A p120-catenin–CK1ε complex regulates Wnt signaling

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Summary

p120-catenin is an E-cadherin-associated protein that modulates E-cadherin function and stability. We describe here that p120-catenin is required for Wnt pathway signaling. p120-catenin binds and is phosphorylated by CK1ε in response to Wnt3a. p120-catenin also associates to the Wnt co-receptor LRP5/6, an interaction mediated by E-cadherin, showing an unexpected physical link between adherens junctions and a Wnt receptor. Depletion of p120-catenin abolishes CK1ε binding to LRP5/6 and prevents CK1ε activation upon Wnt3a stimulation. Elimination of p120-catenin also inhibits early responses to Wnt, such as LRP5/6 and Dvl-2 phosphorylation and axin recruitment to the signalosome, as well as later effects, such as β-catenin stabilization. Moreover, since CK1ε is also required for E-cadherin phosphorylation, a modification that decreases the affinity for β-catenin, p120-catenin depletion prevents the increase in β-catenin transcriptional activity even in the absence of β-catenin degradation. Therefore, these results demonstrate a novel and crucial function of p120-catenin in Wnt signaling and unveil additional points of regulation by this factor of β-catenin transcriptional activity different of β-catenin stability.

Key words: CK1ε, E-cadherin, Wnt, p120-catenin

Introduction

The Wnt pathway plays diverse roles in embryonic development and is implicated in human diseases, including cancer (Raya and Clevers, 2005). A key element in this pathway is the E-cadherin-associated protein β-catenin. When released from the junctional complex, β-catenin translocates to the nucleus, where it interacts with the Tcf family of transcriptional factors and regulates the expression of a variety of genes. The translocation of β-catenin to the nucleus is tightly controlled by the activity of a complex involved in β-catenin degradation. This complex includes the product of the tumor suppressor adenomatous polyposis gene (APC), Axin, and the Thr/Ser protein kinases, CK1α and glycogen synthase kinase 3β (GSK3β) (Liu et al., 2002). As result of the activity of this complex, β-catenin is phosphorylated and degraded by the proteasome. The activity of the degradation complex is blocked by Wnt factors, which activate a signaling pathway stimulating β-catenin transcriptional activity (Raya and Clevers, 2005; MacDonald et al., 2009).

Wnt ligands form a complex with low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) and Frizzled (Fz) receptors (He et al., 2004). Upon Wnt ligand binding, the LRP5/6 cytosolic domain is phosphorylated by casein kinase 1γ (CK1γ), an event depending on the Fz-associated protein Dishevelled (Dvl) (Davidson et al., 2005; Bilic et al., 2007; Zeng et al., 2008). As a consequence of LRP5/6 phosphorylation, Axin and GSK3β are recruited to the complex (Mao et al., 2001; Zeng et al., 2005). Moreover, CK1ε is also activated by Wnt and contributes to the phosphorylation of LRP5/6 and Dvl (Swiatek et al., 2004; Zeng et al., 2005; Price, 2006). Expression of CK1ε has been shown to stabilize β-catenin in *Xenopus* embryos and HEK293 cells and to stimulate β-catenin–Tcf-4-dependent transcription in this same cell line (Peters et al., 1999; Sakanaka et al., 1999). Moreover, addition of CK1ε blocks degradation of β-catenin in a *Xenopus* cell-free system (Gao et al., 2002). Upon phosphorylation of LRP5/6 by the cooperative action Ck1γ, Ck1ε and GSK-3β, the PPPSPxS motif directly binds and inhibits GSK3β (Piao et al., 2008; Wu et al., 2009), preventing phosphorylation of β-catenin by this enzyme and, consequently, increasing β-catenin half-life.

We describe here that CK1ε is constitutively bound to another E-cadherin-associated protein, p120-catenin (also known as catenin δ-1). This catenin binds to a distinct site of E-cadherin that is different from that for β-catenin, and is necessary for the stabilization of E-cadherin at the cell membrane (Davis et al., 2003). Moreover, p120-catenin also interacts with the transcriptional factor Kaiso, through its central armadillo domain (Daniel and Reynolds, 1999), and with Fer or Fyn tyrosine kinases (Kim and Wong, 1995) or RhoA GTPase (Castaño et al., 2007; Yanigisawa et al., 2008), through the 325 amino-acid long N-regulatory domain. Therefore, p120-catenin is a pleiotropic protein that, in addition to controlling E-cadherin stability, modulates Kaiso transcriptional activity, anchors tyrosine kinases to the adherens junctional complex and inhibits the activity of RhoA GTPases (Anastasiadis, 2007). We show that p120-catenin is necessary for the formation of the

Wnt signalosome because it interacts with LRP5/6 and is required for CK1ε activation by Wnt3a.

Results

p120-catenin phosphorylation by CK1 modulates its interaction with E-cadherin

In vitro association assays demonstrated that phosphorylation of p120-catenin by CK1 decreased its interaction with E-cadherin. A fragment of p120-catenin, corresponding to the isoform 3A (amino acids 102-911), the most abundant in epithelial cells, was phosphorylated by the catalytic domain of CK1, common to all the isoforms of this family of kinases (Knippschild et al., 2005). The phosphorylated protein was much less efficiently pulled down by a GST fusion protein consisting of the cytosolic domain of E-cadherin (cytoE-cadh; see Fig. 1A). To characterize the amino acid sequences of p120-catenin involved in this regulation, we constructed a series of deletion mutants lacking part or the entire N-terminus regulatory domain (supplementary material Fig. S1A). This domain contains most of the residues that have been found to be phosphorylated by several protein kinases. The inhibitory effect of CK1 on p120-catenin binding to cyto-E-cadherin was observed with a form of p120-catenin corresponding to isoform 1A (amino acids 1-911), also with the shorter isoform 3A (102-911) and with the deletion mutant (234-911), but not with the 350-911 amino-acid fragment, which comprises the armadillo repeat domain and the C-tail (supplementary material Fig. S1B). Another p120-catenin construct lacking the C-tail and the last three armadillo repeats (amino acids 1-648) was also sensitive to CK1 (supplementary material Fig. S1B). Therefore, these results suggest that the residues involved in the control of p120-catenin binding to E-cadherin are present in the 234-350 amino acid region.

An analysis of p120-catenin phosphorylation by CK1 indicated that, as expected, ^{32}P was mainly incorporated into the first 347 amino acids (Fig. 1B). A proteomic analysis of in vitro phosphorylated p120-catenin identified several modified residues: serines 47, 252, 268, 269, 858, 878 and threonine 879 (Fig. 1C). Of these amino acids, only Ser 252, 268 and 269 are within the 234-350 sequence, previously identified as critical for CK1 regulation of cytoE-cadh binding. Ser252 was not considered since it is not conserved in other species, unlike Ser268 and 269. Mass spectrometry profiles of the peptide corresponding to Ser268 and 269 are shown in supplementary material Fig. S2A-D.

To demonstrate the role of the phosphorylation of these two amino acids in the control of the interaction of p120-catenin with E-cadherin, both serines were replaced by alanines (mutant S→A), so preventing phosphorylation. Unlike wild-type p120-catenin, the S→A mutant was insensitive to CK1 (Fig. 1D), indicating the relevance of these two amino acids. In addition, the two Ser were replaced by Asp, introducing a negative charge that mimics phosphorylation. This p120-catenin mutant (S→D) was less efficiently bound by cytoE-cadh than the wild-type protein (Fig. 1D).

CK1ε binds and phosphorylates p120-catenin in vivo

Since the CK1 domain used in the in vitro study was common to all the members of the CK1 family, we investigated the isoform involved

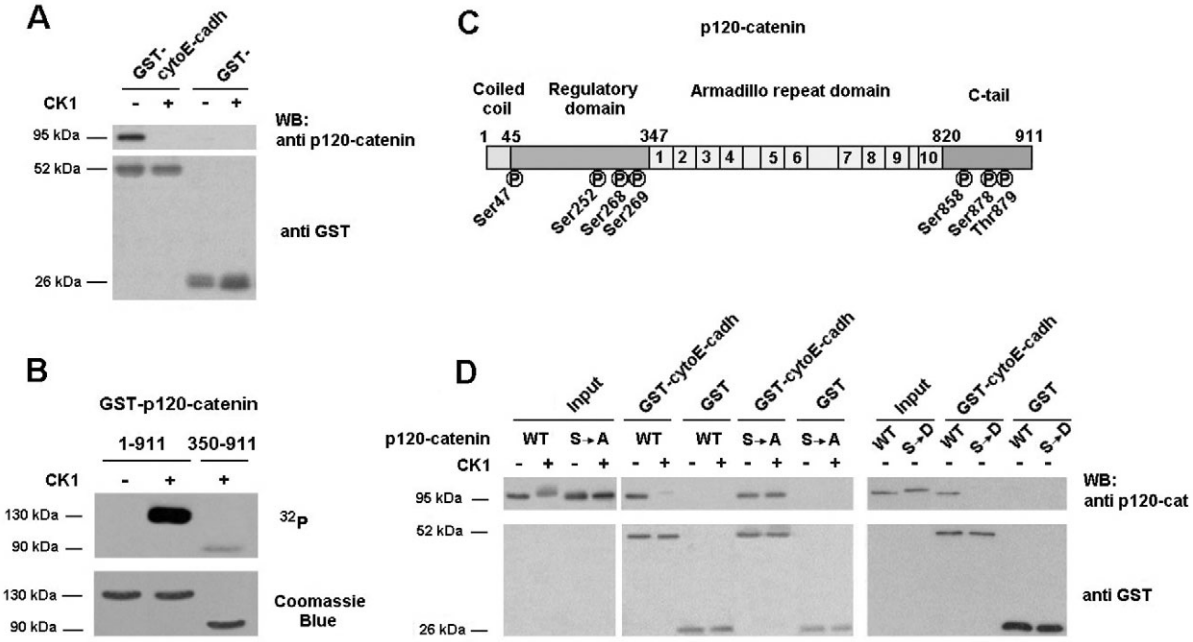

Fig. 1. Phosphorylation of p120-catenin Ser268,269 inhibits p120-catenin-E-cadherin interaction. (**A**) Recombinant p120-catenin (amino acids 102-911) was phosphorylated with CK1 kinase domain. 1 pmol was incubated with GST–cytoE-cadherin, or GST as a control; protein complexes were affinity purified with glutathione-Sepharose and analyzed by western blotting (WB) with anti-p120-catenin. Blots were reanalyzed with anti-GST to ensure that similar amount of fusion protein was present. (**B**) GST–p120-catenin fusion proteins (0.1 pmol) were phosphorylated with CK1 kinase domain (10 mU). Phosphorylation was analyzed by autoradiography and normalized from Coomassie Blue staining. (**C**) Diagram of the different CK1 phosphorylation sites in p120-catenin, identified by mass spectrometry. The different domains forming isoform 1 of p120-catenin are indicated. The epithelial isoform 3 comprises amino acids 102-911. (**D**) p120-catenin binding assays were performed with 1 pmol of p120-catenin (102-911) wild type (WT) or the double point mutants S268A,S269A (S→A) and S268D,S269D (S→D), and 2.5 pmol of GST–cytoE-cadherin, or GST as a control. The different p120-catenin proteins were phosphorylated with recombinant CK1 kinase, as indicated (+). Protein complexes were analyzed by WB with anti-p120-catenin. Control and phosphorylated p120-catenin, WT and point mutants (0.1 pmol), were included as internal references.

in p120-catenin phosphorylation in vivo. First, we examined whether any CK1 isoform immunoprecipitated with p120-catenin 3, the isoform detected in SW-480 cells. As shown in Fig. 2A, CK1ε was detected in an immunocomplex obtained with a p120-catenin monoclonal antibody (mAb) and not with an irrelevant IgG. Other CK1 isoforms, such as CK1α or CK1γ were not observed. Since p120-catenin and E-cadherin associate, we also checked if CK1ε coimmunoprecipitated with E-cadherin. As shown in Fig. 2B, CK1ε was indeed present in the E-cadherin immunocomplex.

Binding of CK1ε was also verified by pull-down assays using different p120-catenin fusion proteins. The association of CK1ε with p120-catenin proteins containing amino acids 1-390 or 234-911 was detected, but not with those containing amino acids 1-347 or 350-911 (Fig. 2C, left panel), suggesting that CK1ε interacts through the 234-390 sequence. Curiously, binding to GST–p120-catenin (1-347) was observed when the 350-end fragment of p120-catenin was added to the assay, indicating that the two halves of the protein interact and reshape the p120-catenin-binding site (Fig. 2C, left panel). Preincubation of GST–p120-catenin with recombinant cytoE-cadh did not affect the binding of CK1ε (Fig. 2C, right panel), suggesting that p120-catenin binds independently to CK1ε and E-cadherin.

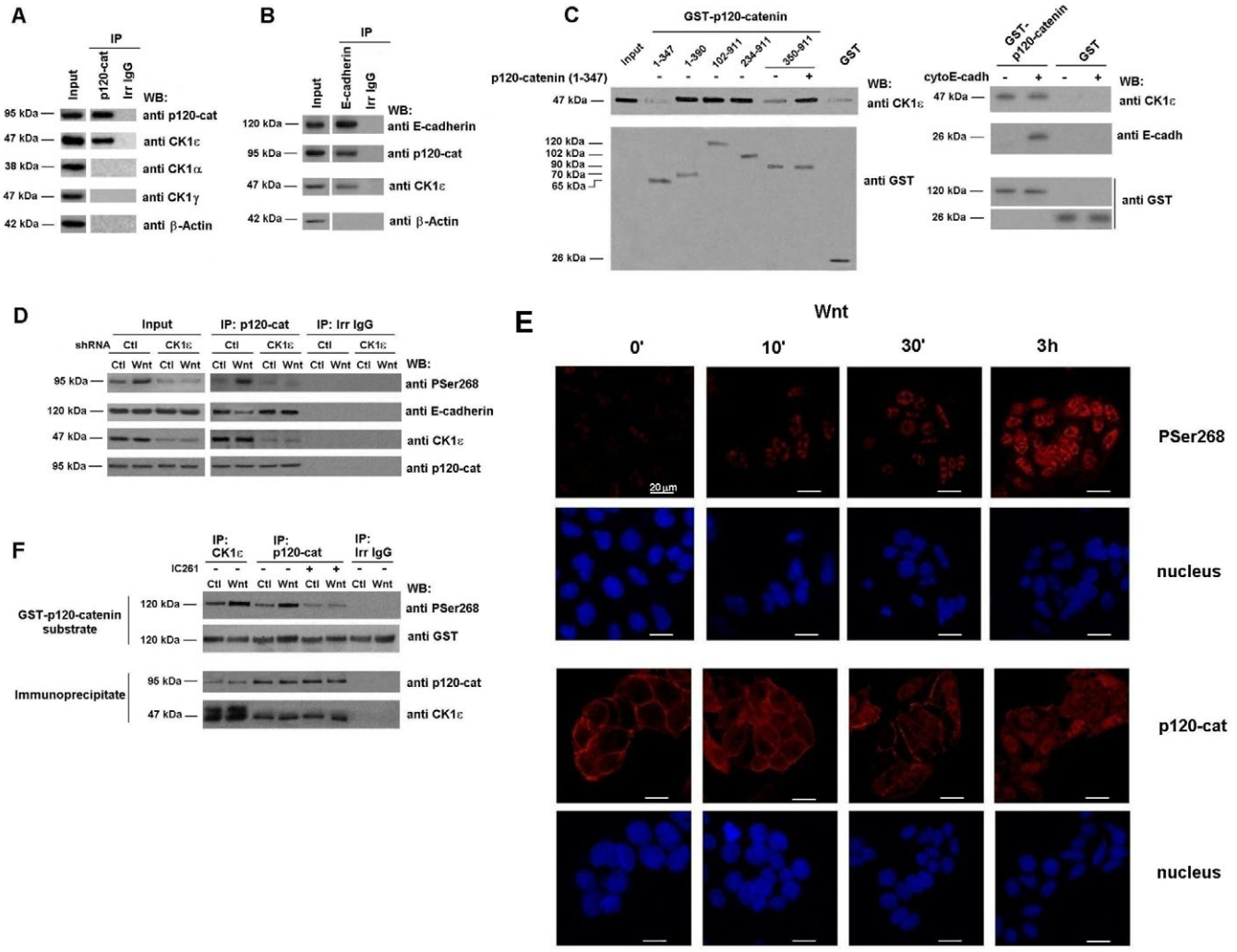

Fig. 2. Wnt3a-activated CK1ε associates with p120-catenin, phosphorylates Ser268 and inhibits p120-catenin interaction with E-cadherin. (**A**,**B**) CK1ε immunoprecipitates with p120-catenin and E-cadherin. p120-catenin (A) or E-cadherin (B) were immunoprecipitated from whole-cell extracts of SW-480 cells and the associated proteins analyzed by western blotting (WB). β-Actin was used as negative control. (**C**) Pull-down assays were performed by incubating 10 pmol of the different GST–p120-catenin deletion mutants with SW-480 cells extracts. Where indicated (+), 20 pmol of p120-catenin (1-347) or cytoE-cadherin were added to the incubation. Protein complexes were purified and analyzed by WB with anti-CK1ε, anti E-cadherin or anti-GST to ensure that similar amounts of fusion proteins were used. (**D**) SW-480 cells were transfected with scrambled or CK1ε-specific shRNA. After selection with 2 μg/ml puromycin, cells were treated with control or Wnt3a-conditioned medium for 4 hours. p120-catenin was immunoprecipitated from total cell extracts and immunocomplexes were analyzed by WB. (**E**) Cells were incubated with control or Wnt3a medium for the indicated times; cells were fixed and analyzed by immunofluorescence with the indicated antibodies. Staining of the nuclei was performed with DAPI. The bar corresponds to 20 μm. (**F**) SW-480 cells were treated with control or Wnt3a-conditioned medium for 4 hours. Total cell extracts were immunoprecipitated with anti-CK1ε or anti-p120-catenin mAbs. Immunocomplexes were analyzed with the indicated antibodies or incubated with 2 pmol of recombinant GST–p120-catenin (102-911) in CK1 phosphorylation conditions, in the presence (lanes 5 and 6) or absence (lanes 3 and 4) of the CK1 inhibitor IC261 (15 μM). Phosphorylation of Ser268 was analyzed by WB with a specific Ser268-*P* antibody. All the data presented in this figure are representative of at least three independent experiments. Irr IgG: an irrelevant IgG used as control in the immunoprecipitation.

Wnt3a modulates p120-catenin phosphorylation by CK1ε and p120-catenin binding to E-cadherin

As CK1ε is activated by Wnt8 (Swiatek et al., 2004), we analyzed whether members of this family of ligands stimulate p120-catenin phosphorylation. To this end, we incubated SW-480 cells with conditional medium from parental L cells or from L cells transfected with a Wnt3a expression plasmid. p120-catenin–Ser268 phosphorylation was determined using a phospho-specific monoclonal antibody (Xia et al., 2004). This Ser268-*P* mAb reacted with p120-catenin phosphorylated by CK1 and not with the non-modified protein (see supplementary material Fig. S3). Moreover, no immunoreactivity was observed when the S→A mutant was phosphorylated (supplementary material Fig. S3), further demonstrating the specificity of this monoclonal antibody.

Wnt3a stimulated Ser268 phosphorylation (Fig. 2D). Concomitantly, and in line with our previous findings, the interaction of p120-catenin with E-cadherin was diminished by Wnt. To check the relevance of CK1ε in Wnt-dependent Ser268 phosphorylation we interfered in the expression of this protein kinase using a specific small hairpin RNA (shRNA). This shRNA did not affect the expression of two other CK1 isoforms analyzed, CK1α and CK1γ (not shown). As shown in Fig. 2D, p120-catenin Ser268 phosphorylation was dependent on the activity of CK1ε, since it was not detected in CK1ε-depleted cells.

A time-course analysis of Ser268 phosphorylation was performed by immunofluorescence (Fig. 2E). Although accumulation of Ser268-*P* was first detected 10 minutes after Wnt addition it was maximal after 3 hours, indicating that it was not an early response to Wnt ligand. This maximal activation correlated with the loss of p120-catenin immunoreactivity in the cell-cell contacts; as shown in Fig. 2E Wnt induced the redistribution of p120-catenin to the cytosol and nucleus, a redistribution only detected at 3 hours of incubation. Phosphorylated p120-catenin was mainly detected in the nucleus (Fig. 2E).

We also checked whether the activity of CK1ε bound to p120-catenin was increased by Wnt3a. For this assay, CK1ε or p120-catenin were immunoprecipitated with a specific mAb and the activity of the immunocomplex on recombinant GST–p120-catenin was assayed, using the Ser268-*P*-specific mAb to detect the modification of this residue. Wnt3a stimulated the Ser268-kinase activity present in both CK1ε and p120-catenin immunocomplexes (Fig. 2F), indicating that both the total and the p120-catenin-associated CK1ε activity were increased by Wnt3a. As an additional control, the protein kinase activity present in the p120-catenin immunocomplex was sensitive to the specific CK1 inhibitor IC261 (Mashoon et al., 2000), indicating that it corresponded to the associated CK1ε.

p120-catenin is required for the interaction of CK1ε with the LRP5/6 co-receptor

We further investigated the mechanism leading to the activation of p120-catenin-bound CK1ε by Wnt3a. A recent report indicated that N-cadherin interacts with the cytoplasmic tail of the Wnt co-receptor LRP5/6 (Hay et al., 2009). We analyzed whether this interaction also occurred with E-cadherin. As shown in Fig. 3A, E-cadherin immunoprecipitated with LRP5/6 and this interaction was sensitive to Wnt3a, since it was disrupted following the addition of this factor. Both p120-catenin and CK1ε were also present in the LRP5/6 immunocomplex and similarly decreased upon Wnt3a binding (Fig. 3A). An inverse experiment, i.e. p120-catenin immunoprecipitation and LRP5/6 western blotting, produced similar results; although some background was observed with the irrelevant IgG used as control, LRP5/6 was detected in the immunoprecipitate, at low levels in cells incubated with Wnt3a (Fig. 3B). Moreover, as shown in previous figures, p120-catenin interaction with E-cadherin was decreased by Wnt3a, whereas that with CK1ε was not modified (Fig. 3B).

The association was also studied by pull-down assays using recombinant GST–cytoE-cadherin or GST–p120-catenin as baits. LRP5/6 was efficiently retained by GST–cytoE-cadherin, but not by GST (Fig. 3C). The cytosolic domain of E-cadherin has been shown to be a substrate of CK1 (Dupre-Cochet et al., 2007). Therefore, we repeated the experiment after phosphorylating GST–cytoE-cadherin in vitro. Incubation of GST–cytoE-cadherin with CK1 induced a shift in the mobility of E-cadherin, probably a consequence of the extensive phosphorylation (Fig. 3C). This phosphorylation of E-cadherin decreased the binding of LRP5/6 without modifying the interaction of E-cadherin with p120-catenin. Since phosphorylation of E-cadherin by CK1 also decreases E-cadherin–β-catenin binding (Dupre-Cochet et al., 2007) (supplementary material Fig. S4) we analyzed whether β-catenin was involved in the association of LRP5/6 to E-cadherin. As shown in Fig. 3D, addition of recombinant β-catenin did not modify the amount of LRP5/6 pulled-down by GST–cytoE-cadherin. Moreover, binding of LRP5/6 to GST–β-catenin was greatly increased by the addition of recombinant cytoE-cadh, suggesting that LRP5/6 interacts with E-cadherin and not with β-catenin.

LRP5/6 was also retained by GST–p120-catenin, although to a lesser extent than by GST–cytoE-cadh (Fig. 3C). In vitro phosphorylation of GST–p120-catenin by CK1 prevented binding of both E-cadherin and LRP5/6 (Fig. 3C). Therefore, these results demonstrate that CK1 exerts a dual role in the stability of the LRP5–E-cadherin–p120-catenin complex, both phosphorylating E-cadherin and disrupting its association with LRP5, and p120-catenin, and decreasing its association with E-cadherin.

Co-immunoprecipitation experiments performed in extracts depleted in p120-catenin or E-cadherin verified these conclusions. Downregulation of p120-catenin decreased the levels of E-cadherin, in agreement with previous reports (Ireton et al., 2002) (Fig. 3E). The amount of LRP5/6 immunoprecipitated with E-cadherin was decreased to a similar extent to that of E-cadherin, when comparing cells transfected with p120-catenin shRNA with respect to the control, suggesting that the association of LRP5/6 with E-cadherin was not mediated by p120-catenin. To better visualize the result of this experiment, equivalent amounts of E-cadherin were immunoprecipitated (Fig. 3E, lower panel); under these conditions the levels of LRP5/6 in the immunoprecipitates were similar. To further demonstrate that E-cadherin mediated the interaction of LRP5/6 with the complex, the levels of E-cadherin were downregulated using a specific shRNA. Elimination of this protein severely affected the interaction of the Wnt co-receptor with p120-catenin, since the amount of this protein immunoprecipitated with LRP5/6 was markedly decreased (Fig. 3F). Similar effects of E-cadherin depletion were observed in the interaction of LRP5/6 with β-catenin, which was markedly lower in the cells where E-cadherin expression had been reduced (supplementary material Fig. S5).

Downregulation of E-cadherin also prevented p120-catenin phosphorylation, estimated by analyzing the phosphorylation of Ser268 in this catenin (Fig. 3F), suggesting that interaction of the E-cadherin–p120-catenin–CK1ε complex with LRP5/6 is required for the phosphorylation of p120-catenin.

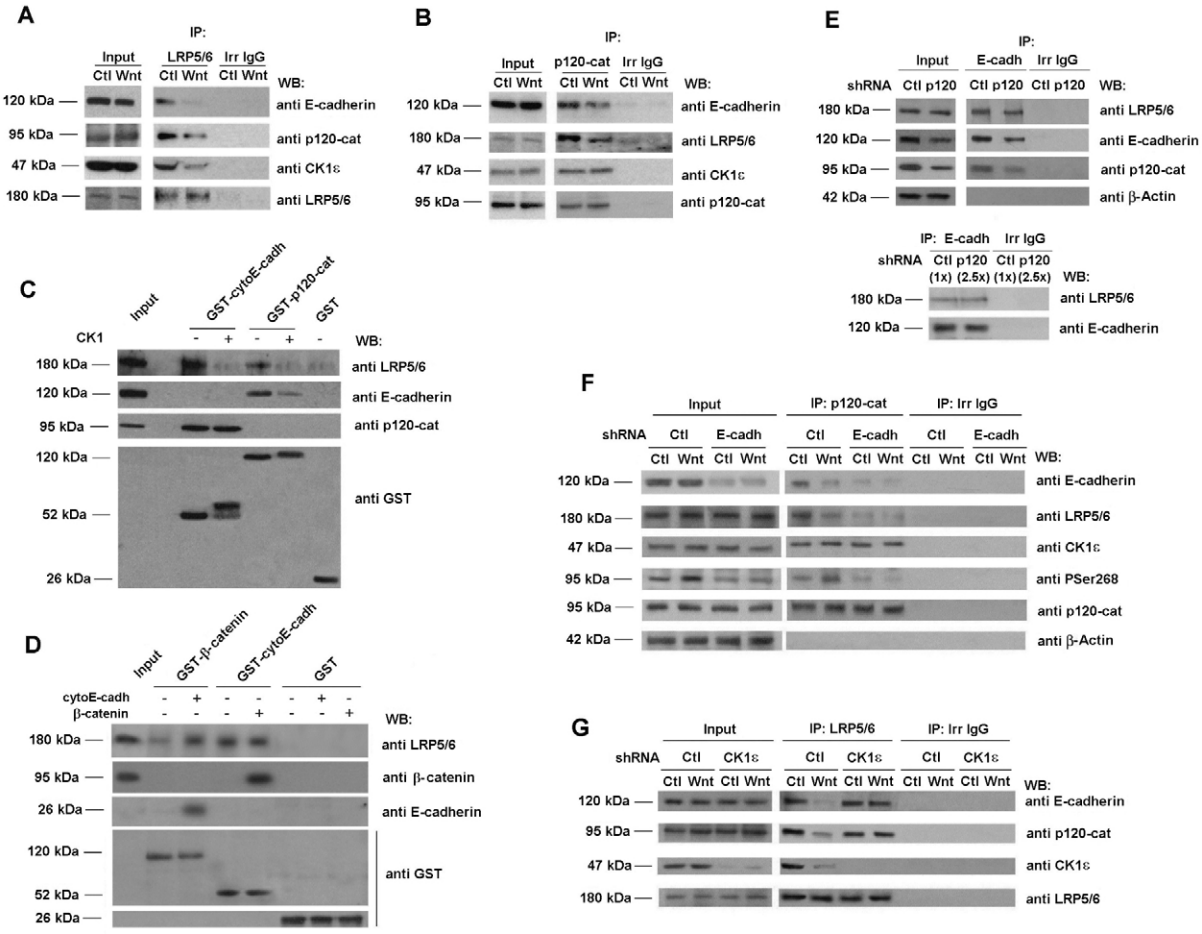

Fig. 3. The interaction of LRP5/6 receptor with p120-catenin and E-cadherin is modulated by CK1ε and Wnt3a. SW-480 cells stimulated or not with Wnt3a-conditioned medium were lysed and LRP5/6 (**A**) or p120-catenin (**B**) were immunoprecipitated with specific antibodies. Associated proteins were analyzed by western blotting (WB). In the 'Input' lane, a sample corresponding to 5% of the lysates used was loaded. (**C**,**D**) Recombinant GST fusion proteins containing cyto-E-cadh, p120-catenin (102-911), β-catenin or GST as a control were phosphorylated in vitro with the CK1 kinase domain, as indicated (+). Pull-down assays were performed by incubating fusion proteins (7.5 pmol) with extracts from SW-480 cells, in the presence of recombinant β-catenin and cytoE-cadh (10 pmol) where indicated. Protein complexes were affinity-purified and analyzed by WB. (**E**,**F**,**G**) SW-480 cells were depleted of p120-catenin (E), E-cadherin (F) or CK1ε (G), using specific shRNAs. Cells were treated with control or Wnt3a-conditioned medium for 4 hours. E-cadherin (E), p120-catenin (F) or LRP5/6 (G) were immunoprecipitated and immunocomplexes analyzed by WB. The immunoprecipitation in E, was repeated (lower panel) using 2.5-fold more extract from cells treated with shRNA p120-catenin than from cells transfected with scrambled shRNA, in order to immunoprecipitate similar amounts of E-cadherin. All the data presented in this figure are representative of at least three independent experiments.

Finally we verified the role of CKε in the regulation of the association of the p120-catenin–E-cadherin complex with LRP5/6 in cells. Depletion of CK1ε prevented the Wnt3a-induced downregulation in the association of LRP5/6 with E-cadherin or p120-catenin (Fig. 3G), indicating that CK1ε controls the interaction of LRP5/6 with E-cadherin.

Therefore, these results indicate that E-cadherin mediates the interaction of the p120-catein–CK1ε complex with the Wnt co-receptor LRP5/6 and that the CK1ε-dependent phosphorylation of E-cadherin and p120-catenin disrupts these associations, decreasing the interaction of E-cadherin with LRP5/6 and that of p120-catenin with E-cadherin.

p120-catenin is required for CK1ε activation by Wnt3a

The lack of phosphorylation of Ser268 in p120-catenin upon Wnt treatment in E-cadherin-deficient cells suggested that the p120-catenin–E-cadherin complex was required for CK1ε activation by Wnt3a. To check this hypothesis, CK1ε was immunoprecipitated and the activity of this protein kinase was measured using recombinant GST–p120-catenin as substrate, and analyzed by measuring phosphorylation on Ser268. As shown in Fig. 4A, elimination of p120-catenin using a specific shRNA greatly affected the activity of this protein kinase in non-stimulated and Wnt3a-treated cells. CK1ε was only very slightly stimulated by Wnt in p120-catenin-deficient cells, in contrast to what happened in control cells.

Dvl is a CK1 substrate frequently used as an early read-out of Wnt activity, since phosphorylation causes a shift in the electrophoretic mobility of this protein (Hino et al., 2003). As shown, depletion of p120-catenin (Fig. 4B) exerted the same effect as downregulation of CK1ε on the phosphorylation of Dvl, and prevented the shift in Dvl induced by Wnt3a. Moreover, we also analyzed Thr1479 phosphorylation in LRP5/6, another rapid response to Wnt3a that requires CK1ε and Dvl activation (Bilic et al., 2007). As shown in Fig. 4B, depletion of CK1ε or p120-catenin prevented Wnt3a-induced stimulation of Thr1479

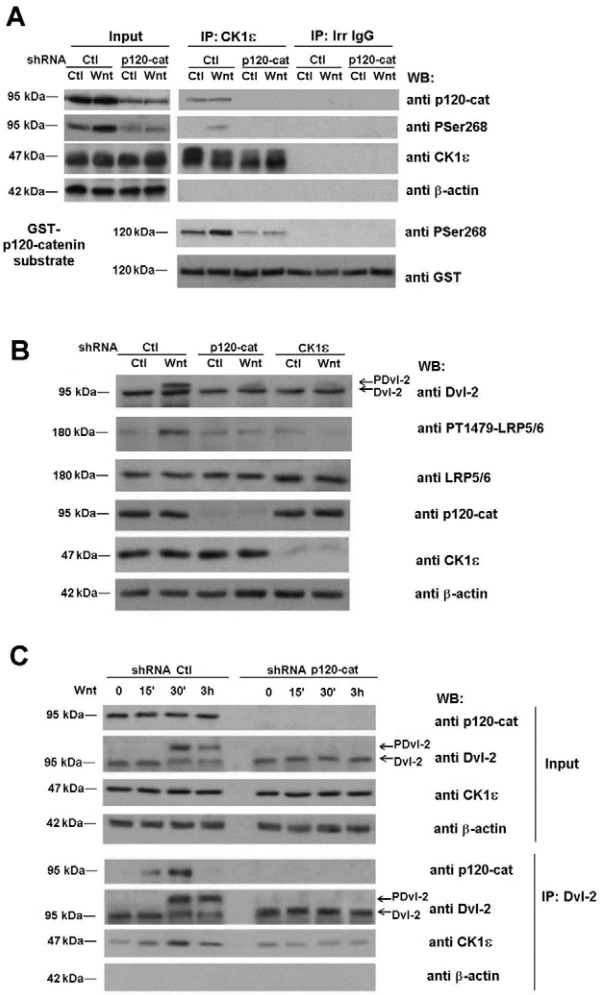

Fig. 4. p120-catenin is required for Wnt3a-induced activation of CK1ε and LRP5/6 and Dvl-2 phosphorylation. SW-480 cells were infected with scrambled or shRNAs specific for the indicated genes and treated with control or Wnt3a-conditioned medium for 4 hours. (**A**) Cell extracts were immunoprecipitated with anti-CK1ε mAb. Immunocomplexes were analyzed with the indicated antibodies or incubated with 2 pmol recombinant GST–p120-catenin (102-911) in CK1 phosphorylation conditions, and the phosphorylation of Ser268 was analyzed by western blotting (WB) with a specific Ser268-*P* antibody as a read-out of CK1 activity. (**B**) Total cell extracts were analyzed with antibodies against Dvl-2, LRP5/6 phosphoT1479, total LRP5/6 or the indicated proteins. The migration of the not-phosphorylated (Dvl-2) or phosphorylated (PDvl-2) forms of Dvl-2 is indicated by arrows. (**C**) SW-480 cells were infected with scrambled or shRNAs specific for p120-catenin and treated with Wnt3a-conditioned medium for the indicated times. Cell extracts were immunoprecipitated with a Dvl-2 pAb and associated proteins were analyzed by WB with the indicated antibodies. All the data presented are representative of three independent experiments.

phosphorylation, detected with a phospho-specific antibody (Bilic et al., 2007).

We also determined the kinetics of the p120-catenin association with Dvl. As seen in Fig. 4C, binding of p120-catenin to Dvl-2 was increased upon addition of Wnt3a: interaction was observed after 15 minutes and was maximal at 30 minutes. The association was transient since it was downregulated at 3 hours (Fig. 4C). Binding of CK1ε to Dvl-2 followed the same pattern, although in this case a basal level of association between CK1ε and Dvl-2 was observed in not-stimulated cells, probably reflecting a direct binding between the two proteins. In any case, the amount of CK1ε associated to Dvl-2 was upregulated by Wnt3a at 30 minutes and returned to the basal levels by 3 hours. Elimination of p120-catenin prevented this increase. Phosphorylation of Dvl-2, determined by the shift in the molecular mass of this protein, was also increased 30 minutes after addition of Wnt3a but, in this case, was maintained at 3 hours (Fig. 4C). As previously shown, p120-catenin depletion prevented this upregulation. The analysis of Dvl-2-associated CK1ε activity also showed an increase in the activity of this enzyme 30 minutes after Wnt addition that did not persist at 3 hours (supplementary material Fig. S6).

The p120-catenin–E-cadherin complex controls assembly of Wnt signaling complex

The influence of the E-cadherin–p120-catenin complex in the formation of the signalosome was also examined. Dvl-2 was associated with the LRP5/6 only in Wnt3a-stimulated cells (Fig. 5A). E-cadherin depletion prevented this association, as well as the interaction of p120-catenin and CK1ε with LRP5/6 (Fig. 5A). The cellular distribution of Dvl-2 was also altered by p120-catenin depletion. By immunofluorescence, Dvl-2 was detected diffusely distributed in the cytosol in not-stimulated cells (Fig. 5B). Two hours after Wnt3a addition, Dvl-2 was mainly localized in the cell membrane, suggesting its recruitment by the Wnt receptor complex. p120-catenin depletion eliminated this translocation; the cellular distribution of Dvl-2 was not modified upon Wnt addition in p120-catenin-deficient cells (Fig. 5B). Similar results were obtained when Axin interaction with Dvl was examined: whereas Wnt3a increased this interaction, p120-catenin depletion markedly prevented upregulation (Fig. 5C).

p120-catenin is required for Wnt3a-induced β-catenin stabilization and transcriptional activity

We also determined whether p120-catenin was also relevant for more distal effects of Wnt3a stimulation, such as the accumulation of β-catenin. For this purpose we used HEK293 cells, since the SW-480 cell line used in our experiments is unable to degrade β-catenin because of an APC mutation. Addition of Wnt3a increased the total levels of β-catenin in HEK293 cells (Fig. 6A,B). Quantification of different experiments indicated that Wnt3a-treated cells showed a 3.9-fold increase in β-catenin protein compared with control cells (Fig. 6C). Inhibition of CK1ε using a shRNA totally prevented this upregulation (Fig. 6A,C). A p120-catenin shRNA that downregulated p120-catenin (by 80%, determined by scanning the autoradiograms) also prevented the β-catenin upregulation caused by Wnt (Fig. 6B,C).

β-catenin stabilization was also analyzed with a monoclonal antibody that recognizes the active form of this protein that is unphosphorylated at Ser37 and Thr41. The amount of active β-catenin was increased by Wnt3a (Fig. 6B); as expected this upregulation was detected in the nucleus by immunofluorescence (Fig. 6D). p120-catenin-depletion abolished the increase in active β-catenin upon Wnt3a stimulation, as determined by western blot and immunofluorescence (Fig. 6B,D).

The effect on β-catenin transcriptional activity was also determined using a β-catenin–Tcf4 responsive plasmid. Elimination of p120-catenin markedly affected the upregulation in this parameter induced by Wnt3a in HEK293 cells (Fig. 6E). Curiously, although SW-480 cells were deficient in β-catenin

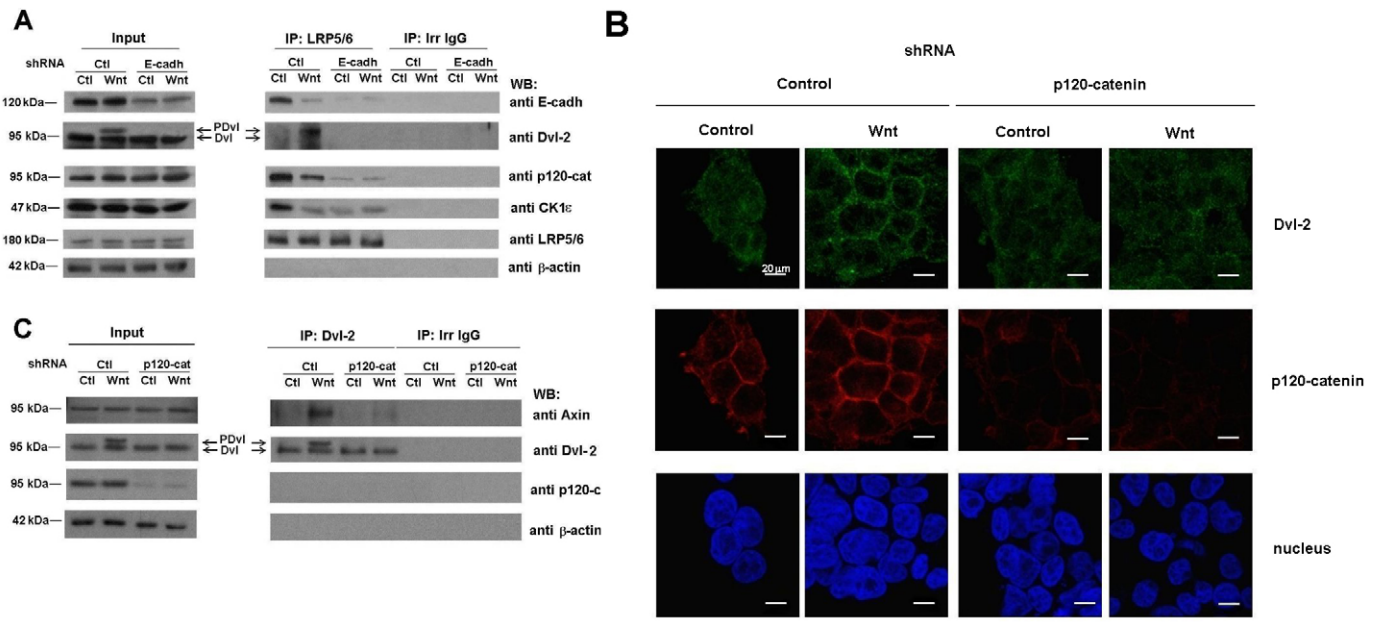

Fig. 5. p120-catenin depletion prevents assembly of the Wnt signalosome. SW-480 cells were infected with scrambled or shRNAs specific for the indicated genes and treated with control or Wnt3a-conditioned medium for 4 hours. Cell extracts were immunoprecipitated with anti-LRP5/6 (**A**) or Dvl-2 (**C**) pAbs and associated proteins were analyzed by WB with the indicated antibodies. The data presented in this figure are representative of three independent experiments. (**B**) The subcellular distribution of Dvl-2 and p120-catenin was determined 30 minutes after the addition of Wnt3a-conditional medium. The analysis was performed by immunofluorescence using a pAb against Dvl-2 and a mAb against p120-catenin. No signal was obtained when the same analysis was performed in the absence of primary antibody. Scale bar: 20 μm.

degradation and levels of this protein were not altered by Wnt3a (data not shown), Wnt3a also increased β-catenin transcriptional activity in these cells (Fig. 6E), although to a lower extent than in HEK293 cells. Comparable effects were observed in another APC mutant cell line, HT-29 M6 (not shown). In both cell lines, elimination of p120-catenin blocked the Wnt3a effect. A similar effect of p120-catenin depletion was observed on the expression of *Myc*, a well established target of β-catenin transcriptional activity (He et al., 1998; Van de Wetering et al., 2002). The increased expression of this gene detected upon Wnt3a stimulation even in SW-480 cells was prevented by p120-catenin depletion (Fig. 6F).

We speculated that the higher β-catenin-dependent transcription upon Wnt3a activation could be a consequence of an enhanced mobilization of this protein; that is, of a higher release from its interaction with E-cadherin at the adherens junctions. Phosphorylation of E-cadherin by CK1 decreases E-cadherin–β-catenin binding (Dupre-Cochet et al., 2007) (supplementary material Fig. S4). In accordance, and considering that CK1ε activity was upregulated upon Wnt3a addition, E-cadherin–β-catenin association was decreased by Wnt3a in SW-480 cells (Fig. 6G). This downregulation was prevented by CK1ε depletion, indicating that p120-catenin- and CK1ε-mediated Wnt signaling not only controls β-catenin stability but also β-catenin interaction with E-cadherin.

Discussion

p120-catenin is a pleiotropic protein with a growing number of actions. It has been reported to be essential for the control of E-cadherin protein stability, the regulation of the activity of Rho and Rac small GTPases and the function of Kaiso transcriptional repressor (Anastasiadis, 2007). We found that p120-catenin is also constitutively associated with CK1ε, a potent activator of Wnt signaling (Price, 2006). CK1ε binding takes place through amino acids 234-390 of p120-catenin, a region in the N-terminal regulatory domain of this protein. Although this sequence is conserved in p120-catenins from different vertebrates, it is not present in lower eukaryotes suggesting that p120-catenin–CK1ε association, and further involvement of p120-catenin in Wnt signaling, is restricted to higher eukaryotes. Preliminary results also indicate that other members of p120-catenin, such as ARVCF (but not δ-catenin) can also interact with CK1ε (data not shown), in accordance with the amino acid similarity in this sequence.

p120-catenin interaction is relevant for CK1ε action, since the cellular elimination of p120-catenin greatly decreases the activity of this protein kinase. It has been shown that auto-phosphorylation of CK1ε C-tail prevents substrate access to the active site, therefore causing the auto-inhibition of the enzyme (Dahlberg et al., 2009). It is possible that binding to p120-catenin limits this auto-phosphorylation reaction. Alternatively, p120-catenin might provide the anchor for the serine phosphatase involved in dephosphorylation and CK1ε activation.

We have studied the involvement of p120-catenin in CK1ε activation and other initial signals triggered by Wnt in SW-480 cells. Although these cells are mutant in APC and have higher levels of β-catenin, they do not show alterations in the initial responses to canonical Wnt, since upon addition of Wnt3a they show a rapid CK1ε activation, Dvl-2 phosphorylation, Axin recruitment to the complex and LRP5/6 Thr1479 phosphorylation. All these Wnt effects are prevented by p120-catenin depletion in these cells. Some of them (Dvl-2 and LRP5 Thr1479 phosphorylation) have been examined in other cellular systems (HT-29 M6, HCT-116, HEK293 cells) and are similarly prevented by p120-catenin downregulation (data not shown).

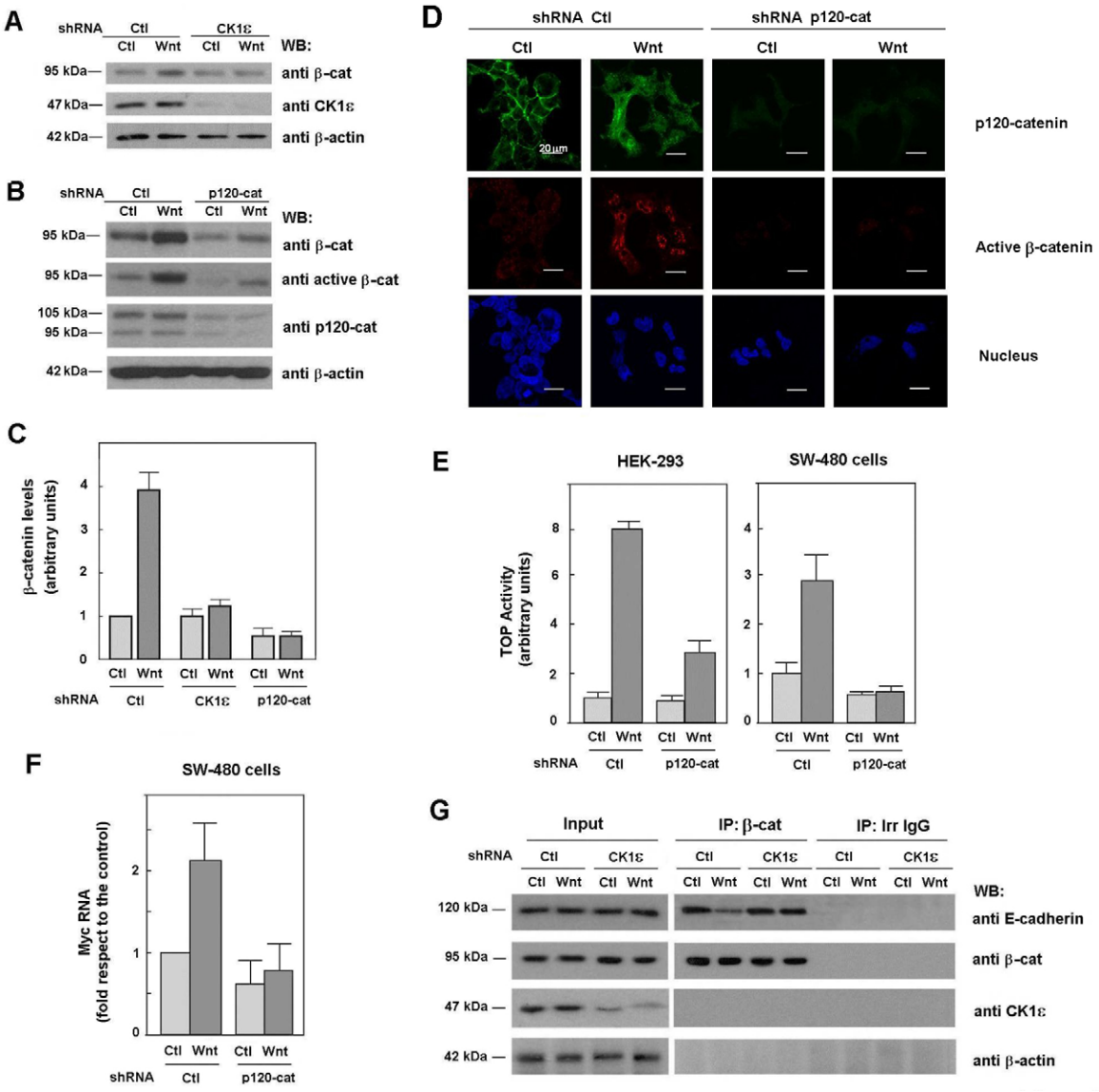

Fig. 6. p120-catenin–CK1ε complex controls β-catenin accumulation and association to E-cadherin. (**A**,**B**,**C**) HEK293 cells were depleted of CK1ε (A) or p120-catenin (B) using specific shRNAs, and treated with control or Wnt3a-conditioned medium. Levels of total β-catenin, active β-catenin or p120-catenin were determined by western blotting (WB). Active β-catenin was determined using a Ab recognizing the unphosphorylated Ser37 and Ser41 in β-catenin. (C) The autoradiograms from the different experiments performed were scanned and the value corresponding to the β-catenin band are presented relative to the value obtained in non-stimulated cells expressing the scrambled siRNA. The values are means ± s.d. of the results of six (cells transfected with scrambled siRNA) or three (cells transfected with CK1ε or p120-cat siRNA) experiments. (**D**) The subcellular distribution of active β-catenin was determined by immunofluorescence in HEK293 cells transfected with a control or p120-catenin-specific shRNA, using a pAb against active β-catenin and a mAb against p120-catenin. Where indicated cells were treated with Wnt3a medium for 4 hours. Scale bars: 20 μm. (**E**) β-catenin transcriptional activity was determined using the TOP reporter plasmid in the indicated cells transfected with scrambled or p120-catenin shRNA. pTK-*Renilla* plasmid was transfected to normalize the efficiency of transfection. Relative luciferase activity was determined 48 after transfection; when indicated cells were treated for the last 16 hours with Wnt3a-conditional medium. The results show the average ± range of two experiments performed in triplicate. (**F**) RNA was isolated from SW-480 cells transfected with control or p120-catenin shRNA, treated with Wnt3a for 48 hours. Expression of *Myc* was assessed by quantitative RT-PCR. The figure shown the average ± range of the results of three experiments. (**G**) Extracts from SW-480 cells were prepared as above from cells expressing the indicated shRNAs and incubated for 4 hours with control or Wnt3a-conditioned medium. β-catenin was immunoprecipitated with a specific mAb and the presence in the immunocomplex of E-cadherin was determined by western blotting. In the input lane, 5% of each total cell extract used. The result of a representative experiment out of three performed is shown.

Our data demonstrate that the p120-catenin–CK1ε complex interacts with the LRP5/6 Wnt co-receptor, an association mediated by E-cadherin. Our model on the involvement of the p120-catenin–CK1ε complex in Wnt signaling, which is depicted in Fig. 7 suggests that this complex is required at the initial steps of receptor activation. In non-stimulated cells (Fig. 7A), E-cadherin forms a complex with the LRP5/6 Wnt co-receptor. p120-catenin associated with CK1ε is also bound to this complex. Upon binding of Wnt ligands and formation of the LRP5/6-Frizzled receptor complex (Fig. 7B), CK1ε is activated by removal of inhibitory phosphates from serines in the C-terminal extension (Price, 2006). Activated CK1ε phosphorylates several proteins in this complex, contributing

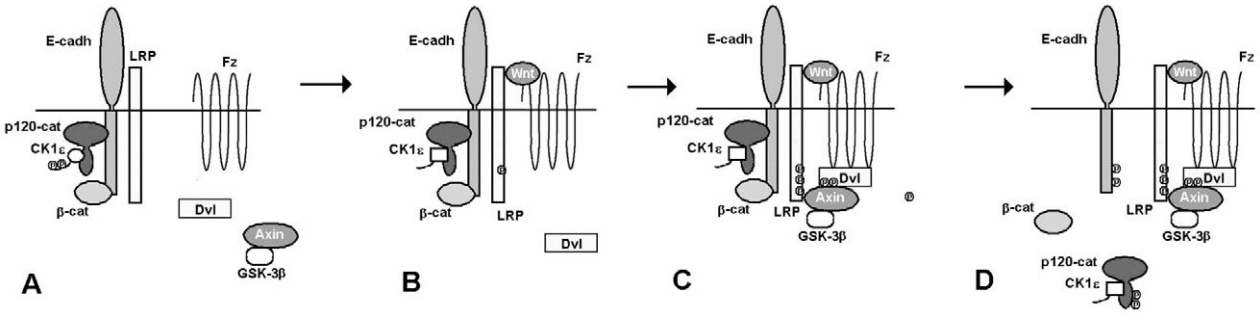

Fig. 7. Model of the involvement of p120-catenin–CK1ε in the Wnt pathway. In resting conditions (**A**), p120-catenin is bound to inactive CK1ε through its N-terminal regulatory domain and to E-cadherin through the armadillo domain. E-cadherin is also associated with the Wnt co-receptor LRP5/6. Upon binding of Wnt ligands and the formation of the LRP5/6-Fz complex, CK1ε is activated by removal of inhibitory phosphates on Ser residues in its C-terminal tail (**B**). Activated CK1ε phosphorylates Dvl-2 (Dvl), favoring its interaction with Axin, and the subsequent phosphorylation of LRP5 by CK1ε and GSK-3β and the inhibition of this kinase (**C**). Finally CK1ε phosphorylates E-cadherin, which loses its association with LRP5/6 and β-catenin, and p120-catenin, releasing it from E-cadherin (**D**).

to Wnt pathway activation by several routes. First, it modifies LRP5/6, probably working cooperatively with CK1γ, and enhances the interaction of the co-receptor with Dvl and the recruitment of Axin and GSK-3β (Fig. 7C). Second, it can phosphorylate Dvl, increasing its affinity for Axin. As a final result, PPPSPxS sequences in LRP5/6 are phosphorylated, GSK-3β is inhibited and β-catenin is stabilized.

CK1ε is also required for other modifications involved in the disruption of the complex. For instance, it can phosphorylate E-cadherin, decreasing its interaction with LRP5/6. It remains to be investigated whether this decreased association is relevant to some of the signals triggered in the Wnt pathway; for instance dissociation of E-cadherin might be required for Wnt receptor clustering (MacDonald et al., 2009). Moreover, CK1-phosphorylated E-cadherin binds β-catenin with lower affinity. This effect will probably cooperate with other modifications in this protein (such as tyrosine modification) in releasing β-catenin from the adherens junctional complex, and enabling its transfer to the nucleus. Therefore, according to this model, Wnt does not only modulate β-catenin protein stability but also mobilizes E-cadherin-associated β-catenin.

CK1ε can also phosphorylate p120-catenin, downregulating the binding to E-cadherin and releasing p120-catenin from the complex (Fig. 7D). Although it is possible that p120-catenin also plays additional roles in the nucleus (see below), disruption of E-cadherin–p120-catenin may be necessary for the finalization of the Wnt signal, since CK1ε would be released from the signalosome and would not be capable of continuing to phosphorylate LRP5/6 and Dvl-2.

We think that this model further refines our current knowledge on the role of Wnt, and demonstrates for the first time that p120-catenin takes part in this pathway, acting as a docking protein for CK1ε. However, the action of this catenin is not unique since its elimination can also indirectly favor β-catenin translocation to the nucleus. p120-catenin depletion decreases E-cadherin protein stability in many cell lines, therefore releasing β-catenin from the adherens junctions. Therefore, the final consequence of p120-catenin depletion would depend on the activity of the β-catenin degradation complex in different cell lines and p120-catenin mutants would not likely present a clear Wnt phenotype. This is not unusual since positive and negative effects on Wnt signaling has also been demonstrated for Axin, another key component of this pathway (MacDonald et al., 2009).

Our results open several potential lines of research. For instance, p120-catenin might be necessary for the recruitment to the Wnt receptor of the Ser/Thr phosphatase responsible for CK1ε activation. In this respect, binding of p120-catenin to Tyr phosphatases has already been reported (Alemà and Salvatore, 2007). Moreover, our results show that Wnt effects on β-catenin-mediated transcription can be observed even in cells deficient for β-catenin degradation (see Fig. 6). These effects, dependent on p120-catenin, are probably due to the increased release of β-catenin from E-cadherin caused by Wnt3a. However, we cannot disregard other potential effects of cytoplasmic p120-catenin on the β-catenin traffic to the nucleus, a step mediated by p120-catenin-sensitive small RhoGTPases (Anastasiadis, 2007; Xu et al., 2008). Moreover, recent data from our laboratory indicates that p120-catenin not bound to E-cadherin modulates the activity of Kaiso, a transcriptional repressor of Wnt signaling (Park et al., 2006) (B.d.V.-P., D.C., A.G.d.H. and M.D., unpublished observations). In any case, our results indicate that p120-catenin acts as the main anchor protein mediating the interaction of CK1ε with the Wnt receptor complex and is required for the activation of this essential component of the Wnt signaling pathway.

Materials and Methods

Cell culture

A subpopulation of SW-480 epithelial cells, established from a primary colon adenocarcinoma, were obtained from our Institute Cell Bank. These cells contain a truncated form of APC and express higher levels of E-cadherin than the parental population (Conacci-Sorrell et al., 2003). Assays were performed in these cells when they were 60-80% confluent. Alternatively, HT-29 M6 intestinal tumor cells, also containing a truncated APC, or HEK293 cells were used. Control L fibroblasts and L fibroblasts stably transfected with a plasmid encoding Wnt3a were obtained from the ATCC and were cultured in medium containing 0.4 mg/ml G-418. Where indicated, cells were treated with conditional medium from control or Wnt3a-expressing cells for 4 hours unless otherwise indicated.

Preparation of DNA constructs

The generation of the bacterial expression plasmid pGEX-6P encoding the GST protein fused to the different p120-catenin regions encompassing amino acids 1-911, 102-911, 234-911, 350-911, or to the cytosolic domain of E-cadherin has been previously described (Castaño et al., 2007; Raurell et al., 2008). To generate the expression plasmid pGEX encoding GST fused to the p120-catenin amino acids 1-648, the cDNA fragment was released from the p120 (1-911)-pGEX-6P3 plasmid by digestion with *Eco*RI-*Xho*I and cloned into *Eco*RI–*Xho*I-linearized pGEX-6P3. pGEX–p120-catenin (1-390) was generated by amplification of the p120-catenin (1-390) fragment from pGEX p120-catenin (1-911) by PCR, adding 5′ *Eco*RI and 3′ *Xho*I restriction sites into the oligonucleotides and cloning into *Eco*RI–*Xho*I-linearized pGEX-6P3. pGEX p120-catenin (1-347) was generated by amplification of this p120-catenin fragment from pGEX p120-catenin (1-911) by PCR, inserting 5′ *Eco*RI and 3′ *Eco*RV restriction sites and cloning into *Eco*RI–*Eco*RV-linearized pGEX-6P3.

p120-catenin point mutants (S268A/S269A and S268D/S269D) were obtained by site-directed mutagenesis using pGEX p120-catenin (102-911) as template and platinum Pfx DNA polymerase (Invitrogen), according to the manufacturer's instructions. The mutagenic sense primers used for the generation of the above-mentioned mutants were, respectively, 5′-GTT CGA GTA GGT GGA **GCC GCT** GTG GAC CTG CAT CGT-3′ and 5′-GTT CGA GTA GGT GGA **GAC GAT** GTG GAC CTG CAT CGT-3′. The product was digested with *Dpn*I (New England Biolabs) prior to bacterial transformation to eliminate the non-mutated original template. Nucleotides modified with respect to the p120-catenin sequence (GenBank accession number Z17804) are indicated in bold. All the constructs were verified by sequencing.

The eukaryotic expression plasmid pEBG-2T encoding the GST protein fused to wild-type p120ctn isoform3 (aa102-911), and the point mutants S268A/S269A and S268D/S269D were generated by PCR amplification of p120-catenin (102-911) inserts from pGEX plasmids, introducing 5′ *Kpn*I and 3′ *Not*I restriction sites. These fragments were cloned into the linearized *Kpn*I-*Not*I pEBG-2T.

Expression of recombinant proteins and pull-down assays

GST fusion proteins were expressed in *E. coli* and purified by affinity chromatography on glutathione-Sepharose as described previously (Castaño et al., 2007). When required, GST was removed by cleaving with Pre-Scission protease (Amersham Biosciences). Where indicated, GST–p120-catenin (7.5 pmol) was phosphorylated using 300 milliunits of recombinant protein kinase CK1 (amino acids 1-237 of CK1δ, corresponding to the catalytic domain; Sigma), in a final volume of 50 µl kinase buffer (9 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 0.5 mM EDTA, 2.5 mM β-glycerol phosphate pH 7.0, 0.1 mM ATP). Reactions were performed for 40 minutes at 30°C. Pull-down assays were performed using purified recombinant proteins fused to GST as bait and SW-480 cell extracts. Glutathione–Sepharose-bound proteins were analyzed by western blotting with specific monoclonal antibodies against p120-catenin, E-cadherin or CK1ε (all from BD Biosciences, references 610134, 610182 and 610445, respectively). The polyclonal antibody for GST was from GE Healthcare. Immunoblots were analyzed using the SNAP protein detection system (Millipore). All binding assays were repeated three times.

Immunoprecipitation assays

Cell extracts were prepared by homogenizing cells in lysis buffer 1 (LB1; 20 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 0.5% Nonidet P-40) or lysis buffer 2 (LB2; 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% digitonin), supplemented with protease inhibitors (0.3 µM aprotinin, 1 µM leupeptin, 1 µM pepstatin, 1 mM Pefabloc) and phosphatase inhibitors (10 mM NaF, 0.1 mM sodium orthovanadate and 2.5 mM β-glycerol phosphate). After passing cells ten times through a 20-gauge syringe, extracts were left on ice for 20 minutes and centrifuged at 14,000 ***g*** for 5 minutes at 4°C. The supernatants contained the cell extracts. Proteins were immunoprecipitated from cell extracts (300-600 µg) using 2 µg/ml of the appropriate antibody, or an irrelevant IgG as control, for 16 hours at 4°C. Tcf-4-HA was immunoprecipitated using 20 µl of anti-HA Affinity Matrix (Roche) for 16 hours at 4°C. Precipitated material was removed by centrifugation at 12,000 ***g*** and the resulting supernatant was incubated for 120 minutes with 20 µl of γ-bind G-Sepharose (GE Healthcare). Immunoprecipitates were washed three times with 0.1% NP-40 and bound proteins eluted with electrophoresis sample buffer. Immunoprecipitated proteins were analyzed by western blotting using mAbs specific for CK1α (Santa Cruz, ref. sc-6477), CK1γ (Abcam, ref. ab64829), Axin (Santa Cruz, ref. sc-14029), β-catenin (BD Biosciences, ref. 65394), active β-catenin (anti-ABC, clone 8E7, Millipore) phosphoThr1479 in LRP5/6 (Abnova, ref. PAB12632) LRP5/6 (Acris, ref. Ap00345PU-N), Dvl-2 (Santa Cruz Biotechnology, ref. sc-13974), HA (Roche, ref. 118767423001) or phospho-Ser268 in p120-catenin (Xia et al., 2004).

Analysis of protein distribution by immunofluorescence

SW-480 cells were cultured on coverslips for 24 hours, treated with control or Wnt3a medium for the indicated times, rinsed three times with PBS, fixed with 4% paraformaldehyde for 30 minutes, washed three times with PBS, permeabilized with PBS-0.2% Triton X-100 for 5 minutes, and washed exhaustively with PBS. Cells were blocked with 3% bovine serum albumin in PBS for 30 minutes at 20°C. A 1/50 dilution of the primary antibodies (mAbs against p120-catenin or PSer268 p120-catenin, pAb against Dvl-2 or active β-catenin) was used to incubate the coverslips for 1 hour. After washing with PBS, cells were incubated with the secondary antibodies Alexa Fluor 555 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) both diluted 1/400 in blocking solution, for 1 hour at room temperature. Cells were washed again and incubated for 10 minutes with DAPI to stain the nucleus. Coverslips were mounted on glass slides with Mowiol and immunofluorescence was viewed with a Leica confocal microscope (LEICA spectral confocal TCS-SL).

Analysis of phosphorylated residues by mass spectrometry

p120-catenin was phosphorylated in vitro by CK1 as described. Coomassie-Blue-stained SDS-PAGE bands corresponding to the phosphorylated protein were excised from the gels, washed, reduced in-gel and Cys-alkylated before digesting with sequencing-grade trypsin (Promega) at a ratio 1:50 (enzyme:protein) and incubated at 37°C for 8 hours. The supernatants were transferred to new tubes, dried using a SpeedVac and re-suspended in 0.1% formic acid in water suitable for mass analysis.

Phosphorylation analysis was carried out as previously described (Casado-Vela et al., 2007). Briefly, peptides were eluted on an RP PepMap C18 column (75 µm i.d. ×150 mm; LC Packings, Amsterdam, The Netherlands) using a 60 minute linear gradient from 5 to 60% solvent B; solvent A being 0.1% formic acid in water and solvent B 0.1% formic acid in acetonitrile. All HPLC runs were performed using an Ultimate 3000 n-HPLC system (LC Packings, Aberdaan, The Netherlands) operated at 280 nl/minute constant flow rate. The peptides were scanned and fragmented with an LTQ linear IT spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a dynamic nano-ESI source (Proxeon, Odense, Denmark) with an electrospray voltage of 1700 V and a capillary voltage of 50 V at 180°C. The LTQ was operated in data-dependent mode using the five most intense precursors detected in survey scans ranging from 400 to 2000 *m/z* (3 µscans) with an isolation width of ±1.5 amu and 35% normalized collision energy. The LTQ was programmed to trigger successive triple mass spectrometry (MS) fragmentation events on precursor ions with enhanced neutral loss (98.0 for singly, 49.0 for doubly and 32.66 for triply charged peptides). All successive tandem or triple MS spectra were manually inspected for diagnostic ions with phosphorylated residues. The following parameters were set for searches using Sequest 3.3.1 SP1: enzyme, trypsin; fixed modifications, carboxyamidomethyl cysteine; variable modifications, oxidation of methionine; peptide tolerance, ±1.50 amu; fragment ion tolerance, ±0.35 amu; number of missed cleavage sites, 1. All fragmentation spectra were searched against the IPI mouse v.3.40 database containing 53825 entries (ftp://ftp.ebi.ac.uk/pub/databases/IPI/current). To address the false-positive rate in the database search using the Sequest engine, we searched the same spectra against the mouse reversed database, created by reversing each individual protein sequence entry, such that the original sequence length and composition were preserved. Only those peptides achieving *X*corr≥1.5 for singly, 2.0 for doubly and 2.5 for triply charged peptides and not identified against the reversed database were considered.

Cell transfection and selection of transfectants

Human shRNA for p120-catenin, CK1ε and E-cadherin were all obtained from Sigma. We selected those with a greatest effect on the expression of the corresponding protein, which were TRCN0000039666, TRCN0000122988 and TRCN0000001837, for E-cadherin, p120-catenin and CK1ε, respectively. For stable expression of p120-catenin shRNA, SW480 cells were infected with lentiviral particles containing an shRNA targeting p120-catenin. Infected cells were selected with puromycin at 1 µg/ml. Control cells were infected with lentivirus bearing a non-targeting shRNA (clone SCH002; Sigma). Stable cell populations of human CK1ε or E-cadherin shRNAs were generated transfecting SW480 cells with specific shRNA using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. After incubation with the reaction mix for 5-6 hours, cells were washed twice with Opti-MEM without antibiotics and then cultured in DMEM, 10% FCS for 72 hours before selection with 2 µg/ml puromycin. Transient expression of ectopic proteins was achieved in 80% confluent cells transfecting the indicated eukaryotic plasmid using Lipofectamine 2000. Cells were analyzed 48 hours after transfection.

Luciferase reporter assays

Cells were transfected as above with TOP-Flash plasmid, a synthetic promoter sensitive to the activity of the β-catenin–Tcf-4 complex, that contains three copies of the Tcf-4 binding site upstream of a firefly luciferase reporter gene. A mutant form of this promoter (FOP plasmid) was used as control. Activity of the product of the *Renilla* luciferase gene under the control of a constitutive thymidine kinase promoter (Promega) was used to normalize transfection efficiency. Assays were always performed in triplicate; the mean ± s.d. of the results of three or four independent transfections is given.

RNA isolation and analysis

RNAs were obtained as previously reported (Solanas et al., 2008) and analyzed by quantitative RT-PCR using the QuantiTect SYBR Green RT-PCR (Qiagen) in triplicate using oligonucleotides specific for Myc or HPRT.

CK1ε activity assay

Proteins were immunoprecipitated from SW-480 total cell extracts with p120-catenin or CK1ε mAbs for 4 hours at 4°C, and then collected using 20 µl of γ-bind G-Sepharose (GE-Healthcare). Immunoprecipitates were washed three times with 0.1% NP-40 and the immunocomplexes were incubated with recombinant GST–p120-catenin in phosphorylation conditions in the presence of 10 µM IC261 where indicated. Specific phosphorylation on GST–p120-catenin Ser268 was analyzed by western blotting with a phospho-specific Ser268–p120-catenin mAb (Xia et al., 2004).

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http://jcs.biologists.org/cgi/content/full/123/15/2621/DC1

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