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The SRC-associated protein CUB Domain-Containing Protein-1 regulates adhesion and motility

CH Benes[1,3], **G Poulogiannis**[1,2], **LC Cantley**[1,2], and **SP Soltoff**[1]

[1]Department of Medicine, Division of Signal Transduction, Beth Israel Deaconess Medical Center, Boston, MA, USA

[2]Department of Systems Biology, Harvard Medical School, Boston, MA, USA

Abstract

Multiple SRC-family kinases (SFKs) are commonly activated in carcinoma and appear to have a role in metastasis through incompletely understood mechanisms. Recent studies have shown that CDCP1 (CUB (complement C1r/C1s, Uegf, Bmp1) Domain-Containing Protein-1) is a transmembrane protein and an SRC substrate potentially involved in metastasis. Here we show that increased SFK and CDCP1 tyrosine phosphorylation is, surprisingly, associated with a decrease in FAK phosphorylation. This appears to be true in human tumors as shown by our correlation analysis of a mass spectrometric data set of affinity-purified phosphotyrosine peptides obtained from normal and cancer lung tissue samples. Induction of tyrosine phosphorylation of CDCP1 in cell culture, including by a mAb that binds to its extracellular domain, promoted changes in SFK and FAK tyrosine phosphorylation, as well as in PKC™, a protein known to associate with CDCP1, and these changes are accompanied by increases in adhesion and motility. Thus, signaling events that accompany the CDCP1 tyrosine phosphorylation observed in cell lines and human lung tumors may explain how the CDCP1/SFK complex regulates motility and adhesion.

Keywords

signaling; mass spectrometry; SRC; adhesion; motility; metastasis

Introduction

CDCP1 (CUB (complement C1r/C1s, Uegf, Bmp1) Domain-Containing Protein-1), also named Transmembrane and Associated with SRC Kinase (TRASK) and Substractive Immunization M+ HEp3-Associated 135-kDa protein (SIMA135), is a substrate and binding protein for several SRC-family tyrosine kinases (SFKs) (Benes *et al.*, 2005; Uekita *et al.*, 2007; Alvares *et al.*, 2008). CDCP1 is a transmembrane protein of undetermined function that is overexpressed and highly tyrosine-phosphorylated in multiple carcinomas, including colon, lung and renal cancers (Scherl-Mostageer *et al.*, 2001; Hooper *et al.*, 2003; Perry *et al.*, 2007; Rikova *et al.*, 2007; Awakura *et al.*, 2008; Ikeda *et al.*, 2009; Spassov *et al.*, 2009).

Correspondence: Dr SP Soltoff, Department of Medicine, Division of Signal Transduction, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, CLS-406, Boston, MA 02215, USA., ssoltoff@bidmc.harvard.edu.
[3]Current address: Massachusetts General Hospital Cancer Center, 149, 13th Street, Charlestown, MA 02129, USA.

Conflict of interest

The authors declare no conflict of interest.

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In addition, the analysis of model systems and patient survival suggest that CDCP1 may be involved in metastasis (Uekita *et al.*, 2007, 2008; Ikeda *et al.*, 2009; Wortmann *et al.*, 2009; Liu *et al.*, 2011), and high levels of CDCP1 expression have been correlated with poor prognosis in pancreatic cancer patients (Miyazawa *et al.*, 2010). Thus, CDCP1 may have a role in SFK-mediated cancer progression. While resistance to anoikis could be involved in the prooncogenic activity of CDCP1 (Uekita *et al.*, 2007), the molecular mechanisms underlying its putative involvement in cancer progression are poorly defined. In fact, there is very little understanding of the function of CDCP1 at the cellular level and of the possible signaling consequences of the CDCP1/SRC complex formation. Understanding the signaling events promoted by the CDCP1/SRC complex is important to further evaluate the role of this complex in cancer.

SFKs participate in a large number of cellular processes, and defective regulation of their activity is likely to have multiple consequences depending on the cell type and extracellular conditions. It is well established that SFKs are implicated in the regulation of proliferation in fibroblasts (Courtneidge, 2002; Bromann *et al.*, 2004); however, it appears that in epithelial cancer cell lines, elevated SFK activity has consequences mostly on migration and adhesion (Brunton *et al.*, 2005; Brunton and Frame, 2008; Vultur *et al.*, 2008). The SRC/focal adhesion kinase (FAK) complex is at the center of current models of the regulation of the integrin-based adhesion system and motility (Sieg *et al.*, 1999, 2000; Mitra *et al.*, 2005; Tilghman *et al.*, 2005), and several SFKs are required for the remodeling of adhesions structures and cellular motility (Klinghoffer *et al.*, 1999; Frame, 2004; Mitra and Schlaepfer, 2006). In particular, SFKs are involved in regulating integrin activation (inside-out signaling) as well as in transmitting signals initiated by integrin engagement (outside-in signaling). SFKs also appear to have a key role in the coordination of multiple signals initiated by growth factors, chemokines, integrin ligands and other extracellular cues that influence the migration of cells (Huveneers and Danen, 2009).

Here, we show that CDCP1 tyrosine phosphorylation in human lung tumor samples is correlated with the activation of SFK and other known SFK-dependent phosphorylation events. Surprisingly, CDCP1/SFK tyrosine phosphorylation is inversely correlated with FAK tyrosine phosphorylation in lung tumors. We show that acutely increasing CDCP1 tyrosine phosphorylation in cell culture also results in the activation of SFK and leads to the inhibition of FAK. Furthermore, we show that inducing CDCP1 tyrosine phosphorylation results in changes in adhesion to fibronectin (FN) and increased migration *in vitro*. Finally, we show that induction of CDCP1 phosphorylation induces changes in cell–cell contacts and actin filament organization.

Based on our findings, we propose that CDCP1 is an SFK-associated receptor that regulates adhesion and motility, and that this may help explain the proposed involvement of CDCP1 in metastasis.

Results

CDCP1 tyrosine phosphorylation and associated events in lung tumors

CDCP1 is highly tyrosine-phosphorylated in a number of carcinomas and was identified as a major phosphotyrosine protein in large-scale mass spectrometric analysis of lung cancers (Scherl-Mostageer *et al.*, 2001; Hooper *et al.*, 2003; Perry *et al.*, 2007; Rikova *et al.*, 2007; Awakura *et al.*, 2008; Ikeda *et al.*, 2009; Spassov *et al.*, 2009) as well as a major target of SRC activity upon expression of activated SRC (Leroy *et al.*, 2009). To explore the phospho-tyrosine signaling events associated with CDCP1 tyrosine phosphorylation in human tissue, we performed a statistical analysis of a mass spectrometric data set obtained from normal and cancer lung tissue samples. This data set consists of phospho-tyrosine

peptide counts obtained after affinity purification using an anti-phospho-tyrosine antibody following complete digestion of tissue lysates by trypsin. The global analysis of this data set was published previously (Rikova *et al.*, 2007). The analysis performed here (described under Materials and methods) revealed over 50 proteins that are differentially phosphorylated on tyrosine between non-small cell lung cell (NSCLC) tumors and normal lung samples (Supplementary Table 1S). This approach identified several SFK and SFK substrates that are differentially phosphorylated in cancer as compared with that in normal tissue, consistent with previous findings (Bolen *et al.*, 1987; Cartwright *et al.*, 1989; Talamonti *et al.*, 1993; Aligayer *et al.*, 2002; Frame, 2002; Serrels *et al.*, 2006). SFK tyrosine phosphorylation is expected to correlate with activity in this study as the majority of the phosphotyrosine peptide counts are from the activation loop of the kinase. CDCP1, a known SFK substrate, was highly phosphorylated on tyrosine in a subset of NSCLCs when compared with normal lung samples (P=0.017) (Figure 1a). A correlation matrix of the phosphotyrosine signal profiles that were detected in ≥50% of the samples was used to identify all the proteins whose tyrosine phosphorylation is strongly correlated with CDCP1 phosphorylation (see Supplementary Figure 1S for a visual representation). The results of this correlation analysis are presented as volcano plots of the correlation factor against the P-value of the corresponding correlation (Figure 1b). The most significant correlation (best P-value) was found between CDCP1 and protein kinase-Cδ (PKCδ). This constitutes an excellent validation of the correlation analysis, as we have shown previously that PKCδ is a direct binder of CDCP1; that this interaction only occurs when CDCP1 is tyrosine phosphorylated; and that binding of CDCP1 to PKCδ promotes the tyrosine phosphorylation of PKCδ (Benes *et al.*, 2005). In addition, there is a positive correlation between the phosphorylation of CDCP1 and the phosphorylation of multiple SFKs, including SRC, LCK, FYN and YES, consistent with CDCP1 being a substrate of SFKs (Benes *et al.*, 2005; Uekita *et al.*, 2007). Surprisingly, while we found a positive correlation between CDCP1 and SFK tyrosine phosphorylation, we also found a negative correlation between CDCP1 phosphorylation and FAK tyrosine phosphorylation. This is somewhat counterintuitive as SFK and FAK are known to regulate each other positively and as FAK has been found phosphorylated on tyrosine concomitantly with SRC activation in multiple other studies, including SRC substrate analysis by mass spectrometry (Leroy *et al.*, 2009). A more comprehensive list of the phospho-tyrosine signals in relation to CDCP1 phosphorylation, which identifies novel correlations of possible significance to the activation and signalling of CDCP1, is provided in Table 1. The best correlations identified with CDCP1 phosphorylation were confirmed when the correlation analysis was assessed separately in normal and tumor samples (Supplementary Tables 2S and 3S). Thus, one of the main conclusions of this correlation analysis is that tyrosine phosphorylation of CDCP1 in lung tissue might in some instances correlate positively with SFK activity but correlate negatively with FAK activity.

SFK activation is induced by the anti-CDCP1 antibody CUB1

To directly investigate the signaling associated with CDCP1 tyrosine phosphorylation, we decided to test the activity of monoclonal antibodies (mAbs) against the extracellular domain of CDCP1. This approach stemmed from the observation of Buhring *et al.* (2004), who showed that an antibody against the extracellular domain of CDCP1 promoted the growth of erythroid colonies established from bone marrow, a response possibly related to the signaling events promoted by this antibody. To test the hypothesis that CDCP1 can transduce a signal by the recruitment of SFK, we added a CUB1 mAb to the culture medium of cells expressing endogenous CDCP1. CUB1 increased the tyrosine phosphorylation of CDCP1 on tyrosine (Tyr)-734, which is the SRC SH2-binding site (Benes *et al.*, 2005), in a time-dependent manner in HCT116 cells (Figure 2a). (The characterizations of the phospho- and the site specificity of the antibodies against CDCP1 are shown in Supplementary Figure

2S.) Importantly, CUB1 promoted a large increase in the phosphorylation of SFKs at their autophosphorylation site in the activation loop (Tyr-416 in SRC, according to the classic numbering of avian SRC). This suggests that CDCP1 could be an upstream activator of SFKs in normal or pathological situations. Confirming the usefulness of antibody stimulation to studying CDCP1 signaling, similar results were reported recently by others using a different antibody (Alvares *et al.*, 2008). As the several members of the SFK family share a common sequence in the activation loop, we sought to identify more specifically which members could be activated by CUB1. We immunoprecipitated specific members of the family and showed that SRC (p60 c-src) itself is activated downstream from CDCP1 under the conditions tested (Supplementary Figure 3S).

We also examined the effects of CUB1 on the tyrosine phosphorylation of SFKs in HCT116 cells over a longer time course. Notably, although short-term exposure of cells to CUB1 produces large increases in the phosphorylation of CDCP1 and activation of SFKs, prolonged exposure of cells to CUB1 leads to loss of the CDCP1 protein (Figure 2b). The total CDCP1 protein levels were reduced substantially as early as 3 h, and almost all of the CDPC1 protein was absent after 5 h of exposure to CUB1. These results do not appear to be because of a change in CDCP1 solubility, as the cells were lysed in boiling 1% sodium dodecyl sulfate-containing Laemmli sample buffer. Notably, SFK protein levels were maintained when CDCP1 levels declined. Importantly, the tyrosine phosphorylation of CDCP1, SFKs and PKCδ were all correlated positively throughout the time course of stimulation with CUB1. We also observed that when HCT116 cells are maintained at confluence in full serum for 48 h there is a large increase in the phosphorylation of CDCP1 at Tyr-734 and phosphorylation of SFKs at the activation loop (Tyr-416) even without the addition of CUB1, suggesting that cell–cell contact at confluence triggers CDCP1 signaling (Figure 2c). Strikingly, when CDCP1 protein levels are downregulated by long-term exposure to CUB1, the confluence-dependent phosphorylation of SFKs is eliminated. These results support a model in which CDCP1 mediates the activation of SFKs in response to prolonged cell confluence.

Altogether, the results presented in Figure 2 suggest that CDCP1 is an upstream activator of SFK in response to cell confluence and potentially under other conditions. The fact that CDCP1 is both a substrate and a binder of SFK is most likely essential for its ability to promote SFK activation, as is the case for other transmembrane proteins without intrinsic tyrosine kinase activity such as multiple chains of the T-cell and B-cell receptors (for a mechanistic analysis of this, see Cooper and Qian (2008)).

Engaging CDCP1 promotes discrete signaling events

To identify additional signaling events initiated by CDCP1 phosphorylation, we compared the tyrosine phosphorylation profile of untreated cells with that of cells exposed to CUB1, and found very few changes in phospho-tyrosine proteins (Figure 3a). This indicated that the CDCP1/SFK complex initiates a discrete number of signaling events rather than widespread tyrosine phosphorylation owing to global SFK activation. We next examined the previously reported substrates of SFKs. CUB1 promoted the tyrosine phosphorylation of SHC, a known SRC substrate (McGlade *et al.*, 1992) (Figure 3b), and induced its association to GRB2 (Figure 3c). CUB1 also induced the association of GRB2 with other tyrosine-phosphorylated proteins (closed circles in Figure 3d), although some GRB2-associated tyrosine-phosphorylated proteins remained unchanged (open circle in Figure 3d). However, we could not detect extracellular signal-regulated kinase (ERK)1/2 activation under these conditions (not shown) and did not study further the role of GRB2/SHC in this context. The tyrosine phosphorylation of SHP2, a regulator of SFKs (Zhang *et al.*, 2004), and the tyrosine phosphorylation of proteins that co-precipitate with SHP2 were also increased in response to

CUB1 (Figure 3e). Altogether, inducing CDCP1 phosphorylation appears to trigger a set of specific signaling pathways downstream from SFK activation.

Acute CDCP1 activation inhibits FAK phosphorylation

Consistent with the statistical correlation between high-phospho-tyrosine CDCP1 and low-phospho-tyrosine FAK in NSCLC tumors (Figure 1b), the tyrosine phosphorylation of FAK was diminished following exposure of HCT116 cells to CUB1 (Figure 4a). There was an inverse correlation between CDCP1 phosphorylation at Tyr-734 and FAK phosphorylation at Tyr-577/576 (SFK phosphorylation sites) over a range of CUB1 concentrations. Similar results were obtained when monitoring Tyr-397 of FAK, an autophosphorylation site that is also an SFK SH2 domain-binding site (Supplementary Figure 4S). Moreover, in cells that were detached and subsequently acutely plated on FN, treatment with CUB1 (added 15 min before plating) increased the Tyr-734 phosphorylation of CDCP1 and blocked the FN-dependent increase in FAK phosphorylation at Tyr-397 (Figure 4b). FAK was not phosphorylated in suspended cells with or without CUB1 exposure, but CDCP1 tyrosine phosphorylation was still increased by CUB1 under these conditions. In addition, the dephosphorylation of FAK in response to CUB1 correlated with loss of phosphorylation of p130CAS (Figure 4c) and paxillin (Figure 4d), known substrates of the SFK/FAK complex. FAK dephosphorylation at Tyr-397 was maximal at 60 min, whereas an increase in CDCP1 phosphorylation was observed as early as 2 min and was maximal around 30 min (Supplementary Figure 4S).

The inverse correlation between CDCP1 phosphorylation and FAK phosphorylation was also observed when CDCP1 was activated owing to cell confluence. Consistent with the previously discussed results in Figure 2c, 48 h of cell confluence resulted in tyrosine phosphorylation of CDCP1 and SFKs, and this correlated with a decrease in tyrosine phosphorylation of FAK (Figure 4e). Importantly, knocking down the expression of CDCP1 with a small interfering RNA (siRNA) resulted in a decrease in the phosphorylation of SFKs and an increase in the phosphorylation of FAK at Tyr-397. These results indicate that CDCP1 mediates an increase in SFK activation and a paradoxical decrease in FAK activation in response to cell confluence. Thus, the unexpected reduction of FAK phosphorylation concomitant with activation of SRC seen in tumor samples can be recapitulated upon acute stimulation of CDCP1 and reversed upon depletion of phosphorylated CDCP1. Interestingly, the FAK inhibitor PF573228 induced an increase in CDCP1 phosphorylation, confirming that CDCP1 and FAK phosphorylation states are coordinated (Supplementary Figure 5S).

Cell attachment to FN is impaired by CUB1

As FAK is involved in the establishment of cell–extracellular matrix adhesion, the suppression of FAK phosphorylation in response to CDCP1 phosphorylation prompted us to investigate whether CDCP1 engagement could negatively regulate adhesion. Consistent with previous reports showing that FAK activity and its phosphorylation on Tyr-397 is important for the establishment of nascent contacts during plating on FN (Serrels *et al.*, 2007), we found that activation of CDCP1 by CUB1 impaired the adhesion of cells to different concentrations of FN (Figure 5). Together with the fact that CUB1 promoted a decrease in FAK phosphorylation in adherent cells (Figure 4), the inhibition of adhesion by CUB1 appears to be a consequence of FAK inhibition rather than loss of FAK activation owing to integrin activation.

CUB1 promotes cell migration

FAK is a critical regulator of cell motility, so we investigated if CDCP1 could regulate this process. The role of FAK activity in cell motility is complex, as both absence of FAK (Ilic *et*

al., 1995) and its hyper-phosphorylation lead to a similar block of motility (Yu *et al.*, 1998; Manes *et al.*, 1999), and both elevated and diminished FAK activity can be correlated with enhanced migration and invasion (Lu *et al.*, 2001; Vitale *et al.*, 2008; Chan *et al.*, 2009; Zheng *et al.*, 2009). Using a Transwell assay in which single cells migrate from one side of a membrane to the other (top chamber to bottom chamber), we observed that CUB1 (present in both chambers) increases cell migration in two epithelial cell lines, DLD-1 (colon cancer) and immortalized, non-tumorigenic MCF10A breast cells (Figure 6). These results show that CDCP1 regulates cell motility.

The increase in migration induced by CUB1 was blocked by SFK inhibition, suggesting that SFK could mediate the effect of CUB1 on migration (Supplementary Figure 6S). As SFKs regulate focal adhesion turnover, their inhibition might block all migration processes regardless of the involvement of the CDCP1/SRC complex. Whereas CUB1 induces a decrease in FAK activity and an increase in migration, a FAK inhibitor suppressed the increase in migration induced by CUB1. Because FAK activity is known to regulate cell migration, similar to the result with the SFK inhibitor, it is difficult to conclude whether or not the inhibitors' effect on migration are specific to CDCP1–SRC–FAK signaling or instead suppress migration potential independently of this pathway (Supplementary Figure 6S).

Overall, our data are consistent with a signaling pathway originating at CDCP1 and regulating migration through FAK inhibition. We propose that the increased motility is because of an increased turnover of adhesions to the substratum, although it is possible that increased phosphorylation of SHC or other SFK substrates contributes to this effect. These results are in good agreement with a recent report using siRNA-mediated knockdown of CDCP1 in cells presenting with constitutive phosphorylation of CDCP1 (Miyazawa *et al.*, 2010). In the cell lines used in the present study, CDCP1 is not constitutively phosphorylated and siRNA knockdown does not affect migration or adhesion (data not shown).

CDCP1 phosphorylation affects cell–cell contacts

SFKs regulate actin organization, cell–cell contacts and the functional interaction between the actin cytoskeleton and regions of cell–cell contact. Consistent with a previous report (Alvares *et al.*, 2008), we find that CDCP1 is located at cell–cell contacts (see Supplementary Figure 7S). As the tyrosine phosphorylation of a number of proteins that are involved in cell–cell contacts and actin organization (for example, DSCs, filamin (FLNB), Arp3, plakophilin, nectin, cortactin) correlates positively with the tyrosine phosphorylation of CDCP1 (Table 1), we investigated whether CDCP1 might be involved in cell–cell contacts and actin organization. To investigate these potential relationships, we determined the effects of CUB1 on the organization of adherens junctions in MCF10A cells as judged by the labeling of β-catenin, a direct binder of E-cadherin at the adherens junctions. When MCF10A cells are placed in 0.1% fetal bovine serum (FBS), the β-catenin proteins at the areas of cell–cell contact form spiked structures, indicating misalignment of adherens junctions, and this effect could be partially reversed by addition of CUB1 (Figure 7). Moreover, CUB1 accelerated the formation of calcium-dependent cell–cell adhesions, consistent with improved formation of E-cadherin-based cell junctions upon CDCP1 activation (Supplementary Figure 8S). We have shown previously that PKCδ can bind to CDCP1 and that this leads to its phosphorylation by SRC (Benes *et al.*, 2005). The Tyr-311-phosphorylated form of PKCδ was detected at cell–cell contacts following CUB1 activation (Supplementary Figure 7S). Interestingly, adding the phorbol ester phorbol 12-myristate 13-acetate (PMA) concomitantly with CUB1 to the cells promoted a large increase in the tyrosine phosphorylation of PKCδ, above the levels seen with CUB1 or PMA (Supplementary Figure 9S), and a more dramatic change in cell–cell contact organization

than with PMA or CUB1 alone (Supplementary Figure 10S). In addition, co-stimulation of CUB1 and a number of other receptors did not produce a similar increase in PKCδ Tyr-311 phosphorylation (Supplementary Figure 9S). These results suggest that PKCδ activation and recruitment at cell–cell contacts might be involved in the re-organization of the junctions induced by CUB1. As shown in Figure 8, the effects of CUB1 on cell–cell contacts appear to be related to the re-organization of the actin cytoskeleton. Activation of CDCP1 with CUB1 leads to the accumulation of actin at cell–cell contacts in fibers parallel to the cell junctions. Dasatinib, an SFK inhibitor, blocked the CUB1-dependent stabilization of the actin cortical network, indicating that the re-organization of actin induced by CUB1 was dependent on SFK activity. In addition, the pan-PKC inhibitor GF109203X blocked at least partially the effect of CUB1 on cell–cell contacts. This result, together with the effects seen with PMA, suggests that PKC activity is involved in regulating cell–cell junctions either downstream from or in coordination with CDCP1 activation. Similar results were obtained in HCT116 (Supplementary Figure 10S) and MCF10A cells (Figures 7 and 8). Although it is still unclear whether the actin re-organization induced by CUB1 is because of the attachment of already polymerized actin bundles to cell–cell contacts or a change in actin polymerization or bundling, all the data presented here are consistent with a model in which downstream signaling of CDCP1/SFK could modulate cell–cell adhesion and cell–matrix adhesion by acting on the recruitment or polymerization of actin at these different adhesion structures.

Taken together, our results showing that CDCP1 activation can affect cell motility and migration, along with results of previous studies indicating an association of CDCP1 with enhanced tumor infiltration (see introduction), argue that the CDCP1/SFK complex plays a critical role in tumor metastasis by modifying cell–cell and cell–matrix adhesion.

Discussion

Previous studies have shown that CDCP1 might regulate cell–matrix adhesion and be involved in metastasis. The results presented here show that CDCP1 phosphorylation is correlated with the phosphorylation of proteins known to regulate adhesion and motility. Furthermore, using antibody stimulation to explore signaling events initiated by CDCP1 phosphorylation, we confirmed its involvement in the regulation of cell adhesion and motility. Most striking is our observation that both in human tissue and upon acute activation in cell culture, the tyrosine phosphorylation of CDCP1 is correlated with SFK activation and with inhibition of FAK.

It is a paradox that CDCP1 activates SFKs, resulting in increased phosphorylation of known SFK substrates such as SHC and SHP2, yet causes a decrease in FAK phosphorylation. Of note, whereas SHP2 is known to regulate FAK tyrosine phosphorylation in other contexts, suppression of SHP2 expression by RNA interference did not diminish the effect of CDCP1 on FAK phosphorylation (data not shown). A possible explanation is that CDCP1 recruits SFKs away from integrins and FAK, and activates these enzymes at a new location in areas of cell–cell contact where distinct substrates are engaged. This model is consistent with the observation that CDCP1 engagement modulates cortical actin at regions of cell–cell contact and decreases interactions with FN. Although siRNA against CDCP1 decreases the phosphorylation of SFKs, it does not completely eliminate SFK phosphorylation, so it is likely that the increased FAK phosphorylation under these conditions is because of a localized pool of active SFKs. Recruitment of SRC to FAK has been shown to be necessary to initiate FAK tyrosine phosphorylation, although SRC activity appears dispensable in at least some instances (Brunton *et al.*, 2005).

The inhibition of adhesion seen here upon acute activation of CDCP1 is consistent with observations that cells overexpressing CDCP1 show a rounded morphology (Bhatt *et al.*, 2005), and with a correlation between CDCP1 tyrosine phosphorylation and loss of adhesion observed in some contexts (Spassov *et al.*, 2009, 2011). Moreover, Liu *et al.* (2011) have shown recently that CDCP1 overexpression reduces cell–matrix adhesion. However, in contrast to some of these other reports, in our experience, detaching cells from their substratum (using a non-enzymatic method that does not induce CDCP1 cleavage) does not lead to CDCP1 phosphorylation within 2 h (see Figure 4). However, we did observe that a high concentration of EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid) (>2mM) induced a rapid and transient activation of CDCP1 tyrosine phosphorylation (unpublished observation). In addition, Alvares *et al.* (2008) reported recently that mAb-induced phosphorylation of CDCP1 could not be induced in suspended cells; however, adhesion status appears to make little or no difference under our conditions (Figure 4b). Although some of these discrepancies are likely because of the use of different cell lines, it would be interesting to understand better the possible crosstalk between CDCP1 activation and cell–matrix adhesion. In a recent study, overexpression of CDCP1 was shown to promote loss of cell–matrix adhesion and FAK phosphorylation, events correlated with impairment in integrin clustering. These results and others in that study addressing the correlation of FAK and CDCP1 phosphorylation are quite consistent with ours (Spassov *et al.*, 2011), and suggest that acute activation of CDCP1 might induce changes in cell–matrix adhesion by affecting integrin clustering. This supports the concept that actin polymerization changes or recruitment of actin filament might underlie a number of functional outcomes of CDCP1 activation. In keeping with this, another recent report has identified cortactin as an interacting partner of PKCδ, and suggests that the PKCδ–cortactin complex mediates changes in migration downstream from CDCP1 phosphorylation (Miyazawa *et al.*, 2010). Although we do observe tyrosine-phosphorylated PKCδ (Supplementary Figure 7S) at cell–cell contacts following CDCP1 activation, our preliminary results have shown that suppression of PKCδ expression only modestly affects actin recruitment at cell–cell contacts in response to CDCP1 activation.

While we observe increased cell motility in Transwell assays upon CDCP1 activation, we also found that CDCP1 activation correlated with the recruitment of actin at the periphery of the cell and faster junction formations in calcium switch experiments. These two sets of events seem somewhat contradictory. As cortical actin accumulation occurs upon maturation of cell–cell contacts, this suggests that CDCP1 activation could reinforce cell–cell adhesion. Interestingly, in the Transwell assay, cells migrate after seeding without prior establishment of a monolayer. Thus, the effect of CUB1 on adhesion to the substratum might overcome the effect on cell–cell contacts in this assay. In fact, activation of CDCP1 had no effect on the healing of the scratched cell monolayer in multiple cell lines (data not shown). This is in contrast to a recent report showing that CDCP1 knockdown in MCF10A cells prevents cell migration in the same setting (Spassov *et al.*, 2011). Moreover, as expected from the fact that CDCP1 is not tyrosine-phosphorylated in unstimulated MCF10A cells or after wounding of an epithelial monolayer, knockdown of CDCP1 does not have any effect on cell migration or cell–cell contacts in our experience (CHB, unpublished studies). It remains unclear at this point why our results and those of Spassov *et al.* (2011) are disparate in that regard, as their study also shows that CDCP1 is not tyrosine-phosphorylated in attached, unstimulated cells.

It is possible that CDCP1 is involved in the coordination of cell–matrix and cell–cell adhesion in the normal epithelia. On the other hand, downregulation of E-cadherin and loosening of cell–cell adhesion, as seen frequently in carcinomas or during epithelial-to-mesenchymal transition, could result in a context where CDCP1 activation produces mainly an increase in cell motility. The endogenous mechanism(s) of CDCP1 activation remain

unknown, although proteolysis by matripase or other serine proteases represents an attractive mechanism (He *et al.*, 2010) especially in the context of wound-healing. In this context, our results suggest that CDCP1 might be involved in coordinating cellular adhesion to the extracellular matrix and to other cells.

Our findings concerning downregulation of CDCP1 by prolonged exposure to anti-CDCP1 antibodies have direct relevance to reports that mAbs against CDCP1 block experimental metastasis (Uekita *et al.*, 2008; Deryugina *et al.*, 2009). Our observations bolster the idea that anti-CDCP1 antibodies could be used to promote the down-regulation of CDCP1 and CDCP1/SFK complexes in some cancers, as these reports suggested, and indicate a mechanism (CDCP1 downregulation) by which this occurs.

A comparison of the results obtained in cell culture assays with the phospho-tyrosine profile from human tissue strongly suggests that the signaling events defined using specific CDCP1 activation in cell culture are indeed operating in human tissue. This supports the notion that biomarkers for SFK activity should be chosen carefully depending on the CDCP1 status. In particular, SFK autophosphorylation, together with the phosphorylation status of FAK and of SRC/FAK substrates, has been proposed as a reporter for SFK activity in tumors, based on the fact that SFK activity is best correlated with changes in migration in cell culture models of carcinomas (Brunton *et al.*, 2005; McLean *et al.*, 2005; Serrels *et al.*, 2006). However, in cases where CDCP1 is involved in the regulation of SFK, high levels of SFK activity might not always correlate well with high levels of FAK and SFK/FAK substrate phosphorylation.

Materials and methods

Statistical analysis

Spectral counts of the phospho-tyrosine proteins of 64 NSCLCs and 102 normal lung samples were measured previously by an immunoaffinity-based phospho-proteomic approach (Rikova *et al.*, 2007). Prior to any statistical computation, data were normalized by the quantile approach under the limma package in R 2.10.0, which ensures that intensities have the same empirical distribution across different samples. The differentially phosphorylated proteins between the normal and cancer samples were computed by an empirical Bayes (eBayes) shrinkage of the standard errors toward a common value approach (Smyth, 2004), and *P*-values were adjusted for multiple comparison. A correlation matrix (Supplementary Figure 1S) was developed to identify the best correlations among phospho-tyrosine signals using Pearson correlation coefficients and the Ward's linkage method for hierarchical cluster analysis. The *P*-values for the Pearson correlation coefficients of CDCP1 phosphorylation were calculated based on the asymptotic confidence intervals given from Fisher's *Z*-transform for at least four complete pairs of observation.

Antibodies

The anti-CDCP1 mouse mAb CUB1 was purchased from BioLegend (San Diego, CA, USA) (cat. no. 324002). Anti-CDCP1 mAb clone 309121 (cat. no. MAB26661) and clone 309137 (cat. no. MAB2666) were from R&D Systems (Minneapolis, MN, USA). The rabbit polyclonal anti-CDCP1 antibodies were raised against the cytoplasmic domain of CDCP1 fused to glutathione *S*-transferase (immunization by Cocalico Biologicals (Reamstown, PA, USA)) or against a C-terminal peptide (cat. no. 4115; Cell Signaling Technology (Beverly, MA, USA)). The phospho-specific antibodies against CDCP1 Tyr-734 or Tyr-743 were developed by Cell Signaling Technology. The other phospho-specific antibodies from Cell Signaling Technology were as follows: Tyr-311 PKCδ, Tyr-416 SFKs, Tyr-397 FAK, Tyr-239/240 SHC and Tyr-317 SHC. Another anti-phospho-Tyr-397 FAK from Invitrogen

(Carlsbad, CA, USA) was also used and gave identical results. The anti-phospho-Tyr-576/577 FAK was from Invitrogen and Cell Signaling Technology. Anti-phosphotyrosine PY100 was from Cell Signaling Technology. The anti-SRC clone GD11 monoclonal agarose-conjugated antibody was from Millipore (Billerica, MA, USA). The anti-PKCδ antibody from BD Biosciences (San Jose, CA, USA) (cat. no.610398) was used for immunoprecipitations and the anti-PKCδ antibody from Santa Cruz Biotechnology (sc-213) was used for western blotting. The anti-P130 CAS antibody conjugated to agarose was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (mAb 35B.1A4; cat. no.sc-20029). The anti-paxillin mAb (clone 349; cat. no.610051) was from BD Biosciences; the anti-paxillin polyclonal rabbit antibody was from Cell Signaling Technology (cat. no. 2542); and the rabbit anti-catenin antibody was from Sigma-Aldrich (St Louis, MO, USA) (cat. no. C2206). Rhodamine phalloidin was purchased from Invitrogen (R415).

Cells

The HCT116 and DLD-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. The MCF10A cells were maintained in DMEM/F12 complemented with 5% heat-inactivated horse serum, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin and 100 ng/ml cholera toxin.

siRNA

We tested five different siRNAs against the CDCP1 coding and untranslated region (UTR) sequence. The most efficient siRNA that we identified was directed against the 3′-UTR sequence of the CDCP1 mRNA. 27-mer oligonucleotides (MWG-Biotech, Huntsville, AL, USA) were used at 80-nM final concentration. Transfection was performed using Dharmafect I (Thermo Fisher Scientific, Lafayette, CA, USA) according to the manufacturer's instructions. The siRNA sequence used was: 5′-UAAAGAAGAGGAAUUAUACA GAAGGAA-3′. As control we used an siRNA matching the CDCP1 mRNA, but that lead to no knockdown as determined by western blotting.

Adhesion assay

Tissue culture plates were coated with FN (Sigma; 0.1% solution stock) in phosphate-buffered saline (PBS) overnight at 4 °C. After three washes with PBS, the plates were blocked with 1% bovine serum albumin in PBS for 1 h at 37 °C followed by extensive washing in PBS.

Cells were detached from their culture plates using a non-enzymatic solution (Cell Dissociation Buffer enzyme-free Hank's-based; Invitrogen; cat. no.13150-016), resuspended in DMEM 10% FBS, washed twice in DMEM 0.1% FBS and incubated in DMEM 0.1% FBS at 37 °C for 60 min. CUB1 (1 μg/ml) or a control antibody (1 μg/ml) was added to the cells 15 min before plating. After 60 min the plates were washed to remove non-adherent cells. The cells were fixed using paraformaldehyde and stained using Diff-Quik Solution 2 (Dade Behring Inc., Newark, NJ, USA). After extensive washing, the retained dye was dissolved in 10% acetic acid and the absorbance of the resulting solution was measured. The results are plotted as the percentage of the maximum absorption obtained corresponding to maximum number of adherent cells (20 μg/ml FN+ control antibody). As control antibody, we used either a non-immune mAb (IgG2b; BD Biosciences; cat. no. 555740; matched to the isotype of the anti-CDCP1 mAb CUB1) or an anti-E-cadherin antibody (BD Biosciences; cat. no. 610182). Identical results were obtained with the two control antibodies, or with no antibody.

Cell migration assay (Transwell)

Cells were seeded at a density of 2000 cells/well in 24-well Transwell chambers (Corning, Lowell, MA, USA; 8 μm pore size) in 0.5% FBS DMEM/F12. The CUB1 mAb and the control mAb (IgG2b; BD Biosciences; cat. no. 555740), both at 1 μg/ml, were added at the time of seeding to the top and the bottom chambers. After 24 h, cell migration was determined by staining the cells on the bottom surface of the porous membrane. The cells were fixed and stained on the filters, and cells on the top surface were scraped off. After extensive washing with water, the dye retained in the cells on the bottom surface was extracted using 10% acetic acid. The absorbance was measured and plotted as a ratio of the absorbance obtained from control wells. Similar results were obtained when the cells were visually counted under a microscope instead of using a dye extraction procedure.

Microscopy

MCF10A cells were grown on glass coverslips. Following treatment, the cells were fixed in paraformaldehyde (3.7% in PBS) and permeabilized using 0.1% Triton X-100. Incubation with primary antibodies was performed overnight at 4 °C. Multiple washes were performed in PBS–5% FBS. The secondary antibodies were from Invitrogen (Alexa Fluor-488 and 568). Images were captured on a Zeiss Axiovert 200M and by using the Axiovision software. All images are representative of at least four different experiments and were chosen to illustrate the average behavior of the entire cell population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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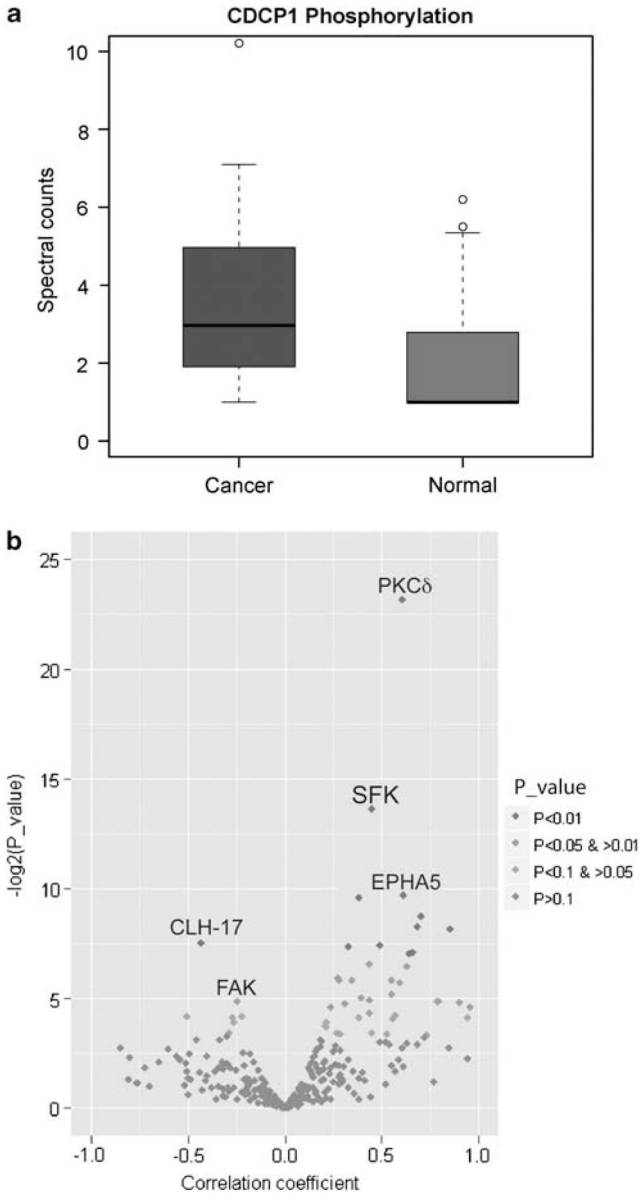

Figure 1.

Correlation analysis of tyrosine phosphorylation of proteins in NSCLCs and normal lung tissue. (**a**) Box-plot diagrams showing the distribution of CDCP1 phosphorylation in lung tumors versus that in normal samples. (**b**) A volcano plot of −log2(*P*-values) (*y*-axis) against the correlation coefficients (*x*-axis) of the phosphotyrosine proteins that were correlated with CDCP1 tyrosine phosphorylation.

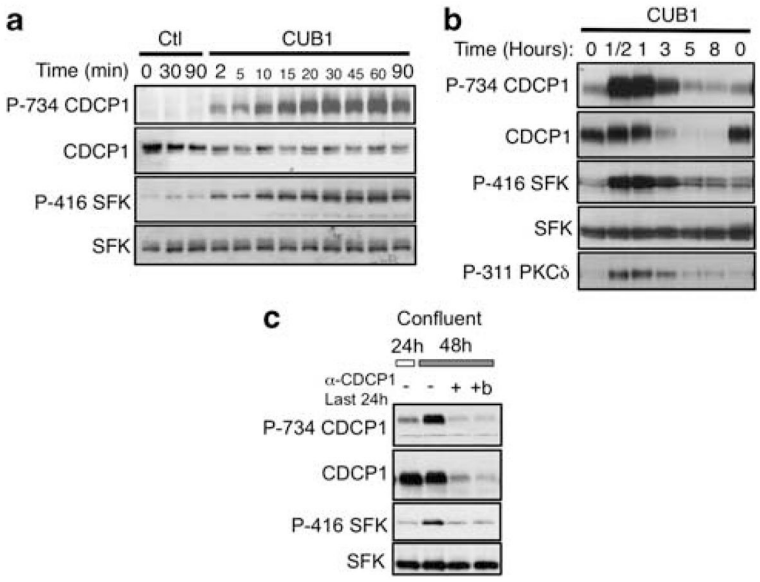

Figure 2.

Regulation of SFK phosphorylation downstream from CDCP1 in HCT116 cells. (**a**) An anti-CDCP1 mAb (CUB1) promotes the tyrosine phosphorylation of CDCP1 and activation of SFK. Cells were stimulated by addition of the CUB1 mAb at 0.5 μg/ml in complete growth medium for 0–90 min. An isotype-matched antibody was used as control (Ctl). The P-734-CDCP1 polyclonal antibody recognizes CDCP1 when phosphorylated on Tyr-734. The P-416 SFK polyclonal antibody recognizes the activation loop of SFKs when phosphorylated on Tyr-416. (**b**) Long-term exposure of cells to the CUB1 mAb downregulates the CDCP1 protein and reduces the phosphorylation of SFK and PKCδ. (**c**) Downregulation of CDCP1 by prolonged exposure to CUB1 mAb blocks the activation of SFKs and the tyrosine phosphorylation of CDCP1 observed in cells maintained at confluence for 48 h. +, CUB1 mAb; + b, anti-CDCP1 mAb RD clone 309131.

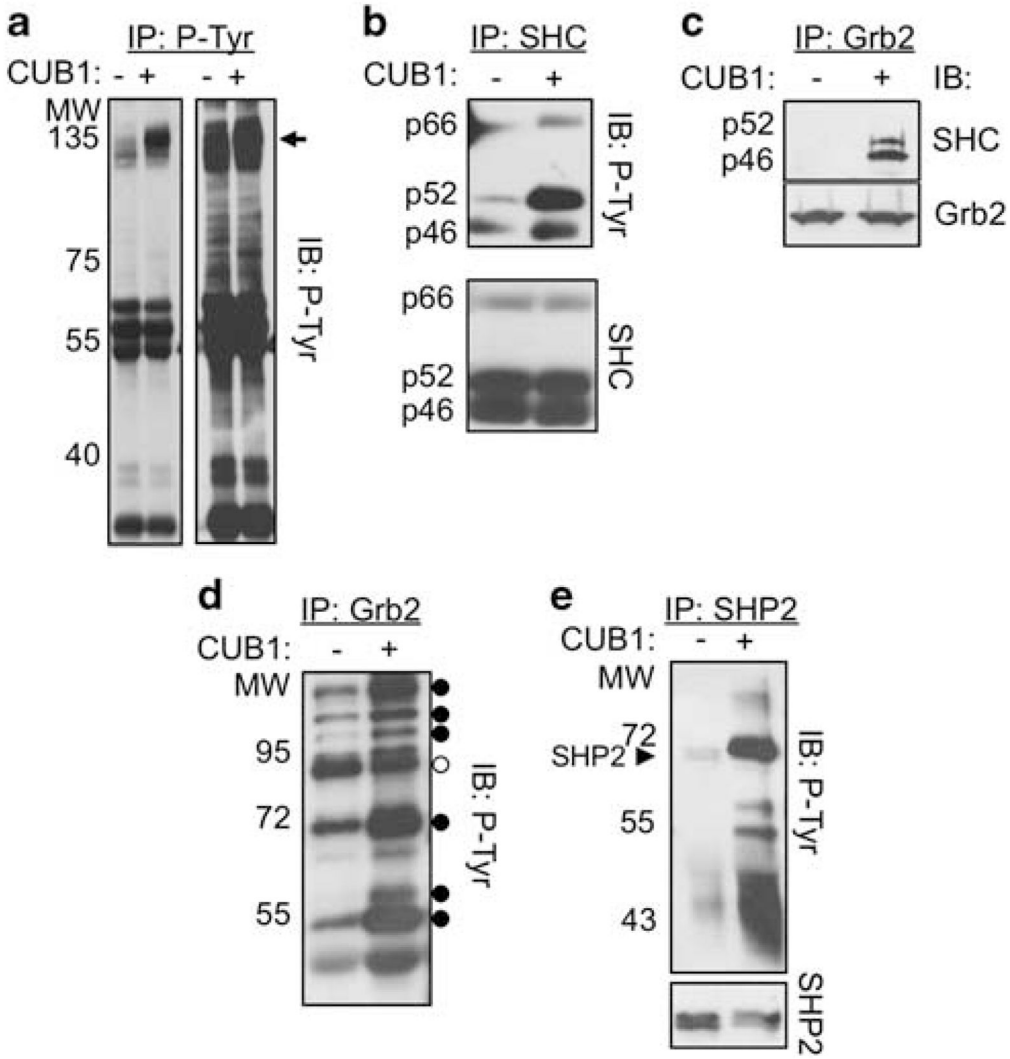

Figure 3.

Signaling events triggered by the CUB1 mAb. In all panels HCT116 cells were stimulated with 0.5 μg/ml CUB1 mAb for 30 min. (**a**) The PY100 monoclonal anti-phospho-tyrosine antibody was used for immunoprecipitation and immunoblotting. (**b**) Tyrosine-phosphorylated p52 and p46 SHC detected in anti-SHC immunoprecipitates. (**c**) SHC binds to GRB2 in response to CUB1. Anti-GRB2 immunoprecipitates were blotted for SHC, and then re-probed with an anti-GRB2 antibody. (**d**) Anti-GRB2 immunoprecipitates were blotted with anti-phospho-tyrosine. (**e**) The anti-SHP2 antibody was used for immunoprecipitation, and immunoblotting was performed as indicated.

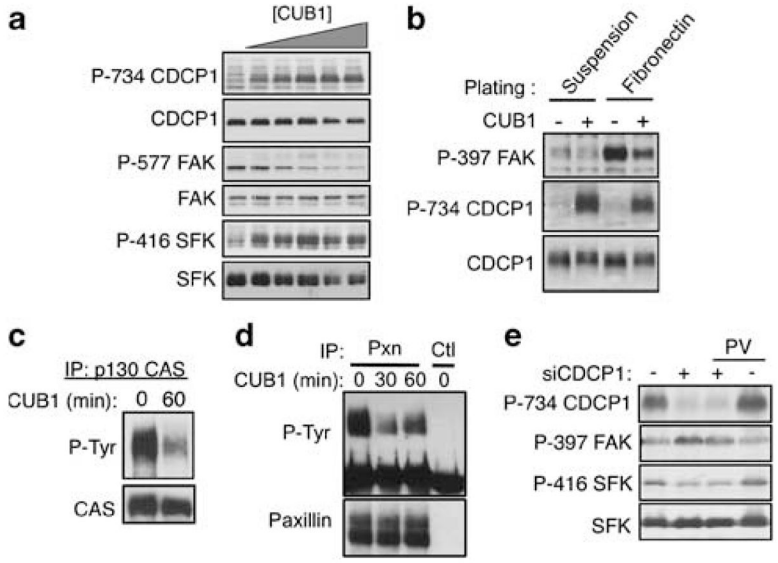

Figure 4.

FAK signaling is inhibited in response to CDCP1 and SFK tyrosine phosphorylation. (**a**) Adherent HCT116 cells were starved overnight in 0.1% FBS and stimulated for 60 min with increasing doses of CUB1 mAb: 0.25, 0.5, 1.0, 5 and 10 μg/ml. FAK tyrosine phosphorylation at the SFK-dependent site 576/577 was determined along with CDCP1 and SRC tyrosine phosphorylation. (**b**) HCT116 cells were detached from culture plates using a non-enzymatic solution (see Materials and methods). Following a 30-min suspension in 0.1% FBS (DMEM/F12), the cells were plated onto an FN-coated surface (coating at 5 μg/ml) or maintained in suspension for an additional 30 min. The CUB1 mAb (1 μg/ml) was added 15 min before plating where indicated, and under all conditions all cells both adherent and suspended were collected. Phosphorylation of FAK and CDCP1 was determined as indicated. (**c, d**) Adherent HCT116 cells were starved (0.1% FBS) overnight and stimulated with 1 μg/ml CUB1 mAb for 60 min as indicated or left untreated (Ctl). P130 CAS (in panel c) or paxillin (Pxn, in panel **d**) were immunoprecipitated, their phosphotyrosine contents were determined using an anti-phospho-tyrosine antibody (P-Tyr) and they were re-blotted for CAS and paxillin. (**e**) HCT116 cells were transfected with siRNA targeting CDCP1 mRNA (+) or a control siRNA (−). The cells were maintained at confluence for 48 h in full serum and lysed 96 h after transfection. The phosphorylation status of FAK, SFK and CDCP1 was determined as indicated. A total SFK immunoblot was used as loading control. FAK and the other proteins analyzed in these experiments were extracted from cells using boiling 1% sodium dodecyl sulfate sample buffer.

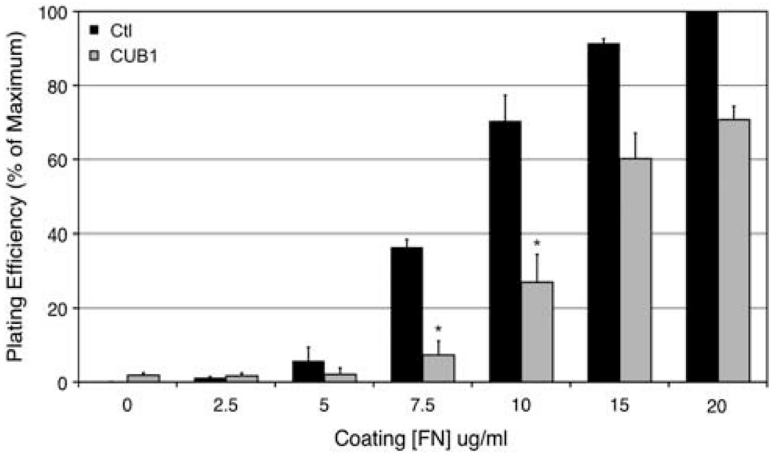

Figure 5.

CDCP1 activation inhibits cell adhesion to FN. HCT116 cells were detached from their culture plates using a non-enzymatic solution. The cells were maintained in suspension for 60 min in 0.1% FBS (DMEM/F12) prior to the addition of CUB1 mAb or an isotype-matched antibody. Fifteen minutes after antibody addition, the cells were added to the dish, with the surface coated with the indicated concentration of FN. After 60 min the number of adherent cells was determined by staining (see Materials and methods). The results are plotted as percentage of maximal adhesion (Ctl mAb, 20 μg/ml FN). The error bars represent the standard deviation of three independent experiments. Student's *t*-test for Ctl versus FN; **P*<0.01.

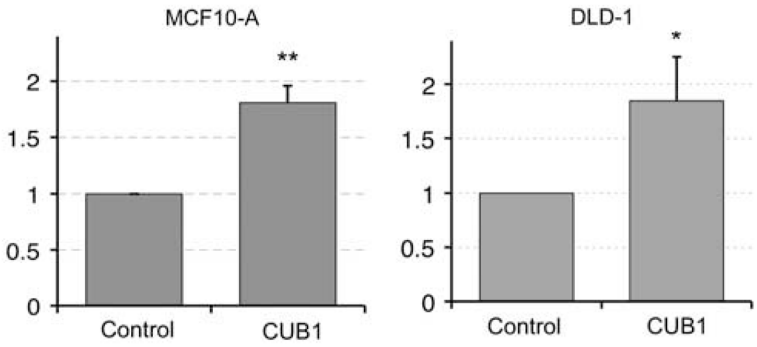

Figure 6.

The migration of epithelial cells is increased by the CUB1 mAb. The CUB1-dependent cell migration (from the top to the bottom of the filter) was calculated relative to the migration of control mAb-treated cells. Each panel is from three independent experiments, each performed in triplicate. The error bars correspond to the standard deviation between experiments. Student's *t*-test for Ctl versus CUB1 migration. MCF10A, $^{**}P<0.01$; DLD1, $^{*}P<0.05$.

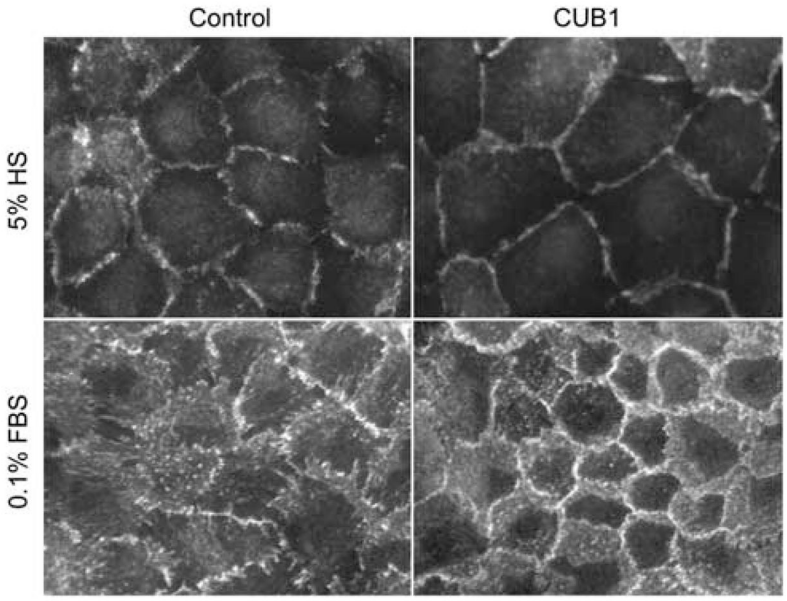

Figure 7.
CDCP1 activation leads to cell-cell contact re-arrangement. MCF10A cells were grown to confluence and either fed full serum or starved in 0.1% FBS (DMEM/F12) for 20 h. CDCP1 tyrosine phosphorylation was induced by CUB1 mAb (60 min, 1 μg/ml) and the effects on cell–cell contacts were characterized by staining adherens junctions using an anti-β-catenin antibody.

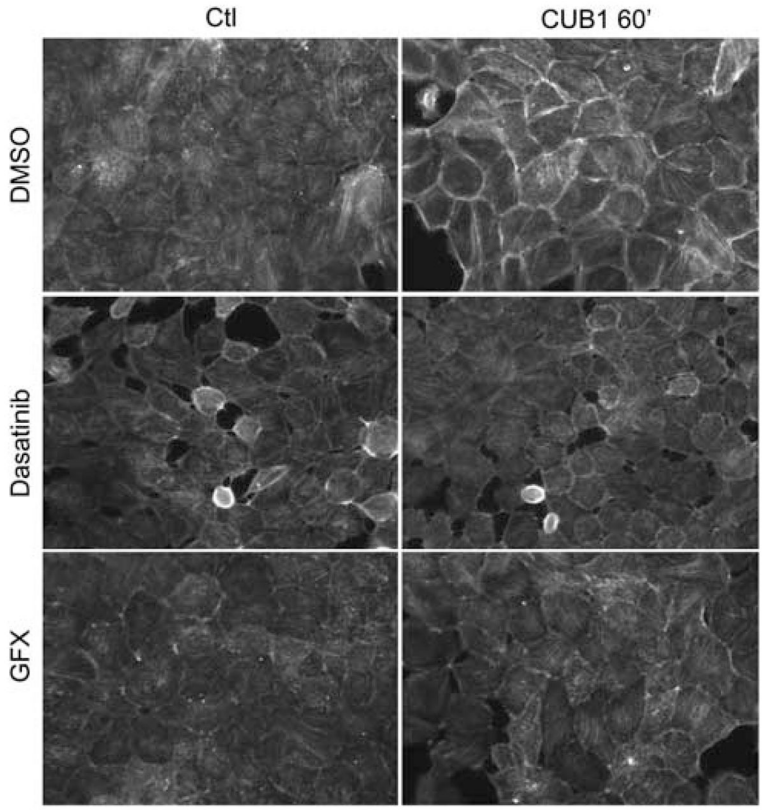

Figure 8.

CDCP1 activation promotes cortical actin fiber accumulation at cell–cell contacts. MCF10A cells seeded on glass coverslips were grown to 90% confluence, incubated with dasatinib (100 nM), GF109203X (5 μM) or DMSO for 15 min, and stimulated with the CUB1 mAb (1 μg/ml) for 60 min. Actin staining was performed using rhodamine phalloidin.

Table 1

Phospho-tyrosine proteins significantly ($P<0.05$) correlated positively or negatively (Pearson correlation) with phosphorylation of CDCP1 in both normal and cancer samples ($n=76$)

Correlation	PTYR protein	Coefficient	*P*-value
Positive	PKCD	0.60	1.06E–07
	SFK[a]	0.45	7.97E–05
	EphA5	0.61	0.001
	SRC	0.38	0.001
	PTRF	0.70	0.002
	DSC3	0.69	0.003
	FLNB	0.85	0.003
	ITGB4	0.49	0.006
	p38-δ	0.33	0.006
	JNK1	0.66	0.007
	JNK3	0.66	0.007
	DSC2	0.64	0.008
	Eps8	0.44	0.011
	Arp3	0.63	0.011
	GRF-1	0.27	0.017
	Plakophilin-1	0.55	0.018
	ERK5	0.35	0.018
	Yes	0.28	0.018
	Plakophilin-3	0.60	0.019
	FLJ32810	0.55	0.028
	APP iso8	0.40	0.031
	EphA2	0.43	0.034
	Desmoplakin-3	0.79	0.034
	K8	0.79	0.035
	Cortactin	0.90	0.036
	KIAA1217	0.31	0.036
	FAM62A	0.24	0.041
	Nectin-1	0.96	0.042
	Axl	0.43	0.049
Negative	CLH-17	−0.43	0.005
	FAK	−0.25	0.034

Abbreviation: CDCP1, CUB (complement C1r/C1s, Uegf, Bmp1) Domain-Containing Protein-1.

[a]The major peptide corresponding to this correlation value is shared by several SFK members (LCK, FYN, YES and SRC).