Adherens junctions and tight junctions are regulated via different pathways by progastrin in epithelial cells

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Summary

Adhesion between neighbouring epithelial cells is a crucial and tightly controlled process. In the gastrointestinal tract, the integrity of cell-cell contacts is essential for the regulation of electrolyte absorption and for the prevention of tumour metastasis. We recently showed that migration of the gastric epithelial cell line IMGE-5 is stimulated by the nonamidated form of the hormone gastrin$_{17}$. Here, we examine the effect on cell-cell adhesion of the prohormone progastrin, the concentration of which is increased in the plasma of patients with colorectal carcinoma.

Progastrin induced the dissociation of both tight junction (TJ) and adherens junction (AJ) complexes in IMGE-5 cells. In progastrin-secreting DLD-1 human colorectal carcinoma cells, expression of an antisense gastrin construct restored membrane localisation of zonula occludens-1 (ZO-1), occludin, β-catenin and E-cadherin. This restoration was reversed by treatment with exogenous progastrin. Endogenous or exogenous progastrin also increased the paracellular flux of mannitol, and induced cell migration of several gastrointestinal cell lines. In addition, progastrin enhanced Src tyrosine kinase activity and induced a spatial delocalisation of protein kinase Cα. Using dominant-negative mutants and pharmacological inhibitors, we showed that the stimulation of Src kinase activity was essential for the regulation of TJs. By contrast, the dissociation of AJs involved phosphatidylinositol 3-kinase, partly through the formation of a complex with protein kinase Cα. We conclude that separate pathways mediate the disruption of AJs and TJs by progastrin. Either pathway may contribute to the co-carcinogenic role of this prohormone in colorectal carcinoma.

Key words: Tight junctions, β-catenin, Progastrin, Src, PI3-kinase

Introduction

In polarised epithelia, specialised structures such as tight junctions (TJs) and adherens junctions (AJs) are responsible for the establishment of contacts between neighbouring cells. TJs maintain the separation between apical and basolateral regions of the plasma membrane and represent a strictly regulated barrier to diffusion across the epithelium. TJs contain the transmembrane proteins occludin and the claudins, which are connected to the cytoskeleton via a network of proteins such as zonula occludens-1 (ZO-1) (for a review, see Tsukita et al., 2001). In the case of (AJs), the transmembrane protein E-cadherin is connected to actin by several submembrane proteins, including β-catenin (Näthke et al., 1994).

The establishment and stability of both AJs and TJs is tightly regulated – in particular, by growth factors, cytokines and hormones (Boyer et al., 2000; Nusrat et al., 2000). Such regulation, although poorly understood, seems essential for the modulation of paracellular permeability in various epithelia (Coyne et al., 2002; Nathanson et al., 1992), for the epithelium-mesenchyme transition (Boyer et al., 2000), and for development, morphogenesis and wound healing (Hellani et al., 2000; Jacinto et al., 2001). The observations that abnormal expression of β-catenin promoted tumour development in adenomatous polyposis coli (APC) mutant mice (Kongkanuntn et al., 1999), that germline mutations of E-cadherin result in familial gastric cancer (Guilford et al., 1998), and that there is a strong correlation between tumour differentiation and the expression of occludin and ZO-1 along the gastrointestinal tract (Kimura et al., 1997), suggest that modulation of both TJs and AJs can also have a significant impact on tumour development and metastasis.

Gastrin has long been known as an important hormone for the development and function of the gastrointestinal tract (Dockray et al., 2001), and the role of amidated and nonamidated progastrin-derived peptides as growth factors (Wang et al., 1996; Koh et al., 1999) and cocarcinogens (Singh et al., 2000a; Aly et al., 2001) has been well documented. Recently, various independent reports have also raised the possibility that progastrin-derived peptides may regulate epithelial cell adhesion or migration. We have shown that glycine-extended gastrin$_{17}$ (Ggly) induces the dissociation of the E-cadherin/β-catenin complex, the delocalisation of β-catenin from the AJ to the cytoplasm and the migration of gastric epithelial cells (Hollande et al., 2001a). Ggly was also shown to promote the invasiveness of the human colon carcinoma cell line LoVo (Kermorgant and Lehy, 2001). Similar dissociating effects of amidated gastrin$_{17}$ (Gam) have been described in Madine-Darby canine kidney (MDCK) cells

transfected with the gastrin/cholecystokinin-B (G/CCK-B) receptor (Bierkamp et al., 2002). An important breakthrough concerning the role of progastrin-derived peptides in cell motility was achieved with the finding, in vivo, that gastrin induced parietal cell migration in mouse gastric mucosa (Kirton et al., 2002).

In this study, we investigated for the first time the direct effect of recombinant human progastrin$_{6-80}$ (Baldwin et al., 2001) on cell-cell adhesion and migration in gastrointestinal epithelial cell lines. The cell lines chosen were the conditionally immortalised nontumorigenic mouse gastric line IMGE-5 (Hollande et al., 2001b) and the human colorectal carcinoma cell line DLD-1 (Dexter et al., 1981). IMGE-5 cells produce no detectable progastrin-derived peptides, whereas DLD-1 cells synthesise and secrete similar amounts of progastrin and Ggly, but little Gam. We now report that treatment of IMGE-5 cells with progastrin, or reduction of nonamidated gastrin production by stable transfection of DLD-1 cells with vectors expressing antisense gastrin (ASG), induced significant changes in the subcellular localisation and association of AJ and TJ proteins, and profoundly affected cell adhesion and motility. We also identified the involvement of Src, phosphatidylinositol 3-kinase (PI3-kinase) and protein kinase Cα (PKCα) in the two separate signalling pathways that mediate the novel effects of progastrin on TJs or AJs.

Materials and Methods

Peptides and radioimmunoassay

Gam was obtained from Research Plus (Bayonne, NJ) and Ggly was from Auspep (Melbourne, Australia). The radioimmunoassays used to detect various molecular forms of gastrin (Hollande et al., 1997) and the production of recombinant progastrin$_{6-80}$ (Baldwin et al., 2001) have been described previously.

Antibodies and cell culture

Polyclonal anti-actin antibody, calphostin C, LY294002 and phalloïdin-FITC were from Sigma-Aldrich (St Louis, MO), and the Src kinase inhibitor PP2 was from Calbiochem (La Jolla, CA). Antibodies against claudin-1, claudin-2, occludin and ZO-1 were from Zymed (San Francisco, CA). The SYM139 monoclonal antibody against symplekin has been described previously (Keon et al., 1996). Antibodies against p125 focal adhesion kinase (p125FAK), E-cadherin, β-catenin, PI3-kinase and phosphotyrosine (PY20) were from Transduction Laboratories (Lexington, KY).

The IMGE-5 (Hollande et al., 2001b) and young adult mouse colon (YAMC) (Hollande et al., 1997) cell lines were generally grown in Dulbecco's modified Eagle's medium (DMEM) containing 1 unit/ml γ-interferon and 5% fetal calf serum (FCS) at 33°C (permissive conditions). For all experiments, cells were transferred to 39°C in the same medium without γ-interferon (nonpermissive conditions), where they show differentiated characteristics, such as expression of functional AJs and TJs. All experiments were performed on cells between passage number 15 and 35. The DLD-1 colorectal carcinoma cell line (Dexter, 1981) was from the American Tissue Culture Collection (ATCC; Manassas, VA).

Preparation of Src kinase dominant-negative mutants and assay of Src kinase activity

The cDNA encoding a constitutively activated Src mutant with the tyrosine in position 527 mutated to a phenylalanine was cloned into the *Eco*RI site of the eukaryotic expression vector pSGT vector, which had been derived from the vector pSG5 (Stratagene, La Jolla, CA) by insertion of a new polylinker containing the following restriction sites: *Eco*RI, *Spe*I, *Bam*HI, *Eco*RV, *Xho*I and *Bgl*II. The Src dominant-negative double mutant ($Src^{-/-}$) was prepared from the constitutively activated mutant by mutation of lysine 295 in the ATP-binding site to alanine with the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA). The primer used was 5′-GTGGCCATCGCGACTCT-GAAGCCC-3′. The double mutant $Src^{-/-}$ lacks kinase activity but maintains an open conformation and hence retains the capacity to interact with substrates and regulators.

Kinase activity in lysates of IMGE-5 and DLD-1 stable transfectants was assessed by measurement of phosphorylation of denatured enolase (Rodier et al., 1995).

Expression of antisense gastrin, dominant-negative mutants and GFP-tagged constructs

DLD-1 cells were stably transfected with the full-length ASG construct (Hollande et al., 1997) in pcDNA3.1. Three clones each of cells transfected with the ASG construct or with pcDNA3.1 only were monitored for Gam, Ggly and progastrin production and secretion, and then used for all experiments. Preparation of cell extracts and conditioned media, as well as radioimmunoassays, for all three gastrin forms were performed as described previously (Hollande et al., 1997).

DLD-1 and IMGE-5 cells were stably transfected with the ΔSH2 p85 mutant of PI3-kinase (kindly provided by W. Ogawa, University of Kobe, Japan). All transfections were performed using Lipofectin® (Life Technologies, Rockville, MD) according to the manufacturer's instructions, and stable transfectants were selected using 500 μg/ml G418.

PKCα-enhanced green fluorescent protein (EGFP) (Clontech, Palo Alto, CA) was transiently expressed using Lipofectin® (Life Technologies, Rockville, MD) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were seeded onto 13 mm glass coverslips and grown overnight, serum-starved for 180 minutes under nonpermissive conditions, treated with various agents as described in the figure legends, then washed once with phosphate-buffered saline (PBS) and fixed for 10 minutes with 2% paraformaldehyde in PBS at room temperature.

Immunocytochemical detection of junction proteins

Immunocytochemistry and bromodeoxyuridine (BrdU) incorporation experiments were performed as described previously (Hollande et al., 2001a). Briefly, cells were grown under nonpermissive conditions on 14 mm glass coverslips in DMEM containing 5% FCS as previously described (Hollande et al., 2001a). Cells were then serum-starved for 24 hours and treated with agents to be tested in DMEM containing 0.1% heat-inactivated FCS for the indicated period of time. Cells were then fixed in ice-cold methanol for 3 minutes at 4°C (for BrdU staining), or in 2% paraformaldehyde in PBS for 10 minutes at room temperature (for immunocytochemistry). After three PBS washes, cells were incubated for 5 minutes in PBS containing 0.2% Triton X-100 followed by PBS containing 0.2% gelatine for 10 minutes. Cells were then incubated with primary antibodies for 2 hours, coverslips were washed three times in PBS, and the appropriate secondary antibody was incubated for 1 hour. After two PBS washes and one rinse in water, coverslips were mounted on slides in cytifluor (Oxford Instruments, Oxford, England). Confocal microscopy was then performed using a Biorad 1024 CLMS System, as described previously (Hollande et al., 2001b).

Immunoprecipitation and western blotting

Cells were grown in 100 mm Petri dishes under permissive conditions until they reached 90% confluence. Cells were then transferred to nonpermissive conditions (Hollande et al., 2001b) and serum-starved

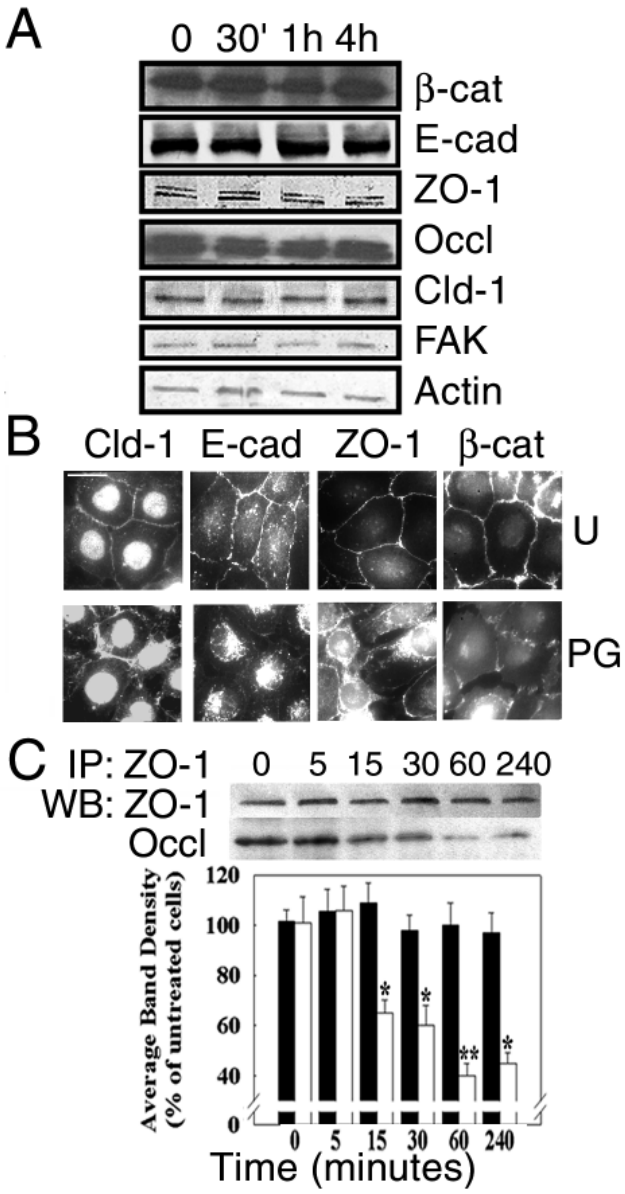

Fig. 1. Progastrin dissociates AJs and TJs. (A) Treatment of the IMGE-5 cell line, which does not produce progastrin-derived peptides, for up to 4 hours with 5 nM recombinant human progastrin$_{6\text{-}80}$ did not affect the expression of β-catenin (β-cat), E-cadherin (E-cad), ZO-1, occludin (Occl), claudin-1 (Cld-1), p125FAK or actin, as assessed by western blotting of cell lysates. (B) Immunostaining for AJ and TJ proteins was observed using a confocal microscope as described in Materials and Methods. Series of horizontal optical sections (0.328 μm each) were collected, and images represent a merging of sections spanning the apical region of IMGE-5 cells (ten sections, 3.28 μm). Claudin-1 (Cld-1), ZO-1 and the AJ proteins E-cadherin (E-cad) and β-catenin (β-cat) were located in the most apical region of the lateral membrane in untreated (U) cells. Treatment with 5 nM progastrin$_{6\text{-}80}$ (PG) for 4 hours induced a partial delocalisation of all four proteins from the membrane to the cytoplasm. Bar, 7 μm. (C) Progastrin treatment also induced a time-dependent partial dissociation of the complex between occludin (white bars) and ZO-1 (black bars), as assessed by densitometric scanning of western blots of ZO-1 immunoprecipitates of cell lysates. Densitometric analysis represents the average of four experiments, and statistical significance was assessed by Student's *t* test. **P*<0.05; ***P*<0.01.

for 24 hours, stimulated with or without the indicated concentration of progastrin$_{6\text{-}80}$ for various time periods with or without a 1 hour preincubation with either 10 μM LY294002 or 0.3 μM calphostin C, and lysed using the procedure described previously (Hollande et al., 2001a). In the case of β-catenin/E-cadherin association studies, 100 μg of protein lysate per sample was immunoprecipitated in Tris/NaCl (pH 7.5) containing 1% Nonidet P40, 100 μM sodium orthovanadate and 1 mM dithiothreitol (DTT) (wash-lysis buffer), using 1 μg of anti-β-catenin antibody for 2 hours at 4°C, followed by 100 μl of 20% protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ) overnight. Samples were washed three times in wash-lysis buffer and centrifuged for 10 seconds at 10,000 ***g***. The pellet was resuspended in loading buffer, denatured for 3 minutes at 95°C, centrifuged for 30 seconds at 10,000 ***g***, and proteins in the supernatant were separated on an 8.5% SDS-polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane using a semi-dry blotting system (Bio-rad, Hercules, CA). Membranes were then incubated with the appropriate primary antibodies, and detection was performed with alkaline phosphatase-coupled anti-rabbit or anti-mouse immunoglobulin G followed by incubation with a 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium solution, pH 9.2 (Sigma, St Louis, MO). Membranes were scanned using a HP ScanJet 5200C and protein bands were analysed densitometrically with Fuji BAS software (Berthold, Bundoora, Australia).

Migration experiments

Wound-healing experiments were performed to assess the effects of progastrin on cell migration. Cells were grown in 12-well plates at 33°C under permissive conditions until they reached 80% confluence; they were then transferred into a 39°C incubator and were serum-starved for 24 hours. The confluent monolayer was then wounded linearly using a pipette tip, washed three times with PBS and treated with or without agents to be tested for the indicated length of time, in the presence of 0.1% FCS. Morphology and migration of cells was then observed and photographed at regular intervals for 24 hours.

Results

Progastrin dissociates AJs and TJs

The IMGE-5 cell line does not produce detectable quantities of progastrin-derived peptides (F.H., unpublished). Treatment of IMGE-5 cells for up to 4 hours with 5 nM recombinant human progastrin$_{6\text{-}80}$ did not affect the expression of a panel of cytoskeleton and junction proteins, including β-catenin, actin, ZO-1, occludin and p125FAK (Fig. 1A). As described previously (Hollande et al., 2001b), immunostaining of untreated IMGE-5 cells confirmed that the AJ proteins E-cadherin and β-catenin (Fig. 1B), as well as the TJ proteins claudin-1, ZO-1 (Fig. 1B) and symplekin (F.H., unpublished), were localised in or along the upper section of the basolateral membrane, consistent with the polarised nature of these cells. Claudin-1 staining was also detected in the nucleus. Progastrin$_{6\text{-}80}$ treatment of confluent IMGE-5 cells induced a strong decrease in the membrane localisation of these proteins (Fig. 1B), along with a decrease in the number of contact points between neighbouring cells. Progastrin treatment also induced a partial dissociation of the complexes between occludin/ZO-1 (Fig. 1C), and between E-cadherin/β-catenin (F.H., unpublished). The delocalisation of junctional proteins and the dissociation of junctional complexes were detected with progastrin$_{6\text{-}80}$ concentrations as low as 50 pM, whereas a maximal effect was reached with 5-10 nM (F.H., unpublished).

Reduction of progastrin expression induces an epithelial-like morphology

In contrast to IMGE-5 cells, DLD-1 colorectal carcinoma cells were found to produce and secrete significant amounts of progastrin and Ggly (Fig. 2A), but negligible amounts of Gam. Expression of an ASG construct was found to reduce significantly the production (Fig. 2A, top panel) and secretion (Fig. 2A, bottom panel) of progastrin and Ggly by DLD-1 cells. When observed by bright-field microscopy the morphology of ASG-expressing cells was much more epithelial-like than that of cells transfected with vector only (VO) (Fig. 2B). In addition, expression of ASG resulted in a delocalisation of actin, with much more cortical actin detectable in confluent DLD-1/ASG cells than in DLD-1/VO controls (Fig. 2C). Treatment of DLD-1/ASG cells with 5 nM progastrin$_{6-80}$ for 4 hours modified their morphology, and resulted in a slight decrease in cortical actin expression on confluent cells (Fig. 2C) and the extension of membrane processes in nonconfluent cells (Fig. 2B).

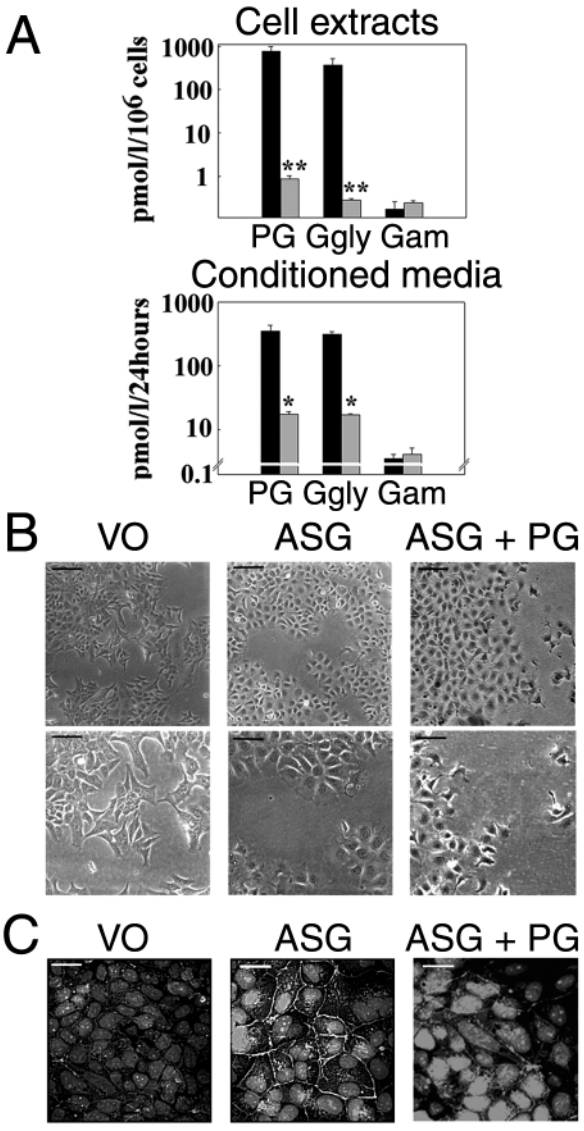

Fig. 2. Reduction of gastrin expression induces an epithelial-like morphology. (A) DLD-1 colorectal carcinoma cells stably transfected with an antisense gastrin construct (ASG, grey bars) produced (cell extracts) and secreted (conditioned media) significantly less progastrin (PG), Ggly and Gam than cells transfected with vector only (VO, black bars), as measured using the radioimmunoassays described previously (Hollande et al., 1997). The statistical significance of the differences between VO and ASG clones was assessed using ANOVA. *P<0.05; **P<0.01; n=3. The reduction of gastrin expression induced an epithelial-like morphology (B), as assessed by bright-field microscopy (bar, 20 μm, upper panels, or 10 μm, lower panels), and increased the expression of cortical actin (C), as assessed by immunofluorescent staining (bar, 7.5 μm). Both parameters were partially reversed by treatment of ASG clones with exogenous progastrin$_{6-80}$ (5 nM) for 4 hours (B,C).

Reduction of endogenous gastrin expression strengthens AJs and TJs

Comparison of ASG and VO DLD-1 cells showed that expression of gastrin gene products reduced the expression of ZO-1, claudin-1, claudin-2 and FAK, but not of β-catenin, E-cadherin and actin (Fig. 3A). Expression of occludin was not significantly decreased in all clones, as clone DLD-1/VO2 showed a similar level of expression as ASG clones. The reason for this higher-than-expected expression is unknown, as this clone behaved similarly to the other VO DLD-1 clones in all other respects. Constitutive expression of progastrin-derived peptides by DLD-1/VO cells greatly reduced or abolished the membrane localisation of ZO-1, occludin, claudin-1 and E-cadherin (Fig. 3B), as well as β-catenin (F.H., unpublished), and induced partial dissociation of the E-cadherin/β-catenin (Fig. 3C) and occludin/ZO-1 complexes (F.H., unpublished). Interestingly, the significant increase in membrane localisation of AJ and TJ proteins seen in ASG clones was largely reversed by treatment with exogenous progastrin$_{6-80}$ (Fig. 3B, bottom panels). Similarly, progastrin$_{6-80}$ also induced a time-dependent decrease of E-cadherin/β-catenin association in DLD-1/ASG clones (Fig. 3C).

Functional consequences of the progastrin-induced disruption of cell-cell adhesion

The effect of progastrin on paracellular permeability was assessed by measuring the flux of [^{3}H]mannitol through confluent monolayers of DLD-1 or IMGE-5 cells. The permeability was reduced by almost 30% over a 24 hour period in DLD-1/ASG cells compared with DLD-1/VO cells (Fig. 4A). Conversely, the mannitol flux was significantly increased from 1 to 4 hours after treatment of IMGE-5 cells (Fig. 4B) or DLD-1/ASG cells (Fig. 4A) with 5 nM progastrin$_{6-80}$.

We also assessed whether the decrease in cell-cell adhesion induced by progastrin in gastrointestinal epithelial cells was coupled to an effect on their motility. When a confluent cell monolayer was wounded using a pipette tip, the spontaneous motility displayed by DLD-1/VO clones (Fig. 4C, columns 1-2) was greatly reduced in clones expressing the ASG construct (Fig. 4C, columns 3-4). Interestingly, motility was partly restored when the latter clones were treated with exogenous progastrin$_{6-80}$ (Fig. 4C, column 5). Similarly, progastrin$_{6-80}$ was found to stimulate the migration of IMGE-5 cells, with a maximal effective dose of 5 nM (Fig. 4D).

Involvement of Src kinase in the progastrin-induced delocalisation of TJ proteins

In DLD-1 colorectal carcinoma cells, basal Src-kinase activity was higher in VO than in ASG clones (Fig. 5A). Furthermore, we found that progastrin$_{6-80}$ induced a three-to-fourfold stimulation of Src kinase activity in ASG clones, whereas it barely affected the same activity in VO clones. The specificity of this effect was illustrated by the failure of progastrin$_{6-80}$ to stimulate cells transfected with a dominant-negative mutant of Src (Fig. 5A). Similar results were obtained on IMGE-5 cells (Fig. 5B). In both cell lines, Src kinase activation was already detectable within 1 minute after treatment, and maximal stimulation was obtained using 5 nM progastrin$_{6-80}$ (F.H., unpublished). Using IMGE-5 cells expressing a dominant-negative Src (IMGE-5/Src$^{-/-}$), we then showed that activation of Src was essential for the effect of progastrin$_{6-80}$ on the delocalisation of the TJ proteins ZO-1 and symplekin (Fig. 5C). By contrast, Src was not essential for the progastrin$_{6-80}$-induced delocalisation of the AJ protein β-catenin. Concomitantly, the partial dissociation of the occludin/ZO-1 complex induced by progastrin$_{6-80}$ was also completely abolished in the IMGE-5/Src$^{-/-}$ clones (Fig. 5D). A similar result was obtained on the weaker association detected between occludin and ZO-1 in DLD-1 cells (F.H., unpublished). However, the expression of Src dominant-negative mutants had no effect on the progastrin-induced dissociation of the E-cadherin/β-catenin complex in IMGE-5 cells (Fig. 5E). Finally, as expected in view of the well-documented proliferative role of Src (Porter and Vaillancourt, 1998), expression of this dominant-negative mutant also slowed down growth in both cell lines (F.H., unpublished).

PI3-kinase is involved in the progastrin-induced cytoplasmic shift of β-catenin

Basal and progastrin$_{6-80}$-stimulated Src kinase activity was assessed in IMGE-5 cells transfected with a dominant-negative mutant of the p85 regulatory subunit of PI3-kinase (IMGE-5/PI3-k$^{-/-}$) (Fig. 6A). Expression of this mutant was found to decrease IMGE-5 cell proliferation (F.H., unpublished), confirming the previously described role of PI3-kinase in cell growth (Porter and Vaillancourt, 1998). Both unstimulated and stimulated Src activity were similar in IMGE-5/PI3-k$^{-/-}$ cells and in cells transfected with vector only (IMGE-5/VO), indicating that the activation of Src is not dependent on PI3-kinase activity in these cells. Furthermore, contrary to the results obtained in IMGE-5/Src$^{-/-}$ cells, expression of the PI3-kinase dominant-negative mutant was found to prevent the progastrin-induced cytoplasmic shift of β-catenin, without affecting the delocalisation of the TJ proteins ZO-1 and

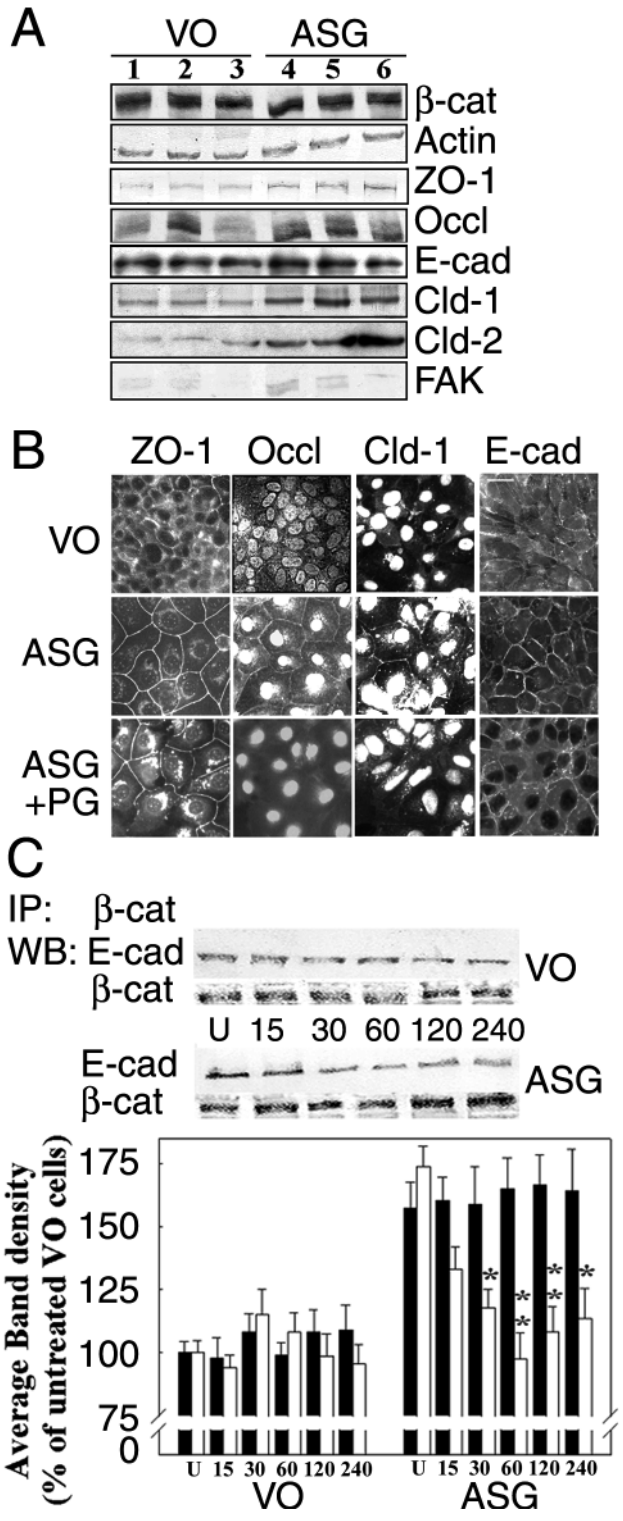

Fig. 3. Reduction of gastrin expression strengthens TJs. (A) Three independent clones of DLD-1 colorectal carcinoma cells stably transfected with an antisense gastrin construct (ASG, columns 4-6) expressed, on average, more ZO-1, occludin (Occl), E-cadherin (E-cad), claudin-1 (Cld-1), claudin-2 (Cld-2) and p125FAK (FAK) than three clones of cells transfected with vector only (VO, columns 1-3), as assessed by western blots of cell lysates. No difference was observed in the amounts of β-catenin and actin. (B) ZO-1, occludin (Occl), claudin-1 (Cld-1) and E-cadherin (E-cad) were localised in the cytoplasm and/or nucleus in VO clones. Membrane staining for these three AJ and TJ proteins significantly increased in ASG clones, and this increase was largely reversed by a 4 hour treatment with 5 nM progastrin$_{6-80}$. Bar, 7.5 μm. (C) Treatment of ASG clones with 5 nM progastrin$_{6-80}$ for up to 240 minutes induced a partial dissociation of the complex between E-cadherin (white bars) and β-catenin (black bars), as assessed by densitometric scanning of western blots of β-catenin immunoprecipitates of cell lysates. Little, if any, dissociation was observed in VO clones after progastrin treatment. Densitometric analysis represents the average of at least three experiments per clone, and statistical significance was assessed by Student's *t* test. **P*<0.05; ***P*<0.01.

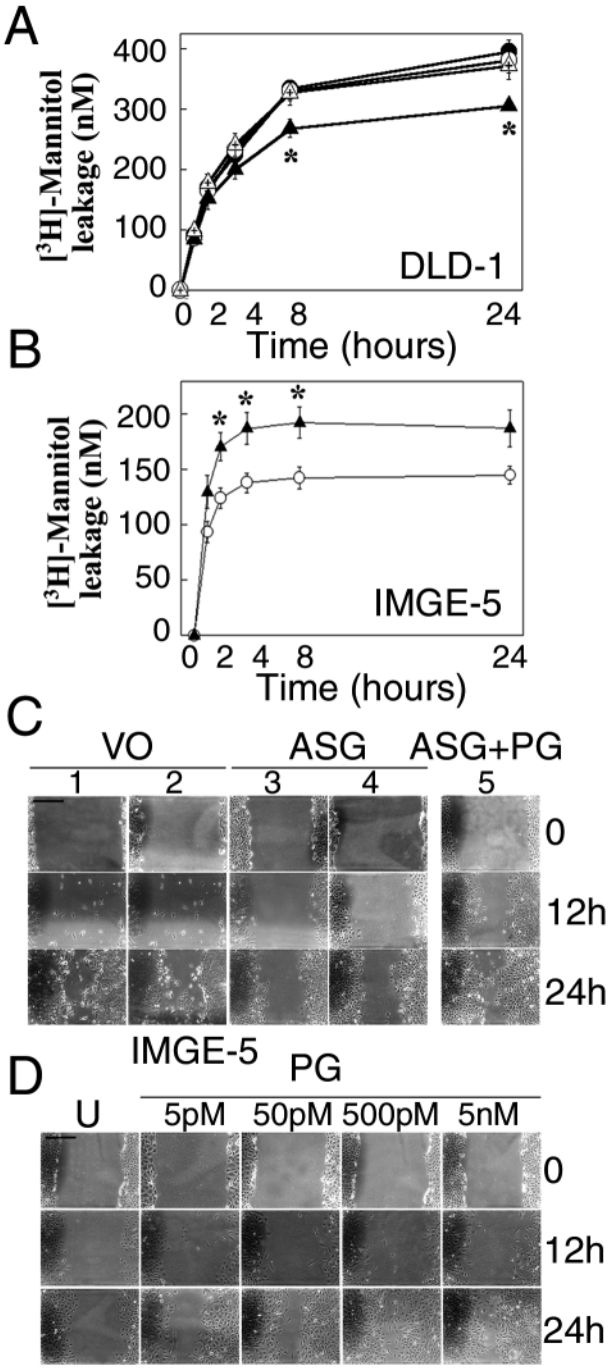

Fig. 4. Functional consequences of the progastrin-induced reduction in cell-cell adhesion. (A) Paracellular permeability, as assessed by [^{3}H]mannitol flux through confluent monolayers, was reduced by almost 30% over a 24 hour period in DLD-1 colorectal carcinoma cells expressing antisense gastrin (▲) compared with control cells transfected with vector only (●). The reduction was reversed by treatment with 5 nM progastrin$_{6\text{-}80}$ (△), which had no effect on vector only cells (○). (B) The permeability through a confluent monolayer of IMGE-5 cells was significantly increased after treatment with 5 nM progastrin$_{6\text{-}80}$ (▲) compared with media alone (○). Significance was assessed by Student's *t* test. **P*<0.05; *n*=4. (C) When a confluent cell monolayer was wounded using a pipette tip, the spontaneous motility of DLD-1/VO clones (VO, columns 1, 2) over a 12 or 24 hour period was greatly reduced in clones expressing antisense gastrin (ASG, columns 3, 4). Motility was partly restored when the latter clones were treated with 5 nM progastrin$_{6\text{-}80}$ (ASG + PG, column 5). Bar, 60 μm. (D) When a confluent IMGE-5 monolayer was wounded using a pipette tip, treatment with 5 pM, 50 pM, 0.5 nM or 5 nM progastrin$_{6\text{-}80}$ (PG) over 12 or 24 hours increased cell motility compared with cells left untreated in DMEM containing 0.1% FCS (U). Bar, 60 μm.

symplekin (Fig. 6B). However, the latter effect was blocked when cells from these same clones were preincubated with the Src kinase inhibitor PP2 before progastrin$_{6\text{-}80}$ treatment. Conversely, the necessity for PI3-kinase activity for mediation of the progastrin$_{6\text{-}80}$ effect on AJs was confirmed by the fact that the PI3-kinase inhibitor LY294002 was able to block the cytoplasmic shift of β-catenin in wild-type (F.H., unpublished) or Src$^{-/-}$IMGE-5 cells (Fig. 6B). Finally, the specificity of PI3-kinase action in mediating the disruptive effect of progastrin on AJs but not TJs was further shown by the failure of the PI3-k$^{-/-}$ mutant to prevent the partial dissociation of the ZO-1/occludin complex induced by progastrin$_{6\text{-}80}$ in IMGE-5 cells, although the mutant completely blocked the dissociation of the β-catenin/E-cadherin complex (Fig. 6C). Although the short-term effects of progastrin$_{6\text{-}80}$ were assessed by stimulation of IMGE-5 cells for up to 4 hours, the long-term effects of progastrin could be assessed in DLD-1 Src$^{-/-}$ and PI3-k$^{-/-}$ mutants, which constitutively secrete progastrin. Interestingly, we noted a significant increase in membrane staining for ZO-1 in DLD-1/Src$^{-/-}$ mutants compared with VO cells (Fig. 6B and Fig. 3B, respectively). By contrast, there was no significant difference in E-cadherin (Fig. 6B) and β-catenin localisation (F.H., unpublished) in DLD-1/ PI3-k$^{-/-}$ cells compared with DLD-1/VO cells (Fig. 3B and F.H., unpublished, respectively).

Involvement of PKC in the progastrin-induced delocalisation of junction proteins

Within 30 minutes of stimulation, progastrin$_{6\text{-}80}$ induced a delocalisation of GFP-PKCα to a perinuclear area, as well as to the plasma membrane (Fig. 7Aa,b,c). Interestingly, only the delocalisation of GFP-PKCα to the membrane was abolished when cells were preincubated with the PI3-kinase inhibitor LY294002 (Fig. 7Af), whereas the Src inhibitor PP2 had no effect (Fig. 7Ae).

Furthermore, in wild-type IMGE-5 cells, progastrin$_{6\text{-}80}$ was found to induce a rapid but transient association of PKCα with a complex co-immunoprecipitating with β-catenin (Fig. 7B). The association was detected 5 minutes after stimulation with 5 nM progastrin$_{6\text{-}80}$, was maximal after about 15 minutes and was not detectable 60 minutes after stimulation.

Although the PKC inhibitor calphostin was found to reverse the progastrin-induced cytoplasmic shift of β-catenin in wild-type (F.H., unpublished) and IMGE-5/Src$^{-/-}$ cells (Fig. 7C), it only had a partial effect on the delocalisation of TJ proteins induced by progastrin in IMGE-5/PI3k$^{-/-}$ cells (Fig. 7C). Similarly, the role of PKC in the progastrin-induced regulation of paracellular permeability was found to be ambiguous. Calphostin C only partially inhibited the stimulation of [^{3}H]mannitol leakage by progastrin$_{6\text{-}80}$. By contrast, progastrin$_{6\text{-}80}$-stimulated mannitol leakage was completely blocked by the Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and

unaffected by the PI3-kinase inhibitor LY294002 (Fig. 7D), thereby confirming the participation of Src in the regulation of TJs by progastrin.

Discussion

The results presented in this manuscript describe the concomitant disruption, through independent signalling pathways, of AJs and TJs by the prohormone progastrin in gastrointestinal epithelial cells. Incubation with selective inhibitors and transfection with dominant-negative mutants revealed that the tyrosine kinase Src was essential for the regulation of TJs in both the conditionally immortalised gastric epithelial cell line IMGE-5 and the human colorectal carcinoma cell line DLD-1. By contrast, the dissociation of AJs involved PI3-kinase, probably in part through its association with PKCα. The importance of autocrine progastrin in the regulation of both TJ and AJ complexes in tumour cells is now shown for the first time by the observation that stable expression of an antisense gastrin construct in DLD-1 human colorectal carcinoma cells resulted in a significant increase in membrane localisation of AJ and TJ proteins, which was largely reversed by treatment with exogenous progastrin$_{6\text{-}80}$.

Our work shows that physiological levels of Src are essential for the disruption of TJs in confluent IMGE-5 and DLD-1 cells. The Src family of tyrosine kinases has previously been implicated in the regulation of cell-cell contacts – for example, during TJ reassembly after an oxidative stress in MDCK cells (Meyer et al., 2001), or after mitogen-activated protein kinase kinase 1 (MEK-1) inhibition (Chen et al., 2000a). Conversely, results on confluent MDCK cells already expressing TJs have shown that tyrosine phosphorylation was essential for TJ dissociation (Collares-Buzato et al., 1998). Interestingly, in post-confluent gastrointestinal tumour (Caco-2) cells, overexpression of an oncogenic form of Src was found to disrupt AJs without affecting the structure and function of TJs (Gomez et al., 1999). In view of our current results and data

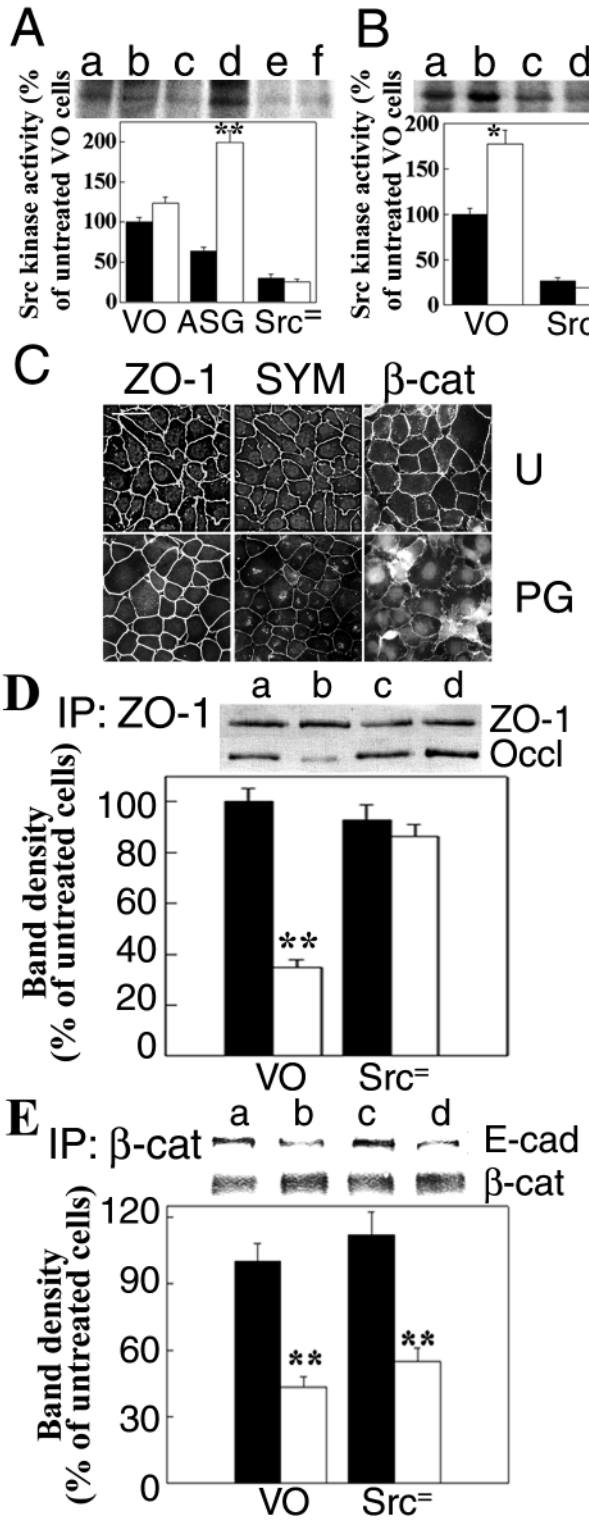

Fig. 5. Involvement of Src kinase in the progastrin-induced delocalisation of junction proteins. (A) In DLD-1 colorectal carcinoma cells basal Src-kinase activity (black bars) in Src immunoprecipitates of cell lysates was higher in VO (a) than in ASG clones (c). Furthermore, 5 nM recombinant human progastrin$_{6\text{-}80}$ (5 minutes) stimulated Src-kinase activity (white bars) in ASG clones (d) but not in VO clones (b). The specificity of this effect was shown by the lack of stimulation of Src kinase activity by 5 nM progastrin$_{6\text{-}80}$ (f) in cells transfected with a dominant-negative mutant of Src (DLD-1/Src$^{-/-}$) (e). (B) In IMGE-5 cells, which do not produce progastrin-derived peptides, basal Src kinase activity (black bars) (a,c) was significantly increased by 5 nM progastrin$_{6\text{-}80}$ (5 minutes) (white bars) in VO cells (b) but not in cells transfected with dominant-negative Src (IMGE-5/Src$^{-/-}$) (d). Bar, 20 μm. (C) Activation of Src was essential for the effect of progastrin on the delocalisation of TJ proteins, as in IMGE-5/Src$^{-/-}$ cells, progastrin$_{6\text{-}80}$ (PG) caused little, if any, delocalisation of ZO-1 and symplekin compared with untreated cells (U). By contrast, in IMGE-5/Src$^{-/-}$ cells, 5 nM progastrin$_{6\text{-}80}$ treatment still caused delocalisation of the AJ protein β-catenin (β-cat). (D) The partial dissociation of the complex between ZO-1 (black bars) and occludin (Occl, white bars) induced in VO clones (a) by 5 nM progastrin$_{6\text{-}80}$ (b) was completely abolished in the IMGE-5/Src$^{-/-}$ cells (c,d), as assessed by densitometric scanning of western blots of ZO-1 immunoprecipitates of cell lysates. (E) By contrast, expression of the Src dominant-negative mutant in IMGE-5 cells (c) did not prevent the dissociation of the complex between β-catenin (β-cat, black bars) and E-cadherin (E-cad, white bars) induced in VO cells (a) by 5 nM progastrin$_{6\text{-}80}$ (b,d), as assessed by densitometric scanning of western blots of β-catenin immunoprecipitates of cell lysates. In A, B, D and E, densitometric analysis represents the average of at least three experiments, and statistical significance was assessed by Student's *t* test. **P*<0.05; ***P*<0.01.

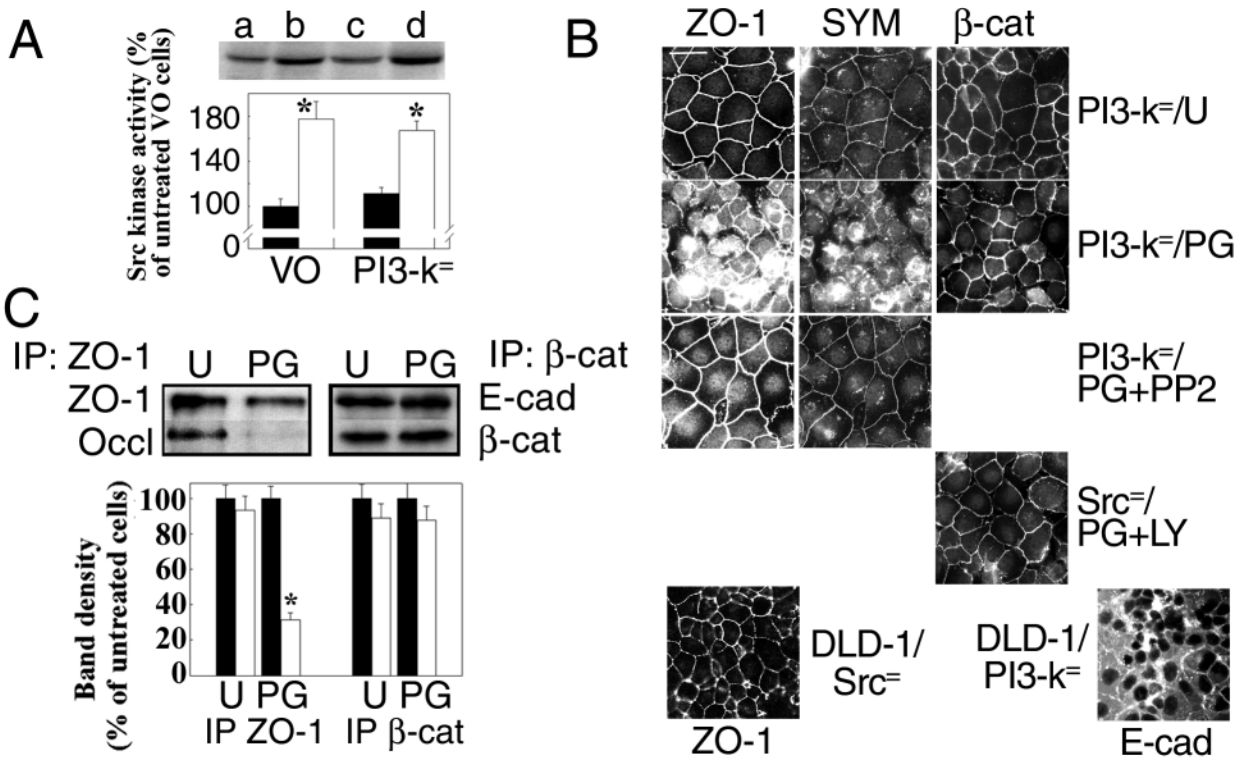

Fig. 6. PI3-kinase is involved in the progastrin-induced cytoplasmic shift of β-catenin. (A) Basal Src kinase activity (black bars), assessed as described in the legend to Fig. 5, in IMGE-5 cells expressing a ΔSH2 dominant-negative mutant of the p85 regulatory subunit of PI3-kinase (IMGE-5/PI3-k$^{-/-}$) (c) was similar to cells transfected with vector only (VO) (a). Furthermore, activation of Src kinase activity by 5 nM progastrin$_{6-80}$ (b,d, white bars) was not PI3-kinase dependent. (B) Treatment of IMGE-5/PI3-k$^{-/-}$ cells with 5 nM progastrin$_{6-80}$ induced a partial or complete disappearance from the plasma membrane of ZO-1 and symplekin (SYM), respectively, compared with untreated (U) controls. The disappearance was prevented by preincubation with the Src kinase inhibitor PP2. Conversely, the cytoplasmic shift of β-catenin (β-cat) induced by progastrin$_{6-80}$ in wild-type or IMGE-5/Src$^{-/-}$ cells was prevented in IMGE-5/PI3-k$^{-/-}$ clones, as well as in IMGE-5/Src$^{-/-}$ cells preincubated with the PI3-kinase inhibitor LY294002. In DLD-1/Src$^{-/-}$ cells, ZO-1 was mostly located at the membrane, whereas DLD-1/PI3-k$^{-/-}$ cells showed a largely cytoplasmic expression of E-cadherin (E-cad). Bar, 20 μm. (C) When compared with untreated cells (U, black bars), 5 nM progastrin$_{6-80}$ (PG, white bars) induced a partial dissociation of the occludin (Occl)/ZO-1 complex, assessed as described in the legend to Fig. 1, in IMGE-5/PI3-k$^{-/-}$ cells (left panel), of similar amplitude to that observed in wild-type or IMGE-5/VO cells. However, dissociation of the E-cadherin (E-cad)/β-catenin (β-cat) complex after progastrin$_{6-80}$ treatment, assessed as described in the legend to Fig. 3, did not occur in IMGE-5/PI3-k$^{-/-}$ clones (right panel). In A and C, densitometric analysis represents the average of at least three experiments, and statistical significance was assessed by Student's *t* test. **P*<0.05.

showing Src involvement in Gam-induced disruption of AJs in MDCK cells (Bierkamp et al., 2002), it is probable that differences in the time-course of activation, in the local microenvironment and in downstream targets, are all responsible for the diversity of Src-mediated effects on cell adhesion and motility in various cell types.

In addition, we also showed that the stimulation of PI3-kinase activity is essential to the disruption of AJs by progastrin, but has no bearing on the regulation of TJs by this peptide. Evidence for the involvement of PI3-kinase in TJ regulation has been limited, although recent results have suggested that it could be involved in the glucocorticoid-induced stimulation of trans-epithelial resistance, without structurally remodelling TJs (Woo et al., 1999). We recently showed in gastric epithelial cells that PI3-kinase was also crucial for the Ggly-induced disruption of AJs (Hollande et al., 2001a), suggesting that progastrin and Ggly share common signalling pathways. These results also corroborate the crucial role played more generally by PI3-kinase in AJ modulation, as shown previously by the direct association between β-catenin and PI3-kinase (Espada et al., 1999). Interestingly, the effect of PI3-kinase activation can clearly differ depending on the conditions in which epithelial cells are kept, and, in particular, on the degree of confluence. Thus, in subconfluent mammary epithelial cells PI3-kinase activation is required for the formation of AJs (Somasiri et al., 2000), but in confluent cells PI3-kinase was found to mediate epithelial cell dissociation induced by hepatocyte growth factor (Royal et al., 1997) and Gam (Bierkamp et al., 2002). The multiple effects of the activation of this enzyme are further shown by the fact that PI3-kinase activation can also be a consequence of the formation of E-cadherin-mediated contacts in MDCK cells (Pece et al., 1999). It remains to be shown whether this flexibility is also connected to a tissue-specificity of the effect of PI3-kinase towards epithelial or nonepithelial AJs, or both.

In view of published data showing that Gam induces translocation of PKCα from cytoskeleton to membrane in colonic cells (Yassin and Little, 1995), and implicating PKCα in the regulation of cell-cell contacts (Chen et al., 2000b; Chen et al., 2002; Vallentin et al., 2001), we investigated the

involvement of PKCα in the progastrin-induced dissociation of TJs and AJs. Our results showed that PKCα behaved as a downstream effector of PI3-kinase in the regulation of AJs by progastrin. Activation of PKC, along with inhibition of glycogen-synthase kinase-3β, seems to be involved in the cytoplasmic accumulation of β-catenin in response to growth factors like Wnt (wingless) (Chen et al., 2000b). Furthermore, a clear colocalisation between β-catenin and PKCα at cell-cell contacts was recently shown in GH3B6 pituitary epithelial cells after phorbol 12-myristate 13-acetate treatment, although no physical association between the two proteins was evident (Vallentin et al., 2001). To our knowledge, the current study shows, for the first time, a physical association between PKCα and β-catenin in epithelial cells, as well as of cooperation between PI3-kinase and PKCα in the regulation of β-catenin localisation at the membrane, although the exact nature of the action of PKCα on AJs needs to be elucidated.

The specific involvement of Src and PI3-kinase in the disruption by progastrin of TJs and AJs, respectively, supports the concept of an independent regulation of both types of junction during the early stages of cell dissociation induced by an extracellular stimulus. Although progastrin induced a disruption of both AJs and TJs in the current study, data from IMGE-5 cells stably expressing Src$^{-/-}$ or PI3-k$^{-/-}$ dominant-negative mutants seem to show that the early stages of TJ and AJ disruption could be independent from one another. Nevertheless, recent results argue in favour of a cross-talk between AJs and TJs, as overexpression of the PDZ domains of ZO-1 (Reichert et al., 2000) or treatment with occludin peptides (Vietor et al., 2001) was found to upregulate the cytoplasmic availability and the transcriptional activity of β-catenin. However, the duration of treatment with progastrin in our study was significantly shorter than the time allowed for measurement of an effect of occludin fragments on the activation of the β-catenin/T-cell factor (TCF)/Lef transcriptional pathway in mammary epithelial cells (Vietor et al., 2001). The occurrence and the importance of cross-talk between TJs and AJs in DLD-1 cells is supported by the greatly decreased membrane localisation of AJ proteins in DLD-1/PI3-k$^{-/-}$ cells. If PI3-kinase specifically mediates the effect of progastrin on AJs, as shown in IMGE-5 cells, any effect of endogenous progastrin on AJs in DLD-1/PI3-k$^{-/-}$ cells is likely to occur indirectly via the presence of higher concentrations of

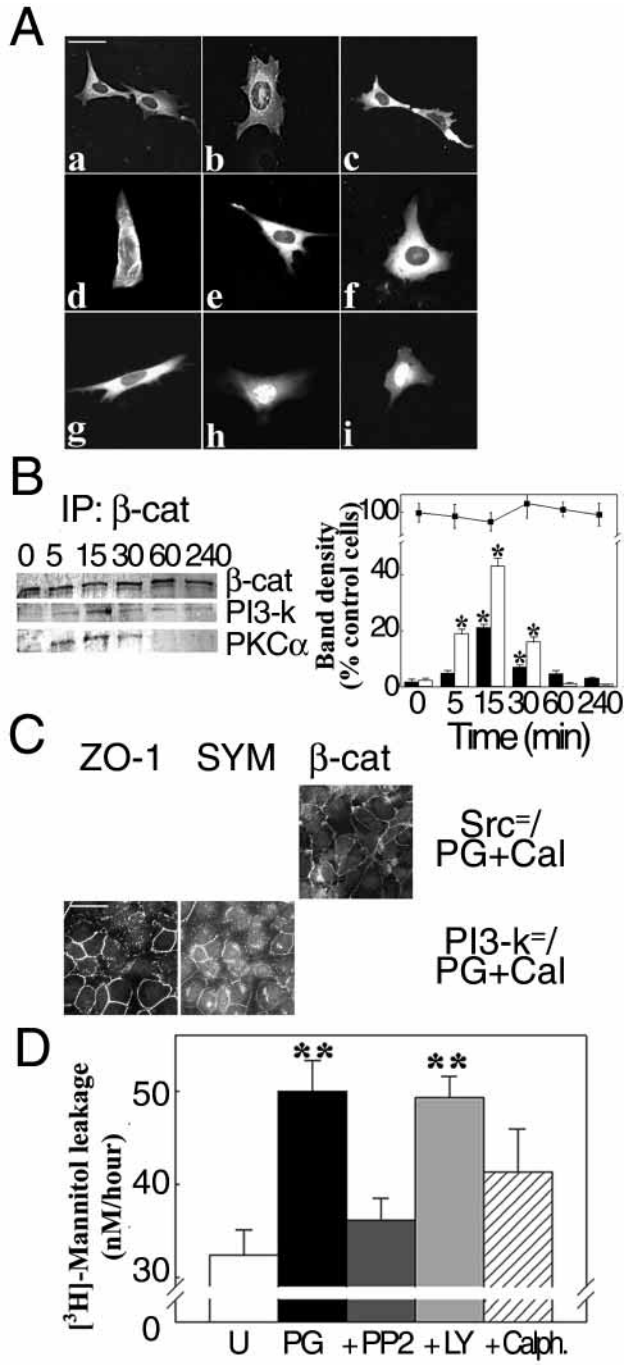

Fig. 7. Involvement of PKC in the progastrin-induced cytoplasmic shift of β-catenin. (A) Of an EGFP-tagged PKCα isoform was transiently overexpressed in wild-type IMGE-5 cells (a). Treatment of EGFP-PKCα transfected cells with 5nM progastrin$_{6-80}$ for 15 minutes (b) or 30 minutes (c), or with phorbol dibutyrate for 30 minutes (d), induced a delocalisation of EGFP-PKCα to a perinuclear area and to the plasma membrane. Preincubation with the PI3-kinase inhibitor LY 294002 (f), for 30 minutes before, and during, progastrin stimulation prevented the delocalisation of EGFP-PKCα to the membrane. Preincubation with the Src kinase inhibitor PP2 (e), or the PKC inhibitor calphostin C (g) had no effect on delocalisation. Control cells were transfected with an empty pEGFP vector similar to the vector used for PKC overexpression (h) and stimulated with 5 nM progastrin$_{6-80}$ for up to 45 minutes (i). Bars, 10 μm for a and c, 5 μm for all other panels. (B) In wild-type IMGE-5 cells, treatment with 5 nM progastrin$_{6-80}$ was found to increase transiently the association between β-catenin (top line), the p85 subunit of PI3-kinase (filled bars) and PKCα (open bars), as detected by densitometric scanning of western blots of anti-β-catenin immunoprecipitates of cell lysates. (C) In IMGE-5/Src$^{-/-}$ cells, preincubation with calphostin C reversed the progastrin$_{6-80}$-induced cytoplasmic shift of β-catenin (β-cat) shown in Fig. 5C. By contrast, the changes in ZO-1 and symplekin (SYM) localisation induced by progastrin$_{6-80}$ were barely affected by preincubation with calphostin C in wild-type (F.H., unpublished) and IMGE-5/PI3-k$^{-/-}$ clones (Fig. 6B). Bar, 20 μm. (D) Preincubation with calphostin C (Calph) partially inhibited the increase in paracellular permeability for [^{3}H]mannitol induced by 5 nM progastrin$_{6-80}$ (PG). The increase in permeability was almost completely blocked by the Src kinase inhibitor PP2, but was unaffected by preincubation with the PI3-kinase inhibitor LY294002. In B and D, densitometric analysis represents the average of at least three experiments, and statistical significance was assessed by Student's *t* test. *P<0.05, **P<0.01.

free TJ proteins, such as ZO-1, in the cytosol. Alternatively, long-term constitutive progastrin stimulation in these DLD-1/PI3-k$^{-/-}$ cells could bypass the PI3-kinase pathway, thereby allowing a resumption of AJ regulation in these cells. By contrast, DLD-1/Src$^{-/-}$ cells did not show a significant disruption of their TJ protein localisation, indicating that the interaction between TJs and AJs during the cell-cell dissociation period in this model could be monodirectional, from the TJ towards the AJ.

The results presented in this study show that tumour cells expressing progastrin and Ggly displayed major perturbations in cell-cell adhesion, as well as spontaneous motility. This shows that such hormone precursors, which are known to be overexpressed during foetal development (Luttichau et al., 1993), as well as in colon carcinoma (Ciccotosto et al., 1995), could act through an autocrine loop to chronically disrupt adhesion and motility of colorectal carcinoma cells. The existence of such a loop could be crucially important in vivo, where progastrin overexpressed in transgenic mice was previously shown to act as a cocarcinogen, following treatment with the colonic carcinogen azoxymethane (Singh et al., 2000b; Singh et al., 2000a). Antibodies raised against a gastrin immunogen were shown to inhibit the spontaneous metastasis of a human colorectal tumour cell line producing progastrin and Ggly when injected into immunodeficient mice (Watson et al., 1999). In humans, a strong correlation was shown between a higher incidence of liver metastasis from colorectal carcinoma and elevated serum gastrin concentrations (>150 pg/ml) in a panel of 140 patients (Kameyama et al., 1993). Furthermore, the role of progastrin-derived peptides on cell motility clearly has other implications than in cancer development, as shown in vivo by a gastrin-induced parietal cell migration in the mouse gastric mucosa (Kirton et al., 2002).

Interestingly, several progastrin-derived peptides have been shown to regulate the morphology, adhesion and motility of gastrointestinal epithelial cells. The fully processed form Gam has recently been shown to induce branching morphogenesis in gastric cancer cells (Pagliocca et al., 2002), loss of adhesion and scattering of G/CCK-B receptor-transfected MDCK cells (Bierkamp et al., 2002), as well as increased matrix metalloproteinase-9 expression in gastric cells in vitro and in the stomach of multiple endocrine neoplasia (MEN-1) patients with elevated concentrations of plasma gastrin (Wroblewski et al., 2002). Ggly has previously been shown to increase the invasiveness of the human colon carcinoma cell line LoVo (Kermorgant and Lehy, 2001) and to induce the dissociation of AJ complexes, as well as the migration of gastric epithelial cells (Hollande et al., 2001a). The current work presents the first description of a regulatory role for the early precursor progastrin in the dissociation and migration of nontumoral and tumoral gastrointestinal cells.

Several observations indicate that the dissociating effect of progastrin is probably mediated by a membrane receptor: (1) no effect of progastrin is detected on cell types where regulation by gastrointestinal peptides is not expected to occur, such as MDCK kidney cells (F.H., unpublished); (2) progastrin$_{6-80}$ is a large peptide (MW 8500) and is therefore unlikely to penetrate membranes quickly, if at all, in order to act on an intracellular target. However, in the current study, the onset of Src kinase activation, as well as dissociation of junctional complexes after progastrin$_{6-80}$ treatment, was very rapid; (3) the progastrin-induced dissociation of AJs and TJs, as well as the stimulation of paracellular permeability and migration, were found to be dose dependent and saturable at the low nanomolar concentrations typical of membrane receptors. The nature of the receptor mediating this effect of progastrin on adhesion and motility is yet to be determined, as we have so far been unable to iodinate progastrin in a reproducible manner on its single tyrosine residue (Baldwin et al., 2001). The receptor could be similar to the only identified progastrin receptor, recently found to mediate proliferation on rat intestinal epithelial cells (IEC) (Singh et al., 2002), and further investigation should determine whether it is related to the receptor involved in the effects of Ggly on cell dissociation and motility (Kermorgant et al., 2001; Hollande et al., 2001a). The affinity of the receptor identified by Singh et al. for progastrin-derived peptides lacking the N-terminal sequence was found to be lower than for the full-length progastrin peptide (Singh et al., 2002). This observation is in agreement with the data presented in this manuscript showing that the maximal effective concentration of progastrin$_{6-80}$, which lacks the six N-terminal amino acids, is slightly higher than the concentration of progastrin$_{1-80}$ inducing maximal proliferation of IEC-6 cells (Singh et al., 2002).

To date, most studies of the effects of progastrin-derived peptides on gastrointestinal cell adhesion and motility have been performed on cell lines in vitro, thereby enabling a more specific description of the molecular and cellular events involved. However, more studies in vivo are clearly necessary to clarify the individual roles and evaluate the impact of progastrin-derived peptides on the regulation of epithelial cell morphology and migration during development, and in the potentiation of the invasive properties of colorectal tumours.

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