The homophilic cell-cell adhesion receptor CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1, CD66a) acts as a regulator of contact-dependent cell survival, differentiation, and growth. It is involved in the control of proliferation in hematopoietic and epithelial cells and can act as a tumor suppressor. In this study, we identify DNA polymerase δ-interacting protein 38 (PDIP38) as a novel binding partner for CEACAM1-L and CEACAM1-S. We show that PDIP38 can occur in the cytoplasm and at the plasma membrane in NBT-II, IEC18, RBE, and HeLa cells and that the distribution in NBT-II cells is influenced by the fluorescence of the cells. We also demonstrate that the interaction of CEACAM1 and PDIP38 is of functional importance in NBT-II cells, which co-express the long and the short CEACAM1 isoform. In subconfluent, proliferating NBT-II cells, perturbation of CEACAM1 by antibody clustering induces increased binding to PDIP38 and results in rapid recruitment of PDIP38 to the plasma membrane. The same treatment of confluent, quiescent NBT-II cells leads to a different response, i.e. translocation of PDIP38 to the nucleus. Together, our data show that PDIP38 can shuttle between the cytoplasmic and the nuclear compartments and that its subcellular localization is regulated by CEACAM1, implicating that PDIP38 may constitute a novel downstream target of CEACAM1 signaling.

The fate of a cell is controlled by its environment, including the extracellular matrix, neighboring cells, and soluble factors. The signals mediated by this environment frequently are transduced by components of cell-matrix or cell-cell adhesion complexes, like integrins, Ig-cell adhesion molecules, or cadherins (1). CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1) is a cell-cell adhesion receptor belonging to the CEA family within the Ig-like cell adhesion molecules. CEACAM1 acts as an important regulator of contact-dependent control of cell survival, differentiation, and growth in various normal and transformed/cancerous cells of epithelial, endothelial, and hematopoietic origin, as reviewed previously (2, 3).

The two major CEACAM1 isoforms are co-expressed in most cells and consist of four extracellular Ig-like domains, a transmembrane domain, and, as a result of differential splicing, a long (L, 71 aa) or a short (S, 10 aa) cytoplasmic domain. Both isoforms mediate cell-cell adhesion via homophilic binding (4, 5). CEACAM1 can also bind to other CEA family members (6) and is used as a receptor by viral and bacterial pathogens (7–9). Recent studies show that there is an important role for CEACAM1 in modulating the immune responses associated with infection, inflammation, and cancer, as reviewed previously (10, 11). CEACAM1 controls differentiation processes (12–14) and apoptosis (13, 15–17) and can act as a tumor suppressor, as reviewed before (2, 18). We have previously demonstrated that CEACAM1 is implicated in the regulation of proliferation and contact inhibition in epithelial NBT-II cells (19,
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The influence on differentiation as well as the tumor suppressor activity may at least partially be due to the inhibitory impact of CEACAM1 on proliferation (21–24).

All these cellular responses originate in the activation or inhibition of CEACAM1 by homophilic or heterophilic interactions. But how does CEACAM1 communicate its signal to the inside of the cell? By now, some mechanisms of signaling by CEACAM1 have been described, e.g. phosphatidylinositol 3-kinase/Akt-dependent (15, 25) and MEK/MAPK-dependent (17, 19, 26) signaling. The CEACAM1-L cytoplasmic domain bears two phosphorylatable tyrosine residues within the immunoreceptor tyrosine-based inhibition and immunoreceptor tyrosine-based switch motifs and binds to protein-tyrosine kinases of the Src family (27, 28) and the protein-tyrosine phosphatases SHP1 and -2 (29, 30). It can also bind to calmodulin (31) and to scaffolding proteins like filamin A and talin (14, 32). Still, little is known about how CEACAM1 accomplishes its effects on the various cellular functions mentioned above, and there might be further mechanisms that link CEACAM1 to the different regulatory mechanisms of gene expression and cell fate control. With this in mind, we performed a yeast two-hybrid screen to search for new CEACAM1-L-interacting proteins that might play a role in CEACAM1 signaling. Thus we identified PDIP38 (DNA polymerase δ-interacting protein, 38-kDa apparent molecular mass, also known as Poldip2 and Mitogenin I) as a novel interaction partner. PDIP38 was originally identified as a PCNA- and DNA polymerase δ-interacting protein, implicating a function in the regulation of gene expression, DNA duplication, or DNA repair (33). PDIP38 thus represents an interesting interaction partner for CEACAM1, because it could link the transmembrane adhesion receptor signaling directly to regulatory processes inside the nucleus.

On the other hand, PDIP38 has been reported to be associated with mitochondria in three previous studies, which might not be congruent with a role in regulation of nuclear functions (34–36). However, these studies were largely performed with transfected GFP-tagged PDIP38, which might give different subcellular localization patterns compared with that of endogenously expressed PDIP38. Therefore, with the aim to determine the subcellular distribution of endogenous PDIP38, we generated a panel of specific antibodies against PDIP38 that could be used for this purpose. We could thus demonstrate that endogenous PDIP38 interacts with both CEACAM1-L and CEACAM1-S and that it localizes to different cellular compartments, i.e. the plasma membrane, the cytoplasm, and the nucleus. We found only a minor amount of endogenous PDIP38 associated with mitochondria. In NBT-II cells, the subcellular localization of PDIP38 depended on the cellular state and was affected by CEACAM1 perturbation using CEACAM1-specific antibody clustering. In proliferating NBT-II cells, this treatment led to rapid recruitment of PDIP38 to the plasma membrane. In contrast, the same treatment applied to quiescent NBT-II cells led to translocation of PDIP38 to the nucleus. The present study accordingly demonstrates that CEACAM1 can regulate the subcellular distribution of PDIP38 in NBT-II cells, suggesting that PDIP38 may represent a novel downstream target of CEACAM1 signaling.

EXPERIMENTAL PROCEDURES

Antibodies, Peptides, Cell Lines, Vectors, and Materials—The monoclonal anti-rat CEACAM1 antibody Be9.2 (37) and the monoclonal anti-mouse CEACAM1 antibody AgB10 (38) (not cross-reacting with rat CEACAM1) were described previously. Other monoclonal antibodies used were: anti-α3 integrin (Ralph3.2), anti-phospho tyrosine (PY99, Santa Cruz Biotechnology, Santa Cruz, CA); anti-PCNA (PC10, Zymed Laboratories Inc.); anti-His-Tag (Merck); and anti-DNA polymerase δ (p125 subunit) and anti-β1 integrin (BD Transduction Laboratories). Polyclonal antibodies used were: rabbit-anti-Histon H3 (Abcam); rabbit-anti-FAK (A-17) and rabbit-anti-TOM20 (FL-145) (Santa Cruz Biotechnology); rabbit-anti-mouse IgG, goat-anti-mouse IgG Alexa Fluor 488-conjugate, goat-anti-rabbit IgG Alexa Fluor 546-, and Alexa Fluor 488-conjugate (Molecular Probes); and peroxidase-conjugated goat-anti-mouse IgG and peroxidase-conjugated goat-anti-rabbit IgG (Jackson ImmunoResearch). Control IgG from rabbit non-immune serum was purified using protein A-Sepharose (Amersham Biosciences). The PDIP38 peptides P3 (NH2-CKAENPAGHG-SKEVKGKTH-CO NH2) and P8 (NH2-CSLESNKDETPPS-GLHW-COOH), as well as the rabbit and guinea pig antiserum against the peptides P3 and P8, were produced by Pineda-Antikörperservice (Berlin, Germany). If not stated otherwise, the IgG fractions of both rabbit antiserum (P8R and P3R) were used, whereas the guinea pig antiserum (P8G and P3G) were used as whole sera only.

Rat brain endothelial (RBE) cells (14) were transfected with rat CEACAM1-L, CEACAM1-S, and CEACAM1-Δcyto (a mutant lacking the cytoplasmic domain) cDNA. The vectors containing these cDNAs were described previously (26). RBE, Hela, and Chinese hamster ovary cells were cultured in RPMI medium, and NBT-II and IEC18 cells in Dulbecco’s modified Eagle’s medium, both containing 10% FBS and penicillin-streptomycin; for transfected cells, 0.6 g/liter G418 was added. All cell culture media and reagents were from Invitrogen.

pGEX-4T and pRSET-A vectors were from Amersham Biosciences. The full-length mouse PDIP38 cDNA (clone IMGAP998F089456) from the IMAGE Consortium (Lawrence Livermore National Laboratory) (39) was obtained from the Deutsches Ressourcenzentrum für Genomforschung (Berlin, Germany). All cDNAs used were verified by sequencing using the Thermo Sequenase primer cycle sequencing kit (Amersham Biosciences). Restriction and DNA-modifying enzymes were from Fermentas or Invitrogen. All other reagents were from Sigma-Aldrich unless otherwise stated.

Yeast Two-hybrid Screen—The yeast two-hybrid MATCHMAKER 3 GAL-4-based system (BD Biosciences Clontech) was used as described earlier (32). Briefly, the yeast strains AH109 and CG1945 were sequentially transformed with pGBKTT7-C1-L-cyto (containing aa 450–519 of rat CEACAM1-L) and the rat liver MATCHMAKER cDNA library (32). Yeast plasmid was extracted using the YEASTMAKER kit (BD Biosciences Clontech). Specificity of reporter gene activation was confirmed by transformation of prey plasmid alone and by one-on-one transformations of prey plasmid pACT2-PDIP38 (aa 296–370) together with pGBKTT7-DNA-BD,
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pGBK7–53, pGBK7-Lam (negative controls), or bait plasmid. Clones were tested for growth on selection medium lacking leucine, tryptophan, and histidine, or were grown on selection medium lacking leucine and tryptophan and tested for β-galactosidase reporter gene activity in filter-lift assays and for α-galactosidase reporter gene activity in liquid assays (substrate: p-nitrophenyl-α-D-galactopyranoside).

Yeast Two-hybrid Analysis of Binding Sites—The following constructs were cloned and tested in one-on-one transformations in AH109 yeast cells as described above. Stop codons were inserted in the pGBK7–CC1-L-cyto and pACT2–PDIP38 plasmids by PCR point mutation using the following primer pairs: (1) CC1-L-cyto–H471stop: CCT CAA CCT CCA GCT AGA ATC TGG GTC CTT (sense), GAA GGA CCC AGA TTC TAG CTG GAG GTT GAG (reverse); (2) CC1-L-cyto–Y488stop: GAC GTC TCA TAG TCT GTC CTG AAC (sense), GTT CAG GAC AGA CTA TGA GAC GTC (reverse); (3) CC1-L-cyto–S503stop: CGA CCA ACT TGA GCC TCC TCA TCA AGC (sense), GCT TGA AGG TCA AGC TTG TCG (reverse); (4) CC1-L-cyto–Y513stop: CAG AAA CAG TTT AGT CGG TAG TC (reverse); (5) PDIP38–Y319stop: GCC TGC ATT CCA GTA GAG CAG CCA T (sense), ATG GCT GCT CTA CTG GAA TGC AGG C (reverse); (6) PDIP38–G330stop: CTG CAA GCT TCC ATG ATG TCA ATG TGC TGG AC (sense), GTG CCC CAC ATG TGT CAA CTG GAA CCA TGC AG (reverse); and (7) PDIP38–V348stop: TCC CAC TTT GAT TAA CGG ATC ATC CCC (sense), GGG TGG GAT CCG TTA ATC AAA GGA AGA (reverse).

Generation and Purification of Recombinant Proteins—The full-length mouse PDIP38 cDNA (IMAGp998F089456) was excised from the pCMV-SPORT6 plasmid and cloned into the EcoRI and Xhol sites of the pGEX-4T-3 plasmid, creating a GST-PDIP38 fusion protein with the GST tag at the N terminus. GST-PDIP38 (65 kDa) and GST (26 kDa) were expressed in E. coli (strain BL21) (Invitrogen) and purified using glutathione-Sepharose (Amersham Bioscience). The generation, expression, and purification of the His-tagged cytoplasmic domain of rat CEACAM1-L (aa 447–519, CC1-L-cyto-His, 14 kDa) and the His-tagged control protein resulting from a frameshift of the UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase cDNA (GNE, 12 kDa, Fig. 2a) was done as described (40). The purified His-tagged cytoplasmic domain of the α3 integrin subunit was kindly provided by Diana Mutz (Berlin, Germany).

Surface Plasmon Resonance Analysis—Analyses were performed in a BIAcore 2000 instrument (BIAcore AB). The His-tagged cytoplasmic domains of rat CEACAM1-L (CC1-L-cyto-His) and of rat α3 integrin (α3-cyto-His, background control) were immobilized on a CM5 sensor chip (Amine Coupling Kit, BIAcore AB). For immobilization, 40 μl of CC1-L-cyto-His or 80 μl of α3-cyto-His (each 200 μg/ml in 10 mM sodium acetate, pH 2.7) were applied. All coupling reactions were carried out at a flow rate of 5 μl/min. Biomolecular interaction analyses were performed in HBS Buffer (150 mM NaCl, 0.005% (v/v) Tween 20, 3.4 mM EDTA, 10 mM HEPES, pH 7.4) at a flow rate of 10 μl/min. Between sample injections the surface was regenerated with 5 μl of 15 mM HCl. Binding constants were calculated using the BIAevaluation software.

Immunoprecipitation, Binding Assays, and Western Blotting—Cells were lysed in HBS (10 mM Hepes, pH 7.2, 150 mM NaCl) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Sigma). When tyrosine phosphorylation was required, phosphatase inhibitors were added (1 mM Na3VO4, 10 mM Na2P2O7, 100 mM NaF). For immunoprecipitations, equal amounts of cleared lysates were incubated with 2–4 μg of the appropriate protein A-Sepharose-bound antibodies or Ig controls, washed and subjected to SDS-PAGE (NuPAGE Novex Bis-Tris Gels, NuPAGE MOPS buffer, Invitrogen). For affinity binding assays, 10 μg of GST or GST-PDIP38 bound to glutathione-Sepharose were incubated with 5 μg of CC1-L-cyto-His or 5 μg of control-His (GNE, frame-shifted), or with RBE-CC1-L/S cell lysate for 2 h at 4 °C, washed, and subjected to Tricine-SDS-PAGE. Alternatively, 3 μg of nickel-Sepharose-bound CC1-L-cyto-His or α3-cyto-His (control) were incubated with NBT-II cell lysates. Protein detection on Western blots using nitrocellulose membrane (Schleicher & Schuell) was performed using the luminol-based SuperSignal West Pico Chemiluminescent Substrate (Pierce). Detection of chemiluminescence and quantification was done using the Fujifilm LAS-1000 digital system. Images were imported into Adobe Photoshop program for processing.

In-gel Digestion and Peptide Mass Fingerprint—After immunoprecipitation using P8R antibody, the protein band with an apparent molecular mass of 39 kDa was excised from a Coomassie G-250-stained gel and cleaved in situ with trypsin (Roche Diagnostics) as described previously (41). Digested samples were desalted using a Zip-Tip-C18 (Millipore) as proposed by the manufacturer, and the peptides were eluted from the tip filter with 10 μl of 50% acetonitrile/0.1% trifluoroacetic acid saturated with α-cyano-4-hydroxycinnamic acid. 1.5 μl of the eluate was immediately spotted onto a MALDI-TOF target and analyzed by MALDI-TOF mass spectrometry in a Bruker reflex mass spectrometer (Bruker Daltonics, Bremen, Germany) in the reflector mode, equipped with pulse-ion extraction and a nitrogen laser (337 nm). Spectra were calibrated with adrenocorticotropic hormone fragment 18–39 and angiotensin II (both Sigma). Searches were performed in Mascot (Matrix Science Ltd.) using the data base NCBI and taxonomy Rattus norvegicus. Mass tolerance was set to 0.5 Da, and one miscleavage was allowed.

Immunofluorescence—Cells were grown on tissue culture-treated 8-well Lab-Tek chamber slides (Nunc). When indicated, cells were treated with antibodies as described, or were incubated with MitoTracker Red CMXRos (1:2000) for 20 min prior to fixation. In some cases, cells were serum-starved for 28 h and then treated with 10% serum prior to fixation. Cells were fixed for 10 min in 4% paraformaldehyde/PBS at room temperature and permeabilized for 10 min in 4% paraformaldehyde/PBS/0.025% saponin. Slides were blocked with 2% bovine serum albumin/PBS, incubated with 10 μg/ml P8R and/or Be9.2 antibody for 2 h or overnight, washed, and incubated with the appropriate secondary antibodies (1:200) for 2 h. Samples were mounted in Elvanol (70% PBS, 25% glycerol, 5% Polyviol, 0.5% β-mercaptoethanol, 1 mg/ml phenylenedia-
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mine). Images were obtained by confocal laser scanning microscopy (Axiocvert 200 inverse microscope equipped with the LSM 510 META system, Zeiss) at 63× magnification (Zeiss Plan-Apochromat, numerical aperture 1.4) or 40× magnification (Zeiss Plan-Neofluar, numerical aperture 0.75). Images were imported from the LSM 5 Image Examiner 3.2 software into Adobe Photoshop program for processing.

Subcellular Fractionation—All steps were performed on ice or at 4 °C, if not stated otherwise. The total volume of the samples was determined in all steps. NBT-II and HeLa cells were washed 1× with hypo-osmolar buffer (10 mM Hepes, pH 7.2, 2 mM KCl, 1.5 mM MgCl₂), scraped into 750 μl of ice-cold hypo-osmolar buffer containing 1 mM phenylmethylsulfonyl fluoride and 1:100 protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, E-64, betastatin, leupeptin, and aprotinin (Sigma)), and incubated for 30 min. Cells were homogenized using a Dounce homogenizer and centrifuged at 9,000 g (10 min). The supernatant was fraction “sn.” The pellet (fraction “in”) was re-pelleted at 600 g (10 min). The resulting mitochondrial enriched fraction (fraction “mi”) was cleared by centrifugation at 17,000 g (10 min). The supernatant was re-pelleted at 600 × g (10 min) and the pellet was discarded. The supernatant (fraction “s1”) was then centrifuged at 10,000 × g (10 min). The resulting cytoplasmic soluble fraction was solubilized in 750 μl of reducing Laemmli buffer (60 mM Tris-HCl, pH 6.8; 10% glycerol (v/v); 2.5% SDS (w/v); 0.006% bromophenol blue (w/v); 10 mM dithiothreitol). The 600 × g supernatant was re-pelleted at 600 × g (10 min) and the pellet was discarded. The supernatant (fraction “s1”) was then centrifuged at 9,000 × g (10 min). The resulting mitochondrial enriched fraction was solubilized in 1 ml of ice-cold hypo-osmolar buffer and re-pelleted. The pellet was dissolved in 500 μl of hypo-osmolar buffer (20 mM Hepes, pH 7.2, 600 mM NaCl, 1 mM MgCl₂) containing 1 mM phenylmethylsulfonyl fluoride, 400 μg/ml DNase I, and 1:100 protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, E-64, betastatin, leupeptin, and aprotinin (Sigma)), and incubated for 30 min. Cells were homogenized using a Dounce homogenizer and centrifuged at 600 × g (3 min). The resulting nuclear pellet was re-homogenized in 1 ml of hypo-osmolar buffer and re-pelleted. The pellet was dissolved in 200 μl of ice-cold hypo-osmolar buffer containing 1 mM phenylmethylsulfonyl fluoride and 1:100 protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, E-64, betastatin, leupeptin, and aprotinin (Sigma)), and incubated for 30 min. Cells were homogenized using a Dounce homogenizer and centrifuged at 600 × g (3 min). The resulting nuclear pellet was re-homogenized in 1 ml of hypo-osmolar buffer and re-pelleted. The pellet was dissolved in 200 μl of ice-cold hypo-osmolar buffer containing 1 mM phenylmethylsulfonyl fluoride and 1:100 protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, E-64, betastatin, leupeptin, and aprotinin (Sigma)), and incubated for 30 min. Cells were homogenized using a Dounce homogenizer and centrifuged at 600 × g (3 min). The resulting nuclear pellet was re-homogenized in 1 ml of hypo-osmolar buffer and re-pelleted. The pellet was dissolved in 200 μl of ice-cold hypo-osmolar buffer containing 1 mM phenylmethylsulfonyl fluoride and 1:100 protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, E-64, betastatin, leupeptin, and aprotinin (Sigma)), and incubated for 30 min. Cells were homogenized using a Dounce homogenizer and centrifuged at 600 × g (3 min). The resulting nuclear pellet was re-homogenized in 1 ml of hypo-osmolar buffer and re-pelleted. The pellet was dissolved in 200 μl of ice-cold hypo-osmolar buffer containing 1 mM phenylmethylsulfonyl fluoride and 1:100 protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, E-64, betastatin, leupeptin, and aprotinin (Sigma)), and incubated for 30 min. Cells were homogenized using a Dounce homogenizer and centrifuged at 600 × g (3 min).

RESULTS

Identification of PDIP38 as a Novel CEACAM1-L-interacting Protein—A yeast two-hybrid screen against a rat liver cDNA library, using the cytoplasmic domain of rat CEACAM1-L (CC1-L-cyto) as a bait, identified PDIP38 as a putative CEACAM1-L-interacting protein. The interacting clone encoded the C-terminal 74 amino acids of the rat PDIP38 homologue (aa 296–370, accession number: XM_001080851, Fig. 1C). The specificity of the interaction was verified by one-on-one transformation in AH109 yeast cells for growth on selection medium lacking histidine and for β-galactosidase reporter gene activity in filter-lift assays. For this purpose, the full-length mouse PDIP38—A yeast two-hybrid screen against a rat liver cDNA library, using the cytoplasmic domain of rat CEACAM1-L (CC1-L-cyto) as a bait, identified PDIP38 as a putative CEACAM1-L-interacting protein. The interacting clone encoded the C-terminal 74 amino acids of the rat PDIP38 homologue (aa 296–370, accession number: XM_001080851, Fig. 1C). The specificity of the interaction was verified by one-on-one transformation in AH109 yeast cells for growth on selection medium lacking histidine and for β-galactosidase reporter gene activity in filter-lift assays. For this purpose, the full-length mouse PDIP38 was co-transfected with pACT2-PDIP38 (aa 296–370) and pGBK77-CCEACAM1-L-cyto or negative control pGBK77-Lam, and tested for β-galactosidase reporter gene activity in liquid assays. To locate the binding site of CEACAM1-L and PDIP38 for each other, the following constructs were tested in one-on-one transformations in AH109 yeast cells for growth on selection medium lacking histidine and for β-galactosidase reporter gene activity in filter-lift assays as indicated: CC1-L-cyto (aa 450–519, bait), CC1-L-cyto-Y513stop, CC1-L-cyto-S503stop, CC1-L-cyto-Y488stop, CC1-L-cyto-H471stop, PDIP38 (aa 296–370, prey), PDIP38-Y319stop, PDIP38-G330stop, and PDIP38-V348stop. The mutant PDIP38 proteins (fraction “me”) were solubilized in 75 μl of reducing Laemmli buffer.

Characterization of the CEACAM1/PDIP38-binding Properties—To further specify the mutual binding sites, we constructed truncated versions of the CEACAM1-L cytoplasmic domain (bait) and the PDIP38 C terminus found in the yeast two-hybrid screen. The different constructs were co-transfected into yeast strain AH109 and tested for β-galactosidase activity and growth on selection medium lacking histidine. The results of these assays are shown in Fig. 1B and summarized in Fig. 1C. The 21 membrane-proximal amino acids of the CEACAM1-L cytoplasmic domain were sufficient to bind PDIP38. In PDIP38, amino acids 330–348 were essential for binding CEACAM1-L.

Direct binding of the two proteins was tested by in vitro binding assays. For this purpose, the full-length mouse PDIP38 cDNA was expressed as a GST fusion protein. The mouse PDIP38 protein is completely identical with the rat PDIP38 protein within the region encoded by the yeast two-hybrid clone. In affinity precipitation studies, purified GST-PDIP38

FIGURE 1. Identification of PDIP38 as a CEACAM1-L-interacting protein by yeast two-hybrid analysis. A, CG1945 and AH109 yeast cells were co-transfected with pACT2-PDIP38 (aa 296–370) and pGBK77-CCEACAM1-L-cyto or negative control pGBK77-Lam, and tested for β-galactosidase reporter gene activity in liquid assays. B, to locate the binding site of CEACAM1-L and PDIP38 for each other, the following constructs were tested in one-on-one transformations in AH109 yeast cells for growth on selection medium lacking histidine and for β-galactosidase reporter gene activity in filter-lift assays as indicated: CC1-L-cyto (aa 450–519, bait), CC1-L-cyto-Y513stop, CC1-L-cyto-S503stop, CC1-L-cyto-Y488stop, CC1-L-cyto-H471stop, PDIP38 (aa 296–370, prey), PDIP38-Y319stop, PDIP38-G330stop, and PDIP38-V348stop. C, partial schematic representation of rat PDIP38 (accession number: XM_001080851).
bound to glutathione-Sepharose specifically precipitated the purified His-tagged cytoplasmic domain of rat CEACAM1-L (CC1-L-cyto-His) (Fig. 2A).

The direct interaction of CEACAM1-L and PDIP38 was confirmed by surface plasmon resonance-based binding measurements. This revealed a concentration-dependent binding of GST-PDIP38 to immobilized CC1-L-cyto-His (Fig. 2B). Binding constants were estimated assuming a 1:1 binding of CEACAM1-L and PDIP38. Using the BiAevaluation software, the following values were calculated: $K_D = 2.4 \times 10^{-5} \text{M}$, $k_{on} = 4.9 \times 10^3 \text{M}^{-1} \text{s}^{-1}$, and $k_{off} = 1.2 \times 10^{-3} \text{s}^{-1}$.

We next tested whether the PDIP38 interaction with CEACAM1-L is restricted to the long CEACAM1-L isoform, or includes also the short isoform, CEACAM1-S, which is identical to CEACAM1-L in the six membrane-proximal amino acids of the cytoplasmic domain. Therefore, we performed pulldown assays using GST-PDIP38 to precipitate CEACAM1-L and CEACAM1-S from transfected rat brain endothelial cells (RBE-CC1-L and RBE-CC1-S) (Fig. 2C). A specific interaction of GST-PDIP38 with either of the two CEACAM1 isoforms could thus be demonstrated.

Characterization of PDIP38-specific Antibodies—To get a tool for further studies, we generated polyclonal antisera (named P8R, P8R, P3G, and P8G) against PDIP38 in two rabbits (R) and two guinea pigs (G) using two different peptides (P3 and P8). The position of both peptides within the PDIP38 protein is illustrated in Fig. 1C. Peptide P3 and peptide P8 are identical with the rat, mouse, human, and dog PDIP38 protein sequence.

Of all four antisera, P8R gave the strongest signal at the expected migration position of ~38 kDa and was therefore used primarily in the following experiments.

In Western blot analysis of P8R immunoprecipitates from NBT-II cell lysates, P8R detected a single band at 39 kDa (Fig. 3A). Also, Coomassie Blue staining revealed, in addition to the light and heavy chains of the P8R IgG, a single specific band occurring at an apparent molecular mass of 39 kDa (Fig. 3B, lane P8R). The band was excised from the gel and analyzed by MALDI-TOF MS as described under “Experimental Procedures.” The precipitated protein was identified as the rat PDIP38 homologue by performing searches in Mascot using the peptide masses obtained by the MALDI-TOF MS analysis ($p < 0.05$, 29% sequence coverage). Identified peptide masses matched in the Mascot search results were: 1027.638, 1172.733, 1313.792, 1322.729, 1461.510, 1480.250, 1502.731, 1664.896, 1766.232, and 1790.926.

The other three anti-PDIP38 antisera also specifically recognized rat and human PDIP38, as shown by immunoprecipitations from NBT-II and HeLa cell lysates using P8R, P3R, P8G, and P3G sera followed by Western blotting with P8R antibody (Fig. 3C). The specificity of P8R for PDIP38 was further confirmed by peptide competition experiments (Fig. 3D). Preincubation of P8R with the peptide used for its generation (P8) at 50-fold molar excess (2 μg of antibody plus 1.25 μg of P8) completely abolished precipitation of PDIP38 from NBT-II cell lysate. The control peptide (P3) had no influence on the P8R binding to PDIP38.

To test for binding properties of the PDIP38 recognized by our antisera, we performed co-immunoprecipitations (Fig. 3E). As described previously (33), a complex of PDIP38, PCNA, and DNA polymerase δ was detected in HeLa cells using either anti-PCNA or P8R antibody for precipitation. In NBT-II cells PCNA co-precipitated PDIP38, and vice versa. However, no co-immunoprecipitation of DNA polymerase δ with PDIP38 or PCNA was detectable. This was probably due to the low sensitivity of the anti-human DNA polymerase δ (p125 subunit) antibody for the rat homologue.

Subcellular Localization of PDIP38 by Indirect Immunofluorescence—To investigate the subcellular distribution of endogenous PDIP38 in NBT-II, HeLa, RBE, and IEC18 cells, we performed immunofluorescence studies using P8R to detect PDIP38. Confocal images revealed a complex staining pattern (Fig. 3F). In all four cell lines, a strong cytoplasmic staining was found, and a portion of the cells also displayed a pronounced nuclear staining. In addition, staining at some, but not all, cell-cell contacts was detected. However, in neither of the four cell lines, any typical mitochondrial staining pattern was observed.

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The localization found for endogenous PDIP38 was thus in contrast to earlier studies performed on cells transfected with HA- or GFP-tagged PDIP38 (34–36). To analyze this further, we performed PDIP38 co-localization studies in NBT-II, HeLa, and IEC18 cells using MitoTracker Red CMXRos as a mitochondrial marker. No significant co-localization of PDIP38 with the mitochondria was observed in any cell line detecting PDIP38 either with P8R (Fig. 4, A and B, and supplemental Fig. S1A) or with P3R (supplemental Fig. S1B). To test for the specificity of P8R and P3R in immunofluorescence, we performed the staining procedure after pre-incubation of both antibodies

FIGURE 3. Characterization of polyclonal PDIP38-specific antibodies and subcellular localization of PDIP38. A, Western blot analysis of immunoprecipitate from NBT-II cell lysate using 2 μg of P8R. The membrane was developed for PDIP38 using P8R (0.25 μg/ml) of peptide:antibody ranges from 200:1 (5 ng) to 1:50 (0.5 ng) as indicated) of the peptide P8 used for the generation of P8R, or with a 5 μg of a peptide covering a different region of PDIP38 (P3, control). The molar ratio of peptide:antibody ranges from 200:1 (5 μg of P8) to 1:50 (0.5 ng of P8). First two lanes show the immunoprecipitation using P8R or control antibody (rabbit normal IgG, rlgG) without added peptides. E, complex formation of PDIP38, PCNA, and DNA polymerase δ. HeLa and NBT-II cell lysates where subjected to immunoprecipitations using anti-PCNA mAb PC10, P8R, or control antibody (rabbit normal IgG, rlgG) (input) were analyzed by Western blot for the presence of DNA polymerase δ (p125 subunit), PCNA, and PDIP38. F, confocal images of representative NBT-II, HeLa, RBE, and IEC18 cells stained for PDIP38 using P8R. Bars: 10 μm.

FIGURE 4. Subcellular distribution of PDIP38 in NBT-II cells and HeLa cells. Confocal images of NBT-II cells (A) and HeLa cells (B) stained for mitochondria using MitoTracker Red CMXRos (red), and for PDIP38 using P8R antibody (green). Right panels: enlarged images of the indicated regions in the merged images (pixel size 80 nm × 80 nm). Bars: 10 μm. C, Western blot analysis of subcellular fractions of NBT-II and HeLa cells prepared by differential centrifugation as described under “Experimental Procedures.” The membranes were developed for PDIP38 (P8R), focal adhesion kinase (FAK), histone H3, TOM20, β1 integrin, or CEACAM1, respectively. s1 = 600 × g supernatant; n = total nuclear fraction (600 × g pellet); sn = insoluble nuclear fraction; in = insoluble fraction; s2 = 9,000 × g supernatant; mi = mitochondrial enriched fraction (9,000 × g pellet); smi = soluble mitochondrial fraction; imi = insoluble mitochondrial fraction; c = cytoplasmic fraction (17,000 × g supernatant), me = small membrane fragment containing fraction (17,000 × g pellet). A representative experiment of two is shown. The intensities of the PDIP38 signals were quantified and are given in % of lane (i.e. % of the sum of all signals). The factor given below (“factor vol.”) is derived by dividing the total fraction volume by the fraction volume examined on the Western blot. D, PDIP38 amounts in the fractions sn, in, smi, imi, c and me were calculated for HeLa and NBT-II cells as follows: the PDIP38 amounts of the fractions (“% of lane,” C) were multiplied with the respective factors (“factor vol.”, C). The sum of the obtained results for the six fractions was set to 100% and the percentages of the single fractions (sn, in, smi, imi, me, and c) was calculated.

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with a 200-fold molar excess of peptide P8 or peptide P3 (supplementary Fig. S1, C–F). Preincubation of P8R and P3R with the respective peptides used for their generation abolished the staining (P8R/P8 and P3R/P3), whereas the respective control peptides showed no effect on the staining (P8R/P3 and P3R/P8).

Subcellular Localization of PDIP38 by Subcellular Fractionation—To verify the results from the indirect immunofluorescence biochemically, we performed subcellular fractionations by differential centrifugation, using NBT-II cells and HeLa cells, as described under "Experimental Procedures" (Fig. 4C). We tested all fractions for the presence of focal adhesion kinase (FAK) as a cytoplasmic marker, histone H3 as a nuclear marker, and translocase of outer membrane (20 kDa subunit, TOM20) as a mitochondrial marker. As marker proteins for plasma membrane fragments, CEACAM1 (NBT-II cells) and β1 integrin (HeLa cells) were used. For the PDIP38 distribution, similar results were obtained in both cell lines. Quantification of the PDIP38 amount in the different fractions revealed a predominant cytoplasmic (c) localization (~68%), but it was also detected in the nuclear (n, ~20%) and the mitochondrial (mi, ~11%) fraction (Fig. 4D). However, the nuclear and the mitochondrial fractions (i.e. the solubilized 600 × g and 9000 × g pellets) were contaminated with plasma membrane fractions, as shown by the presence of CEACAM1 and β1 integrin, respectively. Both fractions were further separated into an insoluble (17,000 × g pellet) and a soluble (17,000 × g supernatant) fraction. Although the nuclear and mitochondrial 17,000 × g pellets contained major amounts of CEACAM1 and β1 integrin, respectively, all supernatants were free of these membrane marker proteins. In NBT-II and HeLa cells, the major part (~75%) of nuclear PDIP38 was present in the soluble fraction (sn), which corresponded to ~14% of the PDIP38 content in the whole cell lysates. On the other hand, <20% of the mitochondrial PDIP38 was present in the soluble mitochondrial fraction (smi) of both cell lines. This corresponded to ~2% of the PDIP38 content in the whole cell lysates. We also found a small but significant amount of PDIP38 in the 17,000 × g pellet (me) obtained from the post-mitochondrial supernatant. The presence of PDIP38 in all 17,000 × g pellets (in, imi, and me) demonstrated that a portion of the cellular PDIP38 pool was present as an insoluble protein.

CEACAM1 Co-immunoprecipitates PDIP38—To test whether endogenous PDIP38 binds to CEACAM1-L and CEACAM1-S, co-immunoprecipitation experiments were performed using RBE cells transfected with CEACAM1-L, CEACAM1-S, or CEACAM1-Δcyto, a deletion mutant lacking a cytoplasmic domain. When precipitated by the rat CEACAM1-specific mAb Be9.2 (directed against the extracellular domain of CEACAM1), CEACAM1-L and CEACAM1-S, but not the Δcyto mutant, co-precipitated PDIP38 (Fig. 5A). PDIP38 was also co-immunoprecipitated by CEACAM1 in NBT-II cells (Fig. 5B), which endogenously co-express both CEACAM1 isoforms.

Clustering of CEACAM1 Recruits PDIP38 to the Plasma Membrane of Proliferating NBT-II Cells—Analysis of confocal images revealed that PDIP38 showed no plasma membrane localization in the majority of unperturbed, subconfluent NBT-II cells (Fig. 6, A and D) but localized to cell-cell contacts in ~10% of the cells (Fig. 6, B and D). PDIP38 at the cell-cell contacts co-localized with CEACAM1 (Fig. 6B).

To test CEACAM1-specific effects on PDIP38, we perturbed CEACAM1 by antibody clustering (Fig. 6C). Subconfluent NBT-II cells were incubated sequentially with the CEACAM1-specific mAb Be9.2 and GaM secondary antibody. This treatment resulted in rapid translocation of PDIP38 from the cytoplasm to the plasma membrane and membrane-proximal regions in ~97% of the cells (Fig. 6, C and D). The translocation occurred within 10 min and lasted for at least 1 h, when the majority of the clustered CEACAM1 molecules were internalized (data not shown).

Clustering of CEACAM1 Induces PDIP38 Translocation to the Nucleus in Quiescent NBT-II Cells—When grown to a confluent monolayer, NBT-II cells enter the G0 stage and remain quiescent. Under these conditions, no nuclear or plasma membrane staining of PDIP38 was observed (Fig. 7A, left panel). To test whether the state of the cells had an effect on the interaction of CEACAM1 and PDIP38, we clustered confluent NBT-II cells with the CEACAM1-specific antibody Be9.2 and GaM. However, in contrast to subconfluent cells, no membrane-recruitment but a translocation of PDIP38 from the cytoplasm to the nucleus occurred in 66% of the quiescent cells within 3 h (Fig. 7, A and C).

Perturbation of CEACAM1 by antibodies has previously been demonstrated to activate the Erk/MAPK pathway in confluent NBT-II cells (19) and is believed to involve tyrosine phosphorylation of CEACAM1-L. Protein kinase C-mediated serine/threonine phosphorylation of the cytoplasmic domains of both CEACAM1-L and CEACAM1-S has also been demonstrated (42, 43) and seems to play important roles in CEACAM1-mediated signal transduction events. To investigate if any of these phosphorylation events might be involved in nuclear translocation or plasma membrane recruitment of PDIP38, we tested a variety of protein kinase inhibitors for their ability to influence the translocation of PDIP38 upon CEACAM clustering by antibodies Be9.2/GaM. However, preincubation of quiescent or proliferating NBT-II cells with 100 μM genistein (broad spectrum tyrosine kinase inhibitor), 10 μM PP2 (inhibitor of Src-family kinases), 20 μM U0126 (inhibitor of MEK1/2), or 1 μM staurosporine (inhibitor of protein kinase C) had no effect on PDIP38 nuclear translocation or plasma membrane recruitment (data not shown). Thus, if any of these phospho-
ylation events are involved they do not seem to act upstream of the CEACAM1-induced translocation of PDIP38.

The exclusive cytoplasmic localization of PDIP38 in unperturbed quiescent NBT-II cells and the antibody-induced nuclear translocation suggested a link between the cell cycle/proliferation and the subcellular localization of PDIP38, because we have demonstrated earlier that perturbation of CEACAM1 in quiescent NBT-II cells with specific antibodies induces cell cycle entrance and DNA synthesis (19, 20). Therefore, we also examined another stimulus of cell cycle entry and DNA synthesis in NBT-II cells, the serum activation of serum-starved cells (19), for a possible effect on PDIP nuclear translocation. Confluent NBT-II cells were serum-starved for 28 h and then incubated with medium containing 10% FBS. Indeed, the serum activation induced translocation of PDIP38 to the nucleus in 86% of the serum-starved NBT-II cells within 3 h (Fig. 7, B and C).

PDIP38 and CEACAM1 Interact Differently in Proliferating and Quiescent Cells—To further investigate the different characteristics of the PDIP38-CEACAM1 interactions in proliferating and quiescent NBT-II cells, and to address the nature/mechanism of CEACAM1 to PDIP38 signaling, we performed additional binding experiments on clustered and non-clustered cells. Using the mAb Be9.2, CEACAM1 was immunoprecipitated from cells clustered with Be9.2/RaM (rabbit anti-mouse IgG), from cells treated with Be9.2 or RaM alone, and from untreated cells, respectively. This showed that the amount of co-precipitated PDIP38 increased 2-fold in clustered proliferating cells, compared with untreated cells, or cells treated with Be9.2 or RaM alone (Fig. 8 A). However, in quiescent cells no co-immunoprecipitation of PDIP38 with CEACAM1 was observed under any of these conditions (Fig. 8 A). This agreed with our observation,
that in quiescent cells no PDIP38 localized to the plasma membrane with or without CEACAM1 clustering (Fig. 7A). To test if PDIP38 had lost its ability to bind CEACAM1 in quiescent cells, we performed pulldown assays using the purified His-tagged cytoplasmic domain of CEACAM1-L (CC1-L-cyto-His). However, PDIP38 was precipitated by CC1-L-cyto-His in equal amounts from lysates of both proliferating and quiescent NBT-II cells, whether clustered or non-clustered (Fig. 8B). This showed that PDIP38 maintained its ability to bind CEACAM1 in quiescent cells.

Another possibility that we explored to some extent is that the inability of CEACAM1 to bind PDIP38 was due to some kind of chemical modification or different organization of CEACAM1 in the quiescent cells. One kind of chemical modification known to be important for signal transduction is phosphorylation of the two tyrosine residues in the cytoplasmic domain of CEACAM1-L (2). Therefore, we tested whether the tyrosine phosphorylation pattern was different in proliferating and quiescent cells. However, no tyrosine phosphorylation of CEACAM1 was detected either in proliferating or quiescent NBT-II cells, that were untreated or CEACAM1-clustered (Be9.2/RaM) for 5, 10, or 15 min (supplemental Fig. S2A). Because PDIP38 has several tyrosine residues, we also analyzed this protein, without detecting any signs of tyrosine phosphorylation under any conditions (supplemental Fig. S2B).

The organization of CEACAM1 with respect to its association with the cytoskeleton has been demonstrated to differ under various conditions. For example, CEACAM1 becomes partially TX-100-insoluble in PC12 cells after clustering (26). Thus, we investigated the TX-100 solubility of CEACAM1 and PDIP38 in the NBT-II cells, before and after clustering (Fig. 8, A and B). All CEACAM1 was soluble in untreated cells, but a substantial fraction became associated with the insoluble pellet after clustering. Co-immunoprecipitation experiments with subconfluent CEACAM1-clustered cells, in which protein G-Sepharose, but no additional Be9.2 antibody, was added to the cleared lysates, resulted in precipitation of CEACAM1 and co-precipitation of PDIP38, showing that a portion of the clustered CEACAM1 remained soluble and was able to bind PDIP38 (data not shown). PDIP38 exhibited a partial TX-100 insolubility under all conditions, and clustering of CEACAM1 had no significant impact on the amount of PDIP38 in the TX-100-insoluble fractions.

In summary, we did not detect any modifications of either CEACAM1 or PDIP38 that could explain why the two proteins co-precipitate in subconfluent, proliferating NBT-II cells, but not in confluent, quiescent cells. We are therefore left with the possibility that the lack of co-precipitation is related to the segregation of the two proteins in the quiescent cells (Fig. 7A).

**DISCUSSION**

Cell adhesion receptors like CEACAM1 influence many different cellular functions, including proliferation and gene transcription. In doing so, they need to interact with proteins that can transmit the information to the signal target, the nucleus. In the present study, we identified the PCNA- and DNA polymerase δ-interacting protein PDIP38 as an interaction partner for both the long and the short CEACAM1 isoforms. The CEACAM1 binding site for PDIP38 was mapped to the six membrane-proximal amino acids of their cytoplasmic domains, this sequence being identical in the two isoforms. For PDIP38, a sequence of 18 amino acids close to its C terminus was found to be a prerequisite for binding to CEACAM1-L, as judged from truncation analysis in yeast two-hybrid studies. To allow analyses of the interaction between endogenously
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expressed CEACAM1 and PDIP38, we produced four different antibodies against two different regions of PDIP38, which we characterized in detail and demonstrated that all of them specifically recognized PDIP38.

The ability of PDIP38 to localize to different subcellular regions like the plasma membrane and the nucleus, as well as its ability to bind to the transmembrane receptor CEACAM1 and the DNA-modifying proteins PCNA and DNA polymerase δ, gave a first hint that this protein might be capable of shuttling between the cytoplasm and the nucleus. Because only a distinct fraction of the cells showed pronounced nuclear staining, the possibility was raised that the subcellular distribution might depend on the cell cycle status. This agrees with data mentioned by Arakaki et al., who found PDIP38 (here called mitogenin I) to be located in the nucleus during the G2/M to the late G1 phase (36). Indeed, we could show that no PDIP38 localized to the nuclei in confluent, non-proliferating NBT-II cells, but that clustering of CEACAM1 induced nuclear translocation of PDIP38 in the majority of the quiescent NBT-II cells. This is interesting, because we have previously demonstrated that antibody-mediated perturbation of CEACAM1 induces cell cycle entry and DNA synthesis in confluent, quiescent NBT-II cells by regulating Erk1/2 activation (19, 20). However, the CEACAM1-induced activation of Erk1/2 is either independent of the PDIP38 signal, or lies downstream of it, as shown here by the inability of the Erk1/2 pathway kinase inhibitors to influence nuclear translocation of PDIP38. Our proposal that the nuclear translocation of PDIP38 might be linked to the position in the cell cycle is supported by our finding that also serum addition to serum-starved quiescent NBT-II cells, which is a strong stimulus for cell cycle entrance (19), induced movement of PDIP38 to the nucleus.

The localization of PDIP38 to the plasma membrane of NBT-II cells also seemed to be regulated, because only a subpopulation of non-confluent, proliferating cells displayed this distribution. Moreover, antibody-mediated clustering of CEACAM1 not only enhanced the physical interaction between the two molecules, but also led to a fast recruitment of PDIP38 to the plasma membrane in almost all cells. PDIP38 was also found at some cell-cell contacts of CEACAM1-negative HeLa and RBE cells, indicating that other membrane components or receptors than CEACAM1 also might interact with this molecule.

What are the mechanisms for the CEACAM1-mediated regulation of the subcellular localization of PDIP38, and what are the reasons for this being different in proliferating and quiescent cells? Clearly, some kind of signal must be transmitted from CEACAM1 to PDIP38, and the nature and/or propagation of this signal must be different between the two cellular states. Our results did not indicate any difference in the biochemical properties of PDIP38 or its ability to bind to CEACAM1 under the different conditions. However, different properties or organization of CEACAM1 remain as possible explanations for the different effects on PDIP38. We could rule out tyrosine phosphorylation of CEACAM1-L being such a factor. However, modification by serine/threonine phosphorylation could be an important factor, because both CEACAM1-L and CEACAM1-S possess phosphorylatable serine and threonine residues within their PDIP38 binding site (42, 43). For technical reasons it was not possible to explore this possibility in the present study. Differences in the association of CEACAM1 or PDIP38 with the cytoskeleton did not seem to be the explanation for the different CEACAM1-to-PDIP38 signaling. However, the different localization of PDIP38 in unperturbed proliferating and quiescent cells might hold a clue to the enigma. Only in proliferating cells, where PDIP38 occurred at the plasma membrane, where also CEACAM1 is located, a co-precipitation between the two molecules was observed. In the quiescent cells, PDIP38 was excluded from the plasma membrane, thus preventing a physical interaction with CEACAM1, which could explain the lack of co-precipitation of the two molecules. A simple explanation for this exclusion could be a different and more dense structure of the submembranous actin network in the quiescent cells. The factors that control the cytoplasmic localization of PDIP38 and its exclusion from the plasma membrane in quiescent cells will be an important issue for future studies. The exclusion of PDIP38 from the plasma membrane, and accordingly from CEACAM1, in quiescent but not in proliferating cells raises the possibility that the signal generated in CEACAM1, which leads to nuclear translocation of PDIP38 in quiescent cells, is blocked in proliferating cells. Could it be that PDIP38 itself is the blocking factor, by binding to the cytoplasmic domain of CEACAM1?

It is tempting to speculate that PDIP38 could link CEACAM1 directly to the regulation of gene expression or to the DNA synthesis and cell cycle control machinery. By its interaction with PCNA and the p50 subunit of DNA polymerase δ, PDIP38 could be implicated in some of the functions attributed to these proteins (44, 45). This might include more than DNA replication, because especially the DNA sliding clamp PCNA has been shown to act on several aspects of DNA modification, including DNA methylation, chromatin remodeling, and translesion DNA synthesis.

In addition to immunofluorescence, the subcellular distribution of PDIP38 was determined by differential centrifugation and immunohistochemical analysis of homogenates of unperturbed, proliferating NBT-II and HeLa cells, because it has been claimed in previous reports that PDIP38 primarily is a mitochondrial protein (34–36). In all fractionation experiments, the majority of PDIP38 was found in the cytoplasmic fractions (~68%), and to a lesser degree also in the nuclear fractions (~20%). The amount of PDIP38 found in the mitochondrial fractions was 10–12%. A small, but significant amount of PDIP38 was also found in the insoluble 17,000 × g pellet obtained from the post-mitochondrial fraction. This pellet contained plasma membrane fragments, as judged from the plasma membrane marker proteins β1 integrin and CEACAM1. Most importantly, both the nuclear and the mitochondrial fractions also contained these plasma membrane markers, which were enriched in the insoluble 17,000 × g pellets of all the fractions. Thus, subcellular fractionation by simple differential centrifugation does not unambiguously demonstrate the presence of PDIP38 in nuclei or mitochondria. However, when these results are taken together with the localization observed by confocal immunofluorescence microscopy, it seems clear that PDIP38 is present in the cytoplasm, and under certain conditions both at
the plasma membranes and in the nuclei. In contrast, the mitochon-
dria only seem to contain a minor fraction of the total cellular PDIP38.

The strongest argument for a mitochondrial localization in the studies by Xie et al. (34) and Arakaki et al. (36) was their finding of an almost exclusive mitochondrial localization of transfected PDIP38 that was tagged with GFP in the C-terminal end. However, there is reason to be cautious with conclusions from localization of GFP-tagged proteins. Indeed, Simpson et al. (46) in a comprehensive study of a large number of proteins, showed that PDIP38, and several other proteins, tagged with yellow fluorescent protein in the C-terminal end gave an exclusive mitochondrial localization, whereas tagging with cyan fluorescent protein in the N-terminal end caused exclusive nuclear localization. In agreement with this, Xie et al. (34) reported that PDIP38 tagged with GFP in the N-terminal end, in contrast to tagging in the C-terminal end, gave a diffuse localization in the entire cell. Xie et al. found a putative mitochondrial signal sequence in the N terminus of PDIP38 and concluded that tagging of the N terminus blocked the function of this signal sequence. However, there might also be sites important for subcellular localization in the C terminus, the blocking of which might interfere with the localization. Indeed, we demonstrate in the present study that PDIP38 has a binding site for CEACAM1, which is a plasma membrane protein, close to its C terminus. Thus, tagging of either end of PDIP38 might cause erroneous localization in intact cells. Taken together, all reports, including our present results, are in agreement with PDIP38 being a shuttling protein present in the cytoplasmic and the nuclear compartments. A minor portion may also enter the mitochondria. Other proteins with dual localization sites in the nucleus and the mitochondria have in fact been described, e.g. the helicase MDDX28 and the topoisomerase IIIα (47, 48).

In conclusion, our results demonstrate that PDIP38 can both interact with the cell adhesion receptor CEACAM1 at the plasma membrane and shuttle between the cytoplasm and the nucleus. This suggests that PDIP38 can function as a signal mediator, transferring information from the cell surface to the genetic and/or mitotic machineries. Future studies will reveal the putative involvement of PDIP38 in cell surface-controlled regulation of gene expression, DNA synthesis, or other events in the cell cycle.

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