP receptor proteins (49).

The mobilization of calcium ions in addition to the recruitment of FYN play an active role in the outside-in signaling pathway mediated by CD146. Indeed, CD146 engagement leads to Tyr(P) of Pyk2, the calcium-dependent tyrosine kinase related to FAK (reviewed in Ref. 41), by a calcium-dependent mechanism. FYN is also involved in the activation of Pyk2. It is known that several Pyk2 tyrosine residues (Tyr-402, Tyr-579, and Tyr-580 within the catalytic domain and Tyr-881 within the carboxyl terminus domain) create binding sites for the SH2 of the Src-like PTK (50, 51). Pyk2 also associates with and is phosphorylated by FYN during stimulation of T cell antigen receptor (52). Nevertheless, in the case of CD146 engagement, FYN exerts an indirect effect on Pyk2 because no direct association between Fyn and Pyk2 could be found (data not shown). Moreover, Pyk2 Tyr(P) occurs more later than that of FYN, indicating that Pyk2 acts downstream of FYN.

The signaling pathway initiated by CD146 engagement includes the Tyr(P) of FAK and its substrate paxillin (16). p130Cas belongs to the group of proteins that associate with FAK and is involved in cell migration (33). p130Cas tyrosine phosphorylation in response to CD146 engagement strengthens the relationship between CD146 and focal adhesion points by a way that is dependent on FYN recruitment. Indeed, p130Cas Tyr(P) depends on an Src PTK activation as demonstrated by PP1 inhibition and on calcium influx as evidenced by its partial inhibition by the calcium chelators. Src PTKs have been implicated in the Tyr(P) of p130Cas mediated by integrins or shear stress (53, 54). Nevertheless Pyk2 does not associate with p130Cas in anti-CD146-treated HUVECs. The molecular
structures implicated in the association of Pyk2 and p130Cas upon CD146 engagement are not known, but the SH3 domain of p130Cas and the two proline-rich sequences of Pyk2 have been involved in the formation of such a complex (55). p130Cas is a ligand of FAK by its SH3 domain (56) and increases cell migration promoted by FAK (57). Taken together, the fact that p130Cas plays an important role in the organization of the cytoskeletal framework (58, 59) and that Pyk2 forms a constitutive complex with paxillin (42, 60) expands the concept that CD146 localization in focal adhesion plaques.

The association with p130Cas and paxillin and will allow their localization in focal adhesion plaques. FYN plays a crucial function in the initiation of the actin reorganization has never been observed (data not shown).

In summary, the present study thus demonstrates that engagement of CD146 induces a complex signaling pathway that includes Ca2+ influx as a second messenger involved in cytoskeleton dynamics. FYN plays a crucial function in the initiation of CD146 signal transduction. Upon engagement, FYN is recruited and phosphorylated by CD146. FYN allows the activation of PLC-δ which in turns can hydrolyze membrane phosphoinositides. The binding of inositol 1,4,5-trisphosphate to its receptor would induce the release of Ca2+ from internal stores and initiate a store-dependent entry of extracellular Ca2+[61]. In addition, FYN regulates the Tyr(P) of targets of CD146 localization after its engagement with its ligand. Further studies are necessary to determine the in vivo physiological relevance of the signaling pathways linked to CD146.

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