The Protein Tyrosine Phosphatase PTP1B Is Required for Efficient Delivery of N-Cadherin to the Cell Surface

Mariana V. Hernández, Diana P. Wehrendt, and Carlos O. Arregui

INTRODUCTION

N-cadherin is a calcium-dependent cell–cell adhesion molecule expressed at the surface of several neuronal and non-neuronal cells (Derycke and Bracke, 2004). N-cadherin function depends on interaction of its cytoplasmic domain with catenins (α-, β-, and p120-catenin), a process modulated by tyrosine phosphorylation (Lilien and Balsamo, 2005; Alema and Salvatore, 2007). Although the binding of β-catenin and p120 is direct, that of α-catenin is indirect (Ozawa and Kemler, 1992; Hinck et al., 1994; Pokutta and Weiss, 2007).

Protein tyrosine kinases of the Src family, EGFR and c-Met phosphorylate β-catenin decreasing its binding to cadherin (Kinch et al., 1995; Roura et al., 1999; Piedra et al., 2003; Lilien and Balsamo, 2005). In contrast, phosphorylation of p120 by Fer, Src, and Fyn increases the binding to cadherin (Roura et al., 1999; Piedra et al., 2003; Castano et al., 2007). PTP1B is a nonreceptor protein tyrosine phosphatase associated with the cytosolic face of endoplasmic reticulum (ER) membranes by a hydrophobic amino acid sequence of the C-terminus (Frangioni et al., 1992). The catalytic domain of PTP1B is oriented to the cytosol where it can potentially interact with proteins at this compartment. Indeed, ER-bound PTP1B dephosphorylates several growth factor receptors, either during their biosynthetic route to the cell surface or after being endocytosed (Haj et al., 2002; Boute et al., 2003; Romsocki et al., 2004; Cohen et al., 2004). In addition, PTP1B binds directly to the cytosolic domain of mature N-cadherin and dephosphorylates β-catenin in the complex, increasing the stability of the N-cadherin–actin linkage, and promoting N-cadherin–mediated adhesion (Balsamo et al., 1998; Rhee et al., 2001; Xu et al., 2002, 2004; Lilien and Balsamo, 2005). Similar function of PTP1B was observed in epithelial cells expressing E- or VE-cadherin (Sheth et al., 2007; Winter et al., 2008; Nakamura et al., 2008). A role of PTP1B in the dephosphorylation of p120 was recently suggested. Knockdown of PTP1B in Colo 205 cells increases the phosphorylation state of p120 (Ezaki et al., 2007). In addition, a comparative phospho-proteomic analysis between wild-type (WT) and PTP1B-deficient fibroblasts identified peptides derived from p120 that were hyperphosphorylated in PTP1B-deficient cells (Mertins et al., 2008). However, the functional consequences of this regulation were not assessed.

Cadherins are synthesized in ER-bound ribosomes as precursor proteins, with a prodomain at the N-terminus that must be removed to generate mature and adhesive competent proteins (Ozawa, 2002; Koch et al., 2004). Prodomain removal occurs as a post-Golgi event, by a furin subgroup of proprotein convertases (Posthaus et al., 1998; Wahl et al., 2003). Early after biosynthesis, cadherin precursors associate with β-catenin and p120 (Ozawa and Kemler, 1992; Hinck et al., 1994; Wahl et al., 2003; Curtis et al., 2008). Mutations impairing the binding of β-catenin led to retention of E-cadherin in the ER and to an inefficient expression at the cell surface (Chen et al., 1999). On the other hand, mutations impairing the binding of p120 led to delayed recruitment of N-cadherin at cell–cell contacts during calcium-initiated junction reassembly (Chen et al., 2003). This effect was attributed to inefficient coupling of the N-cadherin complex with kinesins, which are required for transport of N-cadherin from Golgi to the cell surface (Mary et al., 2002; Chen et al., 2003; Yanagisawa et al., 2004; Teng et al., 2005). At the cell surface, p120 bound to cadherin prevents its endocytosis.
and degradation (Davis et al., 2003; Xiao et al., 2003, 2007; Kowalczyk and Reynolds, 2004).

When N-cadherin constructs deleted in the PTP1B binding site are transfected in L-cells, which do not express endogenous cadherins, they are not expressed efficiently at the cell surface and accumulate intracellularly (Xu et al., 2002). This prompted us to assess the role of PTP1B in N-cadherin trafficking. Using PTP1B null cells, and derivative lines reconstituted with WT PTP1B (Haj et al., 2002), we have defined a novel regulatory role of PTP1B that promotes trafficking of the N-cadherin precursor through early stages of the secretory pathway. This function of PTP1B does not require its association with the cadherin precursor. We found that PTP1B is required for association of p120 to the N-cadherin precursor, an event that is crucial for progression of the complex through the early stages of the secretory pathway. This function of PTP1B differs from its previously described effect on β-catenin regulation at cell surface N-cadherin complexes (Lilien and Balsamo, 2005).

MATERIALS AND METHODS

Antibodies and Reagents

Monoclonal antibodies against PTP1B, β-catenin, phosphotyrosine (clone PY20), and N-cadherin were from BD Transduction Laboratories (Lexington, KY). Polyclonal anti-β-catenin was from W. J. Nelson (Stanford University). Monoclonal anti-p120 (clone 6H13) was from A. B. Reynolds (Vanderbilt University). High-affinity monoclonal anti-hemagglutinin (HA; clone 3F10) was from Roche (Penzberg, Germany). Affinity-purified biotin conjugated goat anti-mouse, streptavidin-garosarago and monoclonal antibodies against HA (clone HA-7), c-myc (clone 9E10), β-catenin, and the ectodomain of N-cadherin (clone CC-4) were from Sigma-Aldrich (St. Louis, MO); mab specific for chicken N-cadherin (NCD-2; Balsamo et al., 1998) was a gift from Jack Lilien (University of Iowa). Monoclonal anti-Xpress was from Invitrogen (Carlsbad, CA). Rhodamine-phalloidin, Alexa-488 – and Alexa-568 – conjugated fluorescein isothiocyanate goat secondary antibodies were from Molecular Probes (Eugene, OR). Protein G-Sepharose was from GE Healthcare (Uppsala, Sweden). Horse-radish peroxidase (HRP)-conjugated goat anti-mouse and aminomethylcoumarin acetate (AMCA)-conjugated streptavidin were from Jackson Immunoresearch (West Grove, PA).

DNA Constructs

PTP1B constructs used in this work were previously described (Hernandez et al., 2006). HA-N-cadherin-GFP was obtained by PCR, adding the HA epitope (YPDYVPDYA) into the propeptide (between Ala-30 and Thr-31) of chick N-cadherin fused at the C-terminus with the enhanced green fluorescent protein (eGFP-NL1, Clontech BD Biosciences, Mountain View, CA). To obtain N-cadherin(Δ707-991)-GFP, a fragment KpnI/Apa containing the deletion was excised from the pCMV-N-cadherin(Δ707-991) and replaced into the N-cadherin-GFP (Xu et al., 2002). To obtain the HA-N-cadherin(Δ991-991)GFP, a fragment KpnI/Apa containing the deletion was excised from pCMV-N-cadherin(Δ991-991) (Xu et al., 2002) and replaced into the HA-N-cadherin-GFP construct. Murine N-cadherin-3A-YFP was prepared by PCR insertion of HA epitope between Ala-25 and Ser-26 of the propeptide. The cDNAs encoding JMP and JMP-3A were amplified by PCR using chick N-cadherin-GFP and mouse N-cadherin-3A-YFP as templates, respectively. PCR fragments were subcloned into EcoRI and BamHI digestion buffer and boiled at 100°C for 10 min. Then, protein G-Sepharose (1.5 h). Immunocomplexes were washed with lysis buffer and boiled in SDS-PAGE sample buffer. Supernatants were fractionated by SDS-PAGE and transferred to polyvinyl difluoride membranes (Millipore, Bedford, MA). Blots were probed with primary antibodies followed by HRP-conjugated second antibodies and revealed by enhanced chemiluminescence. For stripping, blots were incubated (30 min, 55°C) with Tris-buffered saline (TBS) containing 5% 2-mercaptoethanol and 2% SDS, blocked with 5% milk, and reprobed. Soluble proteins from metabolically labeled and cell surface–biotinylated cells were immunoprecipitated with a monoclonal anti-N-cadherin. To isolate the fraction of cell surface N-cadherin, half of the immunoprecipitated beads were boiled 3 min in lysis buffer containing 1% SDS, the supernatant was diluted with 900 μl of TBS, and the biotinylated N-cadherin was pulled down using streptavidin-agarose. Total and cell surface–N-cadherin was analyzed by SDS-PAGE followed by fluorography using DMSO-PPO (2,5-diphenyloxazole). Semiquantitative analysis of the signal intensity of the bands was performed after scanning X-ray films. Integrated optical densities of bands were determined using the routine to analyze one-dimensional electrophoretic gels from ImageJ (http://rsb.info.nih.gov/ij/; Wayne Rasband, NIH, Bethesda, MD).

Endoglycosidase-H Treatments

Forty hours after transfection, cells expressing HA-tagged N-cadherin constructs were processed for immunoprecipitation with anti-HA antibodies. Immunoprecipitates were resuspended in endoglycosidase-H (endo-H) denaturing buffer (0.5% SDS, 40 mM DTT) and heated at 100°C for 10 min. Then, 1/10 volume of 0.5 M sodium citrate, pH 5.5, was added. Samples were split into halves and incubated with without 500 U of endo-H according to the manufacturer’s instructions (New England Biolabs, Beverly, MA). Cells transfected with VSV-G tsO45-myc were incubated for 16 h at 46°C. Then, temperature was shifted to 32°C, and the cells were incubated for the times indicated. VSVG tsO45-myc was immunoprecipitated and processed as described previously.

Immunofluorescence

Cells grown on fibronectin-coated coverslips (20 μg/ml) were fixed with 4% paraformaldehyde in PBS for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 3% BSA in PBS for 60 min. Primary antibodies were incubated overnight at 4°C, and fluorescently conjugated secondary antibodies 45 min at room temperature. Cells were mounted in Vectorshield (Vector Laboratories, Burlingame, CA). For quantitative assessment, cells were analyzed with a 100×/1.4 NA objective in a Nikon E600 microscope (Melville, NY) coupled to a Spot RT Slide CCD camera (Diagnostic Instruments, Sterling Heights, MI), or with a 60×/1.4 NA objective on a Bio-Rad Microscope. For all cases, objectives were removed, and the microscope was placed under a bright light. For quantitative analysis, cells were analyzed with a 60×/1.4 NA objective in a Nikon TE2000 coupled to a Hamamatsu Orca AG 12-bit camera (Hamamatsu Photonics, Hamamatsu, Japan).
Image Quantifications

Twelve-bit images were processed using ImageJ. Only images with the relevant fluorescence signal below the saturation level were used for quantification. Background fluorescence was subtracted from noncellular regions.

Analysis of ER–Golgi Transport after BFA Washout. To quantify the redistribution of N-cadherin-GFP into the perinuclear Golgi location at different times after BFA washout, we followed the procedure described. At each time point, two regions of interest (ROIs) were drawn at peripheral locations and the corresponding mean fluorescence values were averaged. This value represents an estimation of the N-cadherin-GFP at non-Golgi locations (ER plus plasma membrane). Golgi-associated fluorescence was determined from ROIs drawn at perinuclear regions. Golgi formation was also monitored by cotransfection with Sial-T2, a sialyl transferase that localizes in Golgi (Daniotti et al., 2000).

Ratio Analysis of Trafficking. To estimate trafficking of HA-N-cadherin-GFP and derivatives constructs we analyzed HA/GFP fluorescence ratios from intracellular puncta. Puncta represent trafficking carriers, as judged by time lapse and colocalization analysis (not shown). The HA epitope was detected by immunolabeling using Alexa 568 as fluorescent secondary antibodies (red channel). Because the HA-containing domain is removed as a single step at post-Golgi locations, we predicted that carriers originated before this location. Hence, HA/GFP fluorescence ratios than post-Golgi carriers ROIs were manually drawn around puncta, and the mean intensity values were calculated for both the red (HA label) and green (GFP label) channels. Images with pixel misalignments were discarded. The fluorescence contribution of the N-cadherin-GFP located at the plasma membrane was estimated averaging the mean intensity values of two to three ROIs drawn at ER-free thin peripheral areas of the cell. This value was subtracted from all ROIs.GFP. Values from all ROIsHA and ROIsGFP pairs were transferred to Excel sheets (Microsoft, Redmond, WA) for calculation of HA/GFP ratios. In the case of BFA-treated cells, in which puncta were not visible, ROIs were drawn over ER tubules at the cell periphery using the HA image. HA/GFP ratios were analyzed in frequency plots.

Analysis of Functional Cadherin. To evaluate the effect of JMP and JMP-3A constructs on N-cadherin-GFP distribution, pairs of transfected cells in contact with each other were analyzed. For each cell of the pair two nonoverlapping ROIs were drawn; one that enclosed the intercellular junction (functional fluorescence) and the other included the remaining area of the cell (intracellular fluorescence). Mean fluorescence values were used to calculate junctional/intracellular ratios.

Aggregation Assays

Cells were washed with HBBSK (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM glucose, and 3 mM KCl) and resuspended with 0.01% trypsin in HBSSK containing 1 mM CaCl2. After trypsin neutralization with soybean trypsin inhibitor, cells were washed and collected by centrifugation. The cell pellet was resuspended with HBSSK containing 10 μg/ml DNAse. Dispersed cells were seeded in 24-well plates (1.5 x 105 cells/well) previously blocked with 1% BSA and incubated with or without additives (1 mM CaCl2 or 1 mM EDTA) for 30 min at 37°C with constant rotation at 90 rpm. Samples at the start and at the end of the incubation period were fixed, and the number of particles immediately was assessed by phase-contrast microscopy. The extent of aggregation was represented by the N30/N0 index (Takeichi, 1977), where N30 is the number of particles at t0 and N30 the number of particles after 30 min.

RESULTS

ER-bound PTPIB Associates with N-Cadherin at Cell–Cell Junctions But Not with the Intracellular Precursor

Previous studies have shown that PTPIB binds directly to the cytoplasmic domain of cell surface N-cadherin, ensuring β-catenin dephosphorylation and promoting N-cadherin-mediated adhesion (Balsamo et al., 1998; Rhee et al., 2001; Xu et al., 2002, 2004). Because PTPIB is tail-anchored to the ER, it may also potentially bind to the cytoplasmic domain of N-cadherin precursors while they travel from the ER to the cell surface. To investigate this possibility, we analyzed the colocalization of intracellular N-cadherin with either the WT PTPIB or the substrate trapping mutant PTPIB-D181A. Because of its capacity of forming long-lived complexes with substrates, PTPIB-D181A has proved to be crucial for detection, by FRET and BRET techniques, of direct interactions between PTPIB and different growth factor receptors at the surface of the ER (Haj et al., 2002; Boute et al., 2003; Romsicki et al., 2004). A PTPIB null cell line stably reconstituted with WT PTPIB (WT-cells) shows the typical distribution of PTPIB at the ER (Figure 1A), as previously reported (Haj et al., 2002; Hernandez et al., 2006). However, null cells stably reconstituted with PTPIB-D181A (DA-cells) show that in addition to its ER localization, the PTPIB-D181A also accumulates at cell–cell boundaries (Figure 1B, compare with A). This accumulation is prevented by preincubation with pervanadate, which inactivates the active site of the enzyme (Figure 1C). Because WT- and DA-cells express similar levels of PTPIB (Hernandez et al., 2006), our results suggest the particular distribution of PTPIB-D181A is due to its trapping by substrates at intercellular junctions.

To examine whether PTPIB interacts with intracellular N-cadherin in transit to the cell surface, we analyzed their colocalization in DA-cells transfected with N-cadherin-GFP. When overexpressed, newly synthesized N-cadherin-GFP accumulates to some extent at the Golgi complex (Figure 2, H–K). This intracellular pool of N-cadherin-GFP never colocalized with PTPIB-D181A, in clear contrast to the tight overlap of both proteins at cell–cell boundaries (Figure 1, D–D’). As expected, immunolabeling of β-catenin, which associates with the cadherin precursor shortly after its biosynthesis (Ozawa and Klemler, 1992; Hinck et al., 1994; Chen et al., 1999; Wahl et al., 2003; Curtis et al., 2008), tightly colocalized with N-cadherin-GFP at both Golgi and cell–cell junctions (Figure 1E). These results suggest that PTPIB does not bind to the N-cadherin precursor, but it can be recruited to junctional N-cadherin complexes. Indeed, disruption of junctional N-cadherin with EGTA leads to the dispersal of PTPIB-D181A from cell–cell boundaries (Figure 1F). The ER anchor is also required for the accumulation of the trap at cell–cell boundaries because the distribution of GFP-PTPIB-D181A is381, a truncation mutant that terminates at glutamic acid 381, removing just the ER-targeting sequence (Hernandez et al., 2006), becomes uniform and did not accumulate at intercellular junctions, as it did the full-length counterpart (Figure 1, G and H). Thus, ER anchor serves as a platform to position PTPIB at peripheral regions of the cell (Hernandez et al., 2006; Fuentes and Arregui, 2009).

To confirm the lack of association between PTPIB and the N-cadherin precursor in transit to the cell surface, we prepared a construct with the hemagglutinin (HA) epitope inserted into the N-terminal propeptide of chicken N-cadherin-GFP (HA-N-cadherin-GFP; Figure 2). This construct allows for the selective isolation and detection of the N-cadherin precursor using the HA tag, and of the total pool (precursor plus mature) using the C-terminus GFP tag. Expression of HA-N-cadherin-GFP in WT-cells shows that the GFP fluorescence and the HA immunolabel codistribute in intracellular locations, whereas only the GFP fluorescence is detected at cell–cell boundaries (Figure 2, A–C). The intracellular pool of HA-N-cadherin-GFP colocalizes with the ER marker calnexin and with the Golgi marker GalNacT (Giraudo and Macciocci, 2003; Figure 2, D–K). These results indicate that the HA-containing domain is efficiently removed from N-cadherin before arrival to the cell surface, in agreement with previous findings (Wahl et al., 2003; Koch et al., 2004). Western blot analysis shows that the anti-HA antibody selectively detects a band of ~174 kDa corresponding to the HA-N-cadherin-GFP precursor (147-kDa N-cadherin precursor + 27-kDa GFP) and that equivalent levels of processed chicken N-cadherin-GFP is obtained regardless of the presence of the HA tag (Figure 2L). Immunoprecipitation of HA from HA-N-cadherin-GFP transfected KO- and WT-cells does not show PTPIB associated in the complexes.
In contrast, equivalent amounts of immunoprecipitated mature N-cadherin reveals a consistent amount of PTP1B in the complexes of WT-cells (Figure 2M, right panels). Taken collectively, the colocalization and co-immunoprecipitation analysis show that ER-bound PTP1B associates preferentially to the mature, junctional form of N-cadherin and not to the trafficking precursor.

**PTP1B Is Required for Traffic and Function of N-Cadherin**

Cadherin-deficient L-cells transfected with N-cadherin constructs that bear deletions in the PTP1B binding site showed impaired localization of the constructs at the cell surface and enhanced accumulation at intracellular pools (Xu et al., 2002). To determine whether PTP1B expression affects the distribution of N-cadherin, KO- and WT-cells were transfected with N-cadherin-GFP, and the fluorescence distribution was analyzed by confocal microscopy. In WT-cells, N-cadherin-GFP localizes most prominently at cell–cell junctions, with rare intracellular puncta and occasional accumulation in the Golgi apparatus (Figure 3A). In contrast, N-cadherin colocalizes with β-catenin in junctions (arrowhead in D) and in the Golgi complex (arrow in E). The colocalization of DA and N-cadherin-GFP at the cell periphery is lost by EGTA preincubation (F). KO-cells transfected with GFP fusions of DA (G), or DA truncated at the ER targeting sequence (DAt; H) were immunostained for N-cadherin. Note the loss of accumulation of the trap mutant at junctions when its targeting to the ER is prevented. Bars, 20 μm.

**Figure 1.** ER-bound PTP1B distributes with N-cadherin at cell–cell junctions and not at intracellular locations. Cells stably expressing (A) wild-type (WT) PTP1B or (B and C) substrate trapping mutant D181A PTP1B (DA) were fixed and immunostained for PTP1B. DA accumulates at cell–cell junctions (B, arrowhead), and this effect is inhibited by preincubation of cells with 0.1 mM sodium pervanadate 30 min before fixation (C). DA-cells transfected with N-cadherin-GFP were immunostained for PTP1B (D, D′, D′′, E, F) or β-catenin (E). Note that N-cadherin colocalizes with PTP1B in junctions (arrowhead in D) but not in the Golgi complex (arrow in D). In contrast, N-cadherin colocalizes with β-catenin in junctions (arrowhead in E) and in the Golgi complex (arrow in E). The colocalization of DA and N-cadherin-GFP at the cell periphery is lost by EGTA preincubation (F). KO-cells transfected with GFP fusions of DA (G), or DA truncated at the ER targeting sequence (DAt; H) were immunostained for N-cadherin. Note the loss of accumulation of the trap mutant at junctions when its targeting to the ER is prevented. Bars, 20 μm.
in the Golgi apparatus and in intracellular puncta, in addition to the typical fluorescence in cell–cell boundaries (Figure 3B). A similar fluorescence distribution is observed in WT-cells transfected with two different N-cadherin-GFP constructs deleted in the PTP1B-binding site (Figure 3, C and D). These results suggest that PTP1B is required for traffic and surface expression of N-cadherin-GFP (see below). To evaluate the cell–cell adhesive capacity of WT- and KO-cells, we tested their ability to aggregate in suspension. Although both cell lines form aggregates, those of KO-cells are significantly smaller and less tight than those of WT-cells (Figure 3, E–G, and insets). Thus, expression and binding of PTP1B to the cytoplasmic domain of cadherin promotes the localization and function of N-cadherin at cell–cell junctions.

To determine the effect of PTP1B in traffic and surface expression of N-cadherin, WT- and KO-cells were pulse-labeled for 20 min with [35S]methionine and then chased for different times. At the end of each chase point, cell surface...
proteins were labeled with membrane-impermeable sulfo-NHS-LC-biotin. Total N-cadherin was immunoprecipitated and split into halves; one was further reprecipitated with streptavidin-agarose to isolate the surface-labeled N-cadherin pool. Both display enhanced accumulation of fluorescence in Golgi and in scattered puncta. (E–G) Aggregation assays. Representative phase-contrast fields showing cell suspensions of KO-cells (E) and WT-cells (F) after 30-min incubation in HBSSGK-1 mM CaCl₂. Note that KO-cells form smaller and looser aggregates than WT-cells (E, F, and insets). Quantification of the aggregation in HBSSGK alone (Co), or in the presence of 1 mM CaCl₂ or 1 mM EDTA (see Materials and Methods) is shown in the graph (G). Data are means ± SEM of four experiments. Asterisk denotes significant difference (Student’s t, p = 0.001). Bars, (A–D) 60 μm; (E and F) 150 μm.

Figure 3. PTP1B expression and binding to N-cadherin cytoplasmic domain are required for N-cadherin targeting and function. WT-cells (A, C, and D) and KO-cells (B) transfected with GFP fusions of WT N-cadherin (A and B) or two deletion mutants which cannot bind PTP1B (C and D) were fixed and analyzed by fluorescence confocal microscopy. Note the similar phenotype between KO-cells (B) and WT-cells expressing the deletion mutants (C and D). Both display enhanced accumulation of fluorescence in Golgi and in scattered puncta. (E–G) Aggregation assays. Representative phase-contrast fields showing cell suspensions of KO-cells (E) and WT-cells (F) after 30-min incubation in HBSSGK-1 mM CaCl₂. Note that KO-cells form smaller and looser aggregates than WT-cells (E, F, and insets). Quantification of the aggregation in HBSSGK alone (Co), or in the presence of 1 mM CaCl₂ or 1 mM EDTA (see Materials and Methods) is shown in the graph (G). Data are means ± SEM of four experiments. Asterisk denotes significant difference (Student’s t, p = 0.001). Bars, (A–D) 60 μm; (E and F) 150 μm.

ER-to-Golgi Traffic of N-Cadherin Precursors Requires PTP1B Expression But Not Binding to Their Cytoplasmic Domains

The prodomain removal from N-cadherin precursors is a post-Golgi event (Wahl et al., 2003; Koch et al., 2004; see Figure 2A). In this context, the slower kinetics of N-cadherin maturation occurring in KO-cells suggests that PTP1B regulates an early trafficking step. To examine this possibility, we analyzed the redistribution of N-cadherin-GFP into the Golgi region after BFA recovery. BFA causes redistribution of cis- and medial Golgi components into the ER (Klausner et al., 1992). KO- and WT-cells incubated with BFA shows most of the Golgi Sial-T2 (Supplementary Figure S1; Daniotti et al., 2000) and N-cadherin-GFP dispersed at the ER (Figure 5, A and B). After BFA washout, the kinetics of N-cadherin-GFP accumulation into the Golgi region of WT-cells is faster than that of KO-cells (Figure 5, C–F and I). This difference disappears by 60 min, probably because of the quick export of

![Figure 4. Processing and surface expression of N-cadherin is impaired in KO-cells. (A) KO- and WT-cells were labeled with [35S]methionine and then chased for the indicated times (minutes). At the end of each period, cells were surface biotinylated, lysed, and immunoprecipitated with anti-N-cadherin. Samples were divided in halves with one representing the sum of intracellular and surface pools of N-cadherin (total); the other half was incubated with streptavidin-agarose to isolate biotinylated N-cadherin (surface). All samples were fractionated by SDS-PAGE followed by fluorography. Bands in the films were scanned and processed with ImageJ. (B) The integrated intensity of the signal associated with the band of the precursor (145 kDa) at each time point is expressed as percentage of the total N-cadherin signal (precursor + mature). (C) The integrated intensity of the signal corresponding to mature N-cadherin (130 kDa band) present at the biotinylated fraction (cell surface) is expressed as fraction of the total mature N-cadherin signal (biotinylated + nonbiotinylated). Shown are mean ± SD of three to five independent experiments.](image-url)
N-cadherin-GFP from the trans-Golgi in WT-cells (Figure 5, G–I). We confirmed these results by HA/GFP fluorescence ratio analysis of HA-N-cadherin-GFP transfected in KO- and WT-cells. We reasoned that at pre-Golgi locations (before prodomain cleavage), HA/GFP ratios should be higher than at post-Golgi locations (after prodomain cleavage; see Materials and Methods for details). The frequency plot of HA/GFP fluorescence ratios in WT-cells has a Gaussian shape with a mean of 0.5. Gating values higher than two SDs include ~5% of the whole data set (Figure 5J, bottom plot). As expected, BFA treatment led to a shift of HA/GFP ratios to higher values, with a new mean of 1.9 (Figure 5J, top plot). KO-cells also showed a shift of HA/GFP ratios to higher values, as expected for an accumulation of noncleaved precursors (Figure 5J, middle plot). Applying the previous gate to these distributions includes 82 and 44% of data, respectively.

To confirm by biochemical means that PTP1B is required for trafficking of N-cadherin precursors at pre-Golgi stages, we performed endo-H digestion of the HA-N-cadherin-GFP precursor in KO- and WT-cells. N-linked oligosaccharides added in the ER are sensitive to cleavage by endo-H; however, subsequent modifications occurring in the Golgi complex confer resistance to cleavage (Hubbard and Ivatt, 1981). HA-N-cadherin-GFP transfected in KO- and WT-cells was isolated by HA immunoprecipitation and analyzed before/after endo-H digestion. The fraction of HA-N-cadherin-GFP resistant to endo-H in KO-cells was half of that in KO-cells (Figure 6A). Similar reduction of the endo-H–resistant fraction was observed in WT-cells expressing GFP-PTP1B-C215S (Figure 6B, compare with 6A). PTP1B-C215S is a dominant negative mutant that efficiently impairs the function of WT PTP1B in a variety of cell types (Arregui et al., 1998; Balsamo et al., 1998; Pathre et al., 2001; Rhee et al., 2001; Yigzaw et al., 2003; Chang et al., 2006).

Because our data indicate that PTP1B is required for pre-Golgi trafficking of the N-cadherin precursor without being part of the complex, we predicted that the precursor of the HA-N-cadherin<sub>884-891</sub>-GFP construct, which cannot bind PTP1B because of the deletion of the binding site (Rhee et al., 2001; Xu et al., 2002), would traffic normally when transfected in WT-cells. As expected, the endo-H–resistant fraction of the HA-N-cadherin<sub>884-891</sub>-GFP precursor was similar in magnitude to that of HA-N-cadherin-GFP (Figure 6C). The precursor of this construct binds β-catenin and p120 at equivalent levels as the HA-N-cadherin-GFP construct (Supplementary Figure S2). Collectively, our results indicate that PTP1B expression is required for trafficking of the N-cadherin precursor at pre-Golgi stages; however, association of PTP1B with the precursor is not essential for this function.

To determine whether the function of PTP1B in N-cadherin trafficking applies to other glycoproteins using the secretory pathway, we performed endo-H analysis of the temperature-sensitive mutant VSV-G tsO45 in KO- and WT-cells. At the restrictive temperature (40°C) VSV-G tsO45 is retained at the ER and is endo-H–sensitive. At the permissive temperature (32°C) VSV-G tsO45 is exported from the ER and become endo-H–resistant (Balch and Keller, 1986). At the restrictive temperature, most of the VSV-G tsO45 expressed in KO- and WT-cells was sensitive to endo-H (Figure 6D). After shifting to the permissive temperature, endo-H–resistant fractions of VSV-G accumulated progressively and at similar proportions in KO- and WT-cells (Figure 6D). These results indicate a specific regulation of N-cadherin precursor trafficking by PTP1B.

**Association of Catenins to the N-Cadherin Precursor**

Although our results do not reveal the presence of PTP1B in the N-cadherin precursor complexes, we still considered the possibility that PTP1B may stabilize the binding of β-catenin to the N-cadherin precursor, because it occurs in the mature N-cadherin complexes (Lilien and Balsamo, 2005). Stabilizing the binding of β-catenin to N-cadherin precursors may be required for their trafficking, as it has been shown for E-cadherin in Madin-Darby canine kidney (MDCK) cells (Chen et al., 1999). Isolation of the HA-N-cadherin-GFP precursor from transfected WT- and KO-cells, by HA immunoprecipitation, followed by Western blot analysis revealed similar levels of associated α- and β-catenins in both cell lines (Supplementary Figure S3A). Preincubation of KO-cells...
with pervanadate, a general inhibitor of tyrosine phosphatases, led to an overall increase of phosphotyrosine, as expected (Supplementary Figure S3B). However, this treatment did not change the levels of α- and β-catenin bound to the precursor. In addition, phenylalanine substitution of the critical tyrosine-654 in β-catenin did not affect the binding (Supplementary Figure S3C). These results indicate that in the context of the N-cadherin precursor, the association of β-catenin is not regulated by PTPs, as it happens in the cell surface complexes (Balsamo et al., 1998; Xu et al., 2002).

P120 binds to the N-cadherin precursor before the incorporation of α- and β-catenin into the complex (Wahl et al., 2003). P120 has been implied in post-Golgi trafficking of N-cadherin (Mary et al., 2002; Chen et al., 2003; Yanagisawa et al., 2004; Ichii and Takeichi, 2007). However, there is no evidence that P120 plays a role in ER–Golgi trafficking. Our examination of the levels of p120 associated to the HA-N-cadherin-GFP precursor in WT- and KO-cells reveals a significant reduction in KO-cells, which is about half of that found in WT-cells (Figure 7A). In contrast, p120 is present at equivalent levels in complexes of mature N-cadherin isolated from KO- and WT-cells. Because it has been shown that tyrosine phosphorylation of p120 modulates its binding to E-cadherin (Roura et al., 1999), we sought to determine whether the level of phosphotyrosine in p120 was altered in KO-cells. Immunoprecipitation of total tyrosine-phosphorylated proteins from equal amount of proteins of KO- and WT-cells reveals higher levels of phosphotyrosine of p120 in KO-cells. These results suggest that PTP1B contributes to the association of p120 to the N-cadherin precursor likely through regulation of p120 phosphorylation. However, more complex mechanisms may operate to regulate the binding of p120 to mature N-cadherin complexes.

**Requirement of p120 Catenin in Early Stages of N-Cadherin Precursor Trafficking**

Our findings that in KO-cells the N-cadherin precursor showed impaired ER–Golgi trafficking and reduced association with p120 suggest that both phenomena could be associated. To examine this possibility further, we inserted the HA tag in the prodomain of N-cadherin-3A-YFP, a construct bearing a triple alanine substitution in the juxtamembrane domain, which specifically disrupts its interaction with p120 (Thoresen et al., 2000; Chen et al., 2003), and analyzed the ER–Golgi trafficking by endo-H digestion analysis. This construct does not bind to p120 but recruits β-catenin similarly to the HA-N-cadherin-GFP (not shown). HA-N-cadherin-GFP and HA-N-cadherin-3A-YFP constructs were expressed in WT-cells, and the respective precursors were isolated by HA-immunoprecipitation. Half of each immunoprecipitate was kept as control, and the other half was treated with endo-H and analyzed in Western blots as described before. Although the endo-H–resistant fraction of HA-N-cadherin-GFP was ~46%, that of HA-N-cadherin-3A-YFP was barely detectable (Figure 7C). These results were confirmed by microscopy analyzing the HA/YFP fluorescence ratios. When expressed in WT-cells, HA-N-cadherin-3A-YFP distributes in two distinct populations of fluorescent puncta, one with low HA/YFP fluorescence ratios (~0.5) and one with high ratios (~1.9; Figure 7D). High ratios suggest less processing of the precursor and resemble the condition observed for the HA-N-cadherin-GFP construct in KO-cells or in WT-cells treated with BFA (Figure 5).

We additionally analyzed the effects of perturbing the association of p120 to N-cadherin by competition with small peptides encompassing the p120 binding site in the N-cadherin complex. However, this treatment did not change the levels of α- and β-catenin bound to the precursor. In addition, phenylalanine substitution of the critical tyrosine-654 in β-catenin did not affect the binding (Supplementary Figure S3C). These results indicate that in the context of the N-cadherin precursor, the association of β-catenin is not regulated by PTPs, as it happens in the cell surface complexes (Balsamo et al., 1998; Xu et al., 2002).
Figure 7. P120 association with the N-cadherin precursor is required for ER-to-Golgi trafficking. (A) WT- and KO-cells were transfected with HA-N-cadherin-GFP or with a mock vector. Cell lysates were immunoprecipitated with anti-HA (left panels) and anti-N-cadherin (right panels), respectively. In control immunoprecipitations (Co) using WT-cells, the IP antibodies were omitted. The presence of p120 in the complexes was detected by immunoblotting. Note the reduction of p120 associated to the N-cadherin precursor in KO-cells. (B) Cell lysates from KO- and WT-cells were immunoprecipitated with anti-pY or anti-p120. The amount of p120 and phosphotyrosine was assessed by immunoblotting with anti-p120 and anti-pY, respectively. (C) Cell lysates from WT-cells transfected with HA-N-cadherin-GFP (prowt) or HA-N-cadherin-3A-YFP (pro3A) were immunoprecipitated with anti-HA and processed for endo-H digestion. Sensitive (s) and resistant (r) fractions of the precursor were detected in immunoblots with anti-HA. Note that the precursor of HA-N-cadherin-3A-YFP was mostly endo-H sensitive. (D) WT-cells transfected with HA-N-cadherin-3A-YFP were fixed and immunostained for HA. HA/GFP fluorescence ratios were obtained as described in Materials and Methods and expressed in a frequency plot. Means of populations with low and high HA/GFP ratios were indicated, and correspond to puncta labeled with (a) and (b), respectively, in the picture below. (E–H) CHO-K1 cells were cotransfected with N-cadherin-GFP and JMP (E and F) or JMP-3A (G and H). Note that N-cadherin-GFP accumulates at the cell–cell contact in the presence of JMP-3A (arrowhead) but not in the presence of JMP. Also note the enhanced intracellular localization of N-cadherin-GFP in the presence of JMP. The relative junctional/intracellular fluorescence was estimated for both conditions (I). Data represent means ± SEM of >60 cells for each condition. Bars, (D) 5 μm; (F) 25 μm.

PTP1B Regulates N-Cadherin Trafficking

We used several approaches to study the trafficking of N-cadherin in WT- and KO-cells. Metabolic labeling combined with cell surface biotinylation revealed that in KO-cells the proteolytic processing and arrival of mature N-cadherin at the cell surface occurred with slower kinetics than in WT-cells. We interpreted this result as a consequence of an impaired trafficking of the N-cadherin precursor from ER to the Golgi apparatus. Microscopic and further biochemical analyses support this view. Indeed, most of HA-N-cadherin-GFP precursor was endo-H–sensitive in KO-cells, whereas a fraction close to 40% was resistant in WT-cells. The redistribution of N-cadherin-GFP in the Golgi region after BFA recovery occurred with a slower kinetics in KO-cells than in WT-cells. Furthermore, ratio analysis of HA/GFP fluorescence puncta in KO- and WT-cells expressing HA-N-cadherin-GFP showed that the subset of higher ratios were 44 and 5%, respectively, suggesting that the arrival of the N-cadherin precursor to the post-Golgi compartment where the HA-containing propeptide is removed did not occur efficiently in KO-cells. The PTP1B function on trafficking reveals some degree of specificity for N-cadherin because endo-H analysis of another plasma membrane glycoprotein, the temperature-sensitive mutant of VSV-G, did not show differences between WT- and KO-cells.

PTP1B Regulates ER–Golgi Trafficking of N-Cadherin Precursor through a p120-dependent Mechanism

What could be the mechanism underlying the PTP1B function in N-cadherin precursor trafficking? The catalytic domain of the ER-bound PTP1B is in the right topological orientation for dephosphorylation of substrates at the cytosolic face of the ER, as recently demonstrated (Haj et al., 2002; Boute et al., 2003; Romsicki et al., 2004; Cohen et al., 2003). Expression of endogenous p120 in cells coexpressed with peptides and N-cadherin-GFP in CHO-K1 cells, which do not express endogenous cadherins. In these cells, JMP inhibited the localization of N-cadherin-GFP in intercellular junctions and enhanced the accumulation of intracellular fluorescence (Figure 7, E, F, and I). In contrast, JMP-3A did not perturb the N-cadherin localization at cell–cell contacts, and the intracellular fluorescence was weak (Figure 7, G–I). Together, these results suggest that binding of p120 to the N-cadherin precursor is required for normal trafficking through early stages of the secretory pathway.

DISCUSSION

PTP1B bound to the cytoplasmic domain of surface N-cadherin has been implicated in the regulation of N-cadherin–dependent adhesion through dephosphorylation of β-catenin in the complex (Balsamo et al., 1998; Rhee et al., 2001; Lilien and Balsamo, 2005). In the present work we report a novel function of PTP1B in the regulation of anterograde trafficking of the N-cadherin precursor. This apparently does not require PTP1B binding to the precursor and does not involve β-catenin dephosphorylation and dissociation from the complex. Instead, PTP1B dephosphorylates and promotes the binding of p120 to the N-cadherin precursor complex, facilitating its movement through early stages of the secretory pathway.
2004; Hernandez et al., 2006; Anderie et al., 2007). We considered the possibility that PTP1B regulates the binding of catenins to the cytosolic tail of N-cadherin precursors. A candidate protein whose binding to the E-cadherin precursor is a condition for its export from the ER is β-catenin (Chen et al., 1999). Tyrosine phosphorylation of β-catenin negatively regulates its binding to mature N-cadherin, and PTP1B has been identified as a major phosphatase that dephosphorylates β-catenin and stabilizes the N-cadherin/β-catenin complex (Balsamo et al., 1998; Roura et al., 1999; Lilien and Balsamo, 2005). In KO-cells, however, we did not observe a reduction in the levels of β-catenin associated with the N-cadherin precursor. A possibility is that absence of overt tyrosine phosphorylation in the context of the N-cadherin precursor complexes at the ER turns unnecessary the role of tyrosine phosphatases. Indeed, preincubation of KO-cells with the general inhibitor of tyrosine phosphatases pervanadate did not alter the levels of β-catenin bound to the N-cadherin precursor, even when the phosphotyrosine levels of nonbound β-catenin increased (Supplementary Figure 3B). Nevertheless, we found a reduction of ~50% in the amount of p120 associated to the N-cadherin precursor in KO-cells. P120 is the first catenin that associates with the N-cadherin precursor complexes at the ER (Mary et al., 2002; Chen et al., 2003; Yanagisawa et al., 2004; Ichii and Takeichi, 2007). Is p120 association to the N-cadherin precursor also required for movement of the complex through early stages of the biosynthetic pathway? We addressed this question using different approaches. We detected the accumulation of a population of puncta with high HA/YFP fluorescence ratios in WT-cells expressing HA-N-cadherin-3A-YFP, a construct that cannot bind p120, suggesting that HA-N-cadherin-3A-YFP accumulates at pre-Golgi stages. Further support to this view is the observation that the HA-N-cadherin-3A-YFP precursor was almost 100% sensitive to endo-H. Finally, overexpression of small juxtamembrane competitor peptides that sequester p120 and impair its association to full-length cadherin (Anastasiadis et al., 2000) led to visible intracellular accumulation of N-cadherin-GFP. P120 may be dephosphorylated by ER-bound PTP1B previous to its binding to the N-cadherin precursor. Indeed, we observed that p120 in KO-cells have higher levels of phosphotyrosine compared with WT-cells, as expected for a dominant-negative mutant PTP1B. J. Cell Biol. 143, 861–873.

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