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Suppression of Cellular Invasion by Activated G-Protein Subunits Gαo, Gαi1, Gαi2, and Gαi3 and Sequestration of Gβγ

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ABSTRACT

It was shown previously that platelet-activating factor receptors (PAF-Rs) inhibit invasiveness of colonic and kidney epithelial cells induced by the src and Met oncogenes via a pertussis toxin-sensitive mechanism. Therefore, Madin-Darby canine kidney (MDCKts.src) cells were stably transfected with constitutively activated forms of Gαo, Gαi1, Gαi2, Gαi3 (AGαo/i), two Gβγ sequestering proteins [C-terminal end of β-adrenergic receptor kinase (ct-βARK) and the Gαt subunit of retinal G-protein transducin], and Gβ1–Gγ2 subunits alone or in combination. Cellular invasion induced by src, Met, and leptin was abrogated by the AGαo/i, ct-βARK, and Gαt-positive clones, but was induced by coexpression of Gβ1γ2. In contrast, invasion stimulated by the trefoil factors (TFFs) pS2 and intestinal trefoil factor in MDCKts.src cells or human colonic epithelial cells PCmsrc and HCT8/S11 was insensitive to PAF, AGαo, AGαi1, and AGαi2, but was abolished by AGαi3 and the protease-activated receptor-1 (PAR-1) agonist thrombin receptor-activating peptide. Depletion of free Gβγ heterodimers by ct-βARK resulted in a remarkable decrease of cellular adhesion and spreading on collagen matrix. Our data demonstrate the following: 1) PAF-Rs impair cellular invasion induced by src, Met, and leptin via the activation of Gαo and Gαi1 to -3; 2) invasion induced by TFFs is selectively inhibited by PAR-1 receptors and Gαi3 activation; and 3) Gβγ dimers are required as positive effectors of invasion pathways induced by oncogenes and epigenetic factors. Thus, redistribution of Gαo/Gαi and Gβ/γ heterotrimeric G-proteins by PAF-R and PAR-1 exert differential functions on positive and negative signaling pathways involved in cellular invasion and may serve as potential targets for anticancer therapy.

Persistent dysregulation of signal transduction pathways promote the acquisition of anarchic functions in digestive epithelial cells, adjacent tissues, and immune cells, leading to local inflammation and further susceptibility to the neoplastic progression. Strong experimental and clinical data suggest a close relationship between inflammatory disease and the occurrence of solid tumors in intestine, breast, and prostate (Rudolph et al., 1995a; Emami et al., 2001). Local mediators, cytokines, and inflammatory agents, such as platelet-activating factor (PAF), thrombin, and trefoil factors (TFFs) are involved in the cellular responses to injury and wound repair during human inflammatory processes in the gastrointestinal tract, including peptic ulceration and colitis, Crohn's disease, pancreatitis, and biliary disease (Cirino et al., 1996; Kotelevets et al., 1998; Emami et al., 2001). We have shown previously that functional and specific PAF receptors (PAF-R) are expressed in normal human colonic epithelial crypts, colonic adenoma, and adenocarcinoma cell lines (Kotelevets et al., 1998). Moreover, we found that src- and Met-induced invasion of collagen gels was abrogated by PAF-R activation in Madin-Darbin canine kidney (MDCKts.src) and colonic PCmsrc cells via a pertussis toxin (PTx)-sensitive pathway, suggesting that Gαo/Gαi and Gβ/γ heterotrimeric G-proteins are involved in this process. PTx, which is specific for Gαo and Gαi subunits, prevents the catalysis of GDP-GTP exchange by the receptor, blocking both Gα and Gβγ signaling. The preferred substrates of PTx are Gαo/i subunits associated with Gβγ complexes, whereas monomeric Gα subunits are very poor substrates (Rudolph et

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S.F. and K.R. contributed equally to this work.

ABBREVIATIONS: PAF, platelet-activating factor; TFF, trefoil factor; PAF-R, platelet-activating factor receptor; MDCK, Madin-Darby canine kidney; PTx, pertussis toxin; ITF, intestinal trefoil factor; AGo/i, activated forms of Gα0, Gαi1, Gαi2, and Gαi3; ct-βARK, C-terminal end of the β-adrenergic receptor kinase; Gαt, transducin Gα subunit; PAR-1, protease-activated receptor-1; TRAP, thrombin receptor-activating peptide; HGF, hepatocyte growth factor; RT, reverse transcriptase; PCR, polymerase chain reaction; pAb, polyclonal antibody; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence; PI3′-Kα, activated form of phosphatidylinositol 3-kinase; RGS, regulators of G-protein signaling.

al., 1995b). Redistribution and functional activity of Gβγ subunits released from PTx-sensitive proteins and serpentine receptors are important components of exocytosis and scaffold of molecular complexes at the plasma membrane level and subcellular compartments, including the actin network (Hamm, 1998). We also presented evidence that leptin and the TFFs pS2, spasmolytic polypeptide, and intestinal trefoil factor (ITF) are positive and potent effectors of cellular invasion in premalignant and src-transformed kidney and colonic epithelial cells (Attoub et al., 2000; Emami et al., 2001). Leptin and TFFs are expressed in epithelial cells along the gastrointestinal tract and are considered local and paracrine regulators of mucosal integrity, renewal, inflammation, and neoplastic progression.

This study was therefore conducted to identify the subtypes and cellular functions of the heterotrimeric G-protein subunits linked to the negative control exerted by the serpentine PAF-R on positive invasion pathways controlled by src, Met, and the intestinal mediators leptin and TFFs. For this purpose, we stably transfected MDCKts.src cells by constitutively activated GTPase-deficient forms of Gαo, Gαi1, Gαi2, Gαi3 (AGαo/i), the chimeric C-terminal end of the β-adrenergic receptor kinase (ct-βARK) scavenging Gβγ subunits (Pitcher et al., 1992; Crespo et al., 1994), and transducin Gα subunit (Gαt), another agent known to sequester free G-protein βγ dimers (Federman et al., 1992). To examine further the role of Gβγ subunits on cellular invasion, Gβ1 was also overexpressed, either alone or with Gγ2. The relative contribution of PAF-R, AGαo/i, and Gβγ subunits on cellular invasion and adhesion was then examined with the use of these experimental models, as well as with the human colorectal cell lines PCmsrc and HCT8/S11, which are derived from familial and sporadic tumors, respectively (Behrens et al., 1993; Vermeulen et al., 1995; Empereur et al., 1997). Because trefoil peptides are overexpressed in digestive epithelial cells during inflammatory situations and cancer progression (Emami et al., 2001), we next investigated whether PAF-R can control cellular invasion in MDCKts.src and HCT8/S11 cells stably transfected by the human pS2 cDNA. Results were compared with the functional activity of the thrombin PAR-1 receptor that is specifically activated by the thrombin receptor-activating peptide (TRAP) (Seiler et al., 1996). The PAR-1 receptor has multiple-signaling capacity and is also coupled to the PTx-sensitive G-proteins Gαo, Gαi1, Gαi2, and Gαi3. We report that activated Gαo/i subunits exert an invasion-suppressor role, whereas the Gβγ subunits are required as critical and positive mediators of cellular invasion pathways induced by oncogenes and epigenetic factors.

Materials and Methods

DNA Constructs. The rat cDNAs encoding the constitutively activated forms of the PTx-sensitive Gα protein subunits AGαo/i were generously provided by Dr. E. Peralta (Harvard University, Cambridge, MA). They were cloned in frame into the eukaryotic expression vector pcDNA3.1 recombined with the neo resistance gene (Invitrogen BV, Breda, The Netherlands). The AGαo/i cDNAs were inserted at the cloning sites *Eco*RI/*Xho*I (Q205L–Gαo) or *Eco*RI/*Xba*I (Q204L-Gαi1, -2, and -3). These mutationally activated forms of GTPase-deficient G-proteins were further designated as AGαo/i1, -2, and -3. The structure and function of the expression vectors encoding AGαo/i was checked by direct DNA sequencing. The capture and sequestration of Gβγ subunits was induced by the myc-tagged expression vector pcDNA3 encoding a chimeric protein comprising the membrane-bound CD8 receptor and ct-βARK, as described previously (Pitcher et al., 1992). The human transducin Gαt subunit cDNA cloned into the pcDNA3.1 expression vector was purchased from the American Type Culture Collection (Manassas, VA). The bovine Gβ1 and Gγ2 cDNAs were cloned from the pcDM8–1 vector into the pcDNA3.1 expression vector (Crespo et al., 1994). The Gβγ sequestrant vector ct-βARK and expression vectors encoding Gβ1 and Gγ2 were generously provided by Dr. R. Weitzker (Klinikum der Friedrich-Schiller-Universität, Jena, Germany).

Cell Lines and Culture Conditions. MDCK epithelial cells (MDCKts.src) transformed by a temperature-sensitive mutant of v-src (MDCKts.src, clone 2) were cultured in Dulbecco's modified Eagle's medium (Invitrogen SARL, Cergy Pontoise, France) supplemented with 10% fetal calf serum (Roche Molecular Biochemicals, Meylan, France) plus L-glutamine and antibiotics (Invitrogen), as described previously (Behrens et al., 1993). MDCKts.src cells display an invasive phenotype at the permissive temperature of 35°C for src activity and are not invasive at the restrictive temperature 40°C. The human colorectal cell line PCmsrc was grown in 6-cm diameter Petri dishes. After transfer of the activated c-src oncogene in the premalignant PC/AA/C1 cell line, PCmsrc cells became tumorigenic in the athymic nude mice and are invasive upon addition of hepatocyte growth factor (HGF) (Empereur et al., 1997). The MDCKp110* cell line stably transfected with a constitutively activated form of bovine p110*α by addition of the C-terminal farnesylation signal from Ha-Ras (Khwaja et al., 1997) was a generous gift from Dr. J. Downward (Imperial Cancer Research Fund, London, UK). Kidney MDCKts.src-pS2 cells (clone 2) and colonic epithelial cells HCT-8/S11-pS2 cells (clone 2) stably transfected by the human full-length hpS2 were cultured under standard conditions (Emami et al., 2001).

Stable Transfection of Kidney Epithelial Cells. Approximately 3×10^6 MDCKts.src cells were stably transfected by the activated forms of the PTx-sensitive Gα-protein subunits AGαo/i1, -2, and -3 using the corresponding pcDNA3.1 expression plasmids (3 μg) and 18 μl of the LipofectAMINE Plus reagent (Invitrogen). Control transfections were performed using the empty vector pcDNA3.1. MDCKts.src cells were also transfected under the same conditions using either the Gβγ sequestrant vectors CD8-ct-βARK or Gαt, and the vectors encoding bovine Gβ1 and Gγ2 (see above). After 48 h, cultures were selected for 2 weeks in 1 mg/ml neomycin or 0.5 mg/ml hygromycin (Invitrogen) and MDCKts.src-resistant colonies were ring-cloned as individual colonies or pooled for analysis of ectopic expression of the AGαo/i, CD8–ct-βARK, Gαt, Gβ1, and Gγ2 proteins by immunoblot analysis, indirect immunocytochemistry, and additional functional characterization.

Western Blot Analyses, Immunocytochemistry, and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis. For immunoblotting, cultured cells were homogenized at 4°C in radioimmunoprecipitation buffer containing 0.1 mg/ml phenylmethylsulfonyl fluoride, 100 μM benzamidine, and 100 μM Na_3VO_4 as protease inhibitors. Insoluble material was removed by centrifugation for 15 min at 4°C and 12,000*g*. Proteins were resolved using nonreducing conditions in 12.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride or Hybond-C Extra membranes (Amersham Pharmacia Biotech, Orsay, France). Membranes were blocked overnight in Tris-buffered saline (20 mM Tris-HCl, pH 8, 150 mM NaCl) containing 5% dried skimmed milk. The blots were then probed for 4 h at room temperature with one of the following primary polyclonal or monoclonal antibodies at the indicated dilutions: the rabbit pAb specific for Gαo and Gαi3 (1:2000) was from Euromedex (Souffelweyersheim, France); the rabbit pAb AS for Gαi1 and -2 (1:200) was a generous gift from Professor P. Mazancourt (Hôpital Raymond Poincaré, Garches, France) the rabbit pAb raised against the C-terminal part of Gαi3 (1:2500) was from Calbiochem (Meudon, France); the rabbit pAbs against Gαt transducin K-20 (1:500), Gβ1 C-16 (1:100), and Gγ2 A-16 (1:500), and the

mAb raised against the c-myc epitope (1:1000) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Membranes were then washed in Tris-buffered saline containing 0.1% Tween 20 and probed for 90 min with either a donkey anti-rabbit IgGs pAb (1/2000, Amersham), or a goat anti-mouse IgGs pAb (1/2000, Santa Cruz Biochemicals), and then revealed by enhanced chemiluminescence Western detection (ECL; Amersham).

For immunofluorescence labeling of the c-myc epitope in MDCKts.src cells stably transfected by the CD8–ct-βARK fusion gene, kidney cells were grown on autoclaved glass slides (VWR, West Chester, PA) and processed at 70 to 80% confluence. Cells were rinsed with phosphate-buffered saline (PBS) at room temperature, fixed for 30 min in phosphate buffer containing 4% formaldehyde, and washed 3 times in phosphate buffer. Cells were then made permeable with Triton X-100 in PBS for 10 min, blocked with 3% goat serum at room temperature for 30 min, and incubated overnight at 4°C with primary mAb anti-c-myc (Santa Cruz, 1:500). Then, cells were rinsed with PBS and incubated for 1 h at room temperature with a fluorescein-conjugated goat anti-mouse IgG (1:150) from Immunotech (Roissy, France). Stained cells were rinsed again in PBS, fixed with a solution of glycerol in PBS (9:2, v/v), and viewed by conventional epifluorescence on an Olympus BH2 microscope (Olympus, Tokyo, Japan).

Total RNA was extracted from parental and Gαt-transfected MDCKts.src cells (clones 3–5) using the Trizol reagent (Invitrogen). RT-PCR was performed using the Superscript detection kit (Invitrogen) according to the manufacturer's protocol. The experiment comprised one cycle of 30 min at 55°C and one of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C, with a final extension time of 10 min at 72°C. For human Gαt, we used the following sense and antisense oligonucleotides: 5′-GGCAGACACTATCGAGGAGGGCACGATGCC-3′ and 5′-GAGGTGC-GCCTTCTTGACCTTCTCGAAGAAGAC-3′. PCR products were then separated by electrophoresis in 1.5% agarose gel and detected under UV light. The Gαt primers (Invitrogen) amplified the 519-base-pair fragment specific of the human G-protein transcript. Glyceraldehyde-3-phosphate dehydrogenase messengers (574 base pairs) were also analyzed by RT-PCR to verify the integrity of RNA preparations.

Collagen Invasion and Cell Adhesion Assays. For invasion of collagen gels by renal and colorectal epithelial cells, Petri dishes were filled with 1.35 ml of neutralized type I collagen and incubated overnight at 37°C and allowed to gel. Cells were harvested using Moscona buffer and trypsin/EDTA and were seeded on top of collagen gels. Cultures were incubated for 24 h at the indicated temperature in the presence or absence of the indicated effectors. The depth of cell migration inside the gels was measured with the use of an inverted microscope (Vleminckx et al., 1991). Invasive and superficial cells were counted in 12 fields of 0.157 mm^2. The invasion index is the number of cells invading the gel expressed as a percentage of the total number of cells.

Peptides and Reagents. HGF scatter factor was a generous gift from Professor Paolo Comoglio (University of Turin, Italy). Purified recombinant human ITF and hpS2 were a generous gift from Dr. L. Thim (Novo Nordisk, Bagsvaerd, Denmark) and Professor B. Westley (University of Newcastle upon Tyre, UK). Leptin was from R & D Systems Europe (Oxford, United Kingdom). PTx, PAF, thrombin, and phenylmethylsulfonyl fluoride were from Sigma (Saint Quentin Fallavier, France). The PAR-1 agonist TRAP (SFLLRN) was from Bachem Biochimie (Voisins-le-Bretonneux, France). Collagen type I was from Upstate Biotechnology (Lake Placid, NY).

Results and Discussion

Negative Control of Cellular Invasion Pathways by PAF-R, PAR-1, and the Activated Forms AGαo/i of the PTx-Sensitive G-Proteins. We have recently shown that PAF receptors exert a negative control on src- and HGF-induced cellular invasion in kidney and colonic epithelial cells (Kotelevets et al., 1998). We first re-examined the effect of PTx on positive and negative invasion pathways induced by G-protein -coupled receptors and other transduction elements, including Met, src, and the activated form of phosphatidylinositol 3-kinase (PI3′-Kα) (p110*). As shown in Fig. 1A, 200 ng/ml PTx fully reversed the inhibitory effect of PAF on invasion induced by src in MDCKts.src cells incubated at the permissive temperature 35°C (invasion index, 7.5 ± 0.6%). The A protomer of PTx is a single peptide that ADP-ribosylates cysteine residues of membrane Gαo/i subunits, blocking the heterotrimeric complex in the inactive GDP-bound state and preventing dissociation of Gα and Gβγ. The toxin uncouples the activation of the heterotrimeric complex by PAF-R. In contrast, the same concentrations of PTx (20–200 ng/ml) produced the inverse effect and abolished HGF-induced cellular invasion in MDCKts.src cells incubated at the nonpermissive temperature 40°C (Fig. 1B), directly implicating substrate G-protein α-subunits and released Gβγ subunits in signaling through the Met tyrosine kinase. Similarly, other signals mediated by tyrosine kinase-associated receptors, including epidermal growth factor, insulin-like growth factor-1, basic fibroblast growth factor, erythropoietin Epo receptors, and insulin receptors, can be inhibited or induced by PTx treatment and Gβγ sequestrant because of direct and indirect associations with Gαi, Gβγ, and Gβ subunits (Luttrell et al., 1997; Hallak et al., 2000). Indirect cross-talk and *trans*-activation of the epidermal growth factor receptors was also induced by the G-protein-coupled receptors for thrombin and lysophosphatidic acid (Prenzel et al., 1999). Note that PTx did not abolish invasion induced by the tyrosine kinase src and constitutively activated PI3′-Kα in MDCKts.src and MDCKp110* cells (Fig. 1, C and D), leptin, and TFFs in kidney and colonic epithelial cells. These findings raise the possibility that the PTx-sensitive trimeric subunits on one hand and the Gβγ subunits on the other are selectively and, respectively involved in negative and positive invasion pathways. To explore this possibility, we stably

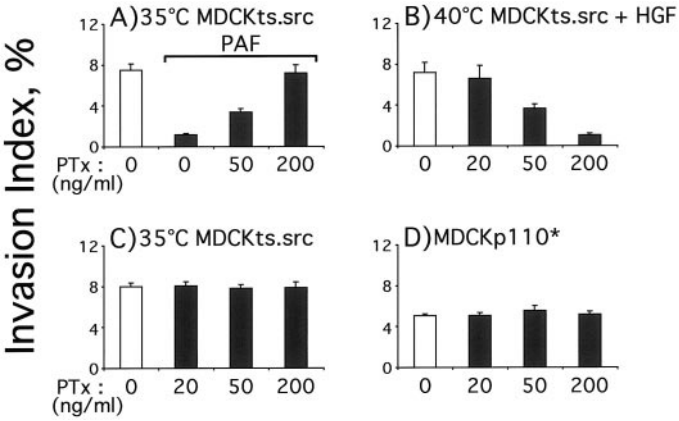

Fig. 1. Differential control by PTx of cellular invasion pathways induced by src, HGF/Met, and the activated form p110* of PI3′-Kα in MDCK cells. MDCKts.src cells were incubated for 24 h in the presence or absence of the indicated effectors [PAF (0.1 μM) or HGF (10 units/ml)] alone or with increasing concentration of PTx (20–200 ng/ml). The percentage of invasive cells in collagen type I gels was measured either at the permissive temperature 35°C for src activation (A and C) or at the restrictive temperature 40°C (B). D, PTx was tested at 37°C in kidney MDCK cells stably transfected by the constitutively activated form of PI3′-Kα (MDCKp110*). Data are means ± S.E. of three or four separate experiments.

transfected MDCKts.src cells with GTPase-deficient mutants of Gαo/i encoding-activated forms (AGαo/i) of these four Gα PTx-sensitive subunits. To confirm that AGαo/i forms are expressed in MDCKts.src cells, immunoblot analysis in Fig. 2 identified several positive clones overexpressing Gαo (clones 1 and 4), Gαi1 (clone 6), Gαi2 (clones 1, 7, 8, and 10) and Gαi3 (clones 1–3). For example, densitometry analysis revealed that the Gαi3 protein was overexpressed 2- and 3-fold in clones 1 and 2, respectively. As shown in Fig. 3, A and B, stable expression of AGαo/i completely abolished cellular invasion induced by HGF and src, suggesting that all four PTx-sensitive Gα subunits are candidate-signaling elements in the negative control of cellular invasion by activated PAF-R. To gain insight into this possibility, we next determined whether PAF and PAR-1 thrombin receptors have similar activities on cellular invasion induced by the trefoil factors pS2 and ITF. The PAR-1 receptor is a seven-transmembrane domain G-protein-coupled receptor that is also connected with the PTx-sensitive proteins Gαo/i (Seiler et al., 1996). As shown in Fig. 4, cellular invasion induced by ITF in kidney MDCKts.src and colonic PCmsrc epithelial cells is resistant to PAF (A and B), and the same situation is observed in kidney MDCKts.src-pS2 and colonic HCT8/S11-pS2 epithelial cells stably transfected by the trefoil factor pS2 (C and D). In contrast, the PAR-1 agonist TRAP abolished ITF- and pS2-induced invasion in the four models, suggesting that the Gαo/i subunits exert a differential control on invasion. In agreement with this interpretation, we observed that thrombin or the exogenous agonist TRAP completely inhibited cellular invasion induced by HGF in MDCKts.src cells and activated PI3-K in MDCKp110* cells in a PTx-sensitive manner (data not shown). Pertussis toxin (200 ng/ml) also completely reversed the inhibitory effect of thrombin or TRAP on HGF-, ITF-, and src-mediated invasion in MDCKts.src cells (not shown). We therefore examined the relative contribution of the activated forms of G-proteins AGαo/i on the negative control exerted by PAF-R and PAR-1 receptors on ITF- and pS2-induced invasion. As shown in Fig. 5, all forms of AGαo/i blocked invasion induced by leptin and HGF in MDCKts.src cells. In contrast, only MDCKts.src cells stably transfected by AGαi3 (clones 1 and 2) seem incapable of responding to ITF and pS2, suggesting that only this Gαi3 subunit can mediate the negative control exerted by activated PAR-1 receptors on cellular invasion induced by the TFFs.

Depletion of Free Gβγ Subunits by the βγ-Sequestering Peptides ct-βARK and Gαt: Consequences for Cellular Invasion and Adhesion. It is becoming increasingly evident that signaling through the Gβγ heterodimers and the individual β and γ subunits released upon activation of PTx-sensitive G-proteins Gαo/i plays a key role in multiple-signaling pathways. For example, inhibition or activation of

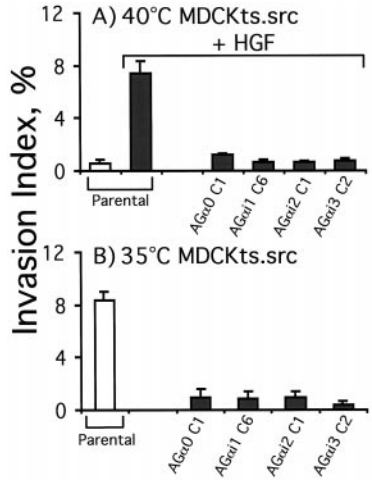

Fig. 3. Negative control of src- and HGF-induced invasion of collagen gels by constitutively activated forms of the AGαo/i1–3 subunits in MDCKts.src cells. Cellular invasion was compared in MDCKts.src cells before (parental cell line) and after stable expression of the constitutively activated forms of Gαo (clone 1), Gαi1 (clone 6), Gαi2 (clone 1), and Gαi3 (clone 2). The effect of the AGαo/i1–3 subunits was tested on invasion induced by HGF at 40°C in MDCKts.src cells (A) and by src in MDCKts.src cells incubated at the permissive temperature 35°C (B). The percentage of invasive cells was determined as described under *Materials and Methods*. Data are means ± S.E. of three separate experiments.

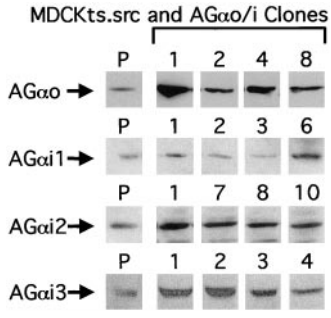

Fig. 2. Expression by Western blot of the constitutively activated forms of the AGαo/i1 to -3 subunits in stably transfected MDCKts.src cells. Parental MDCKts.src cells (P) were stably transfected by the pcDNA3.1 expression vector recombined with the GTPase-deficient forms of Gαo-Q205L (AGo), Gαi1-Q204L, Gαi2-Q204L, and Gαi3-Q204L (AGi1–3). Positive MDCKts.src clones expressing given AGα subunits were identified by immunoblot analysis using the antibodies specified under *Materials and Methods*. Immunoblots were revealed by the ECL Western detection system and quantified by the use of a densitometer. The Gα subunits migrate at 41 kDa (arrow). Results are representative of one other independent experiment.

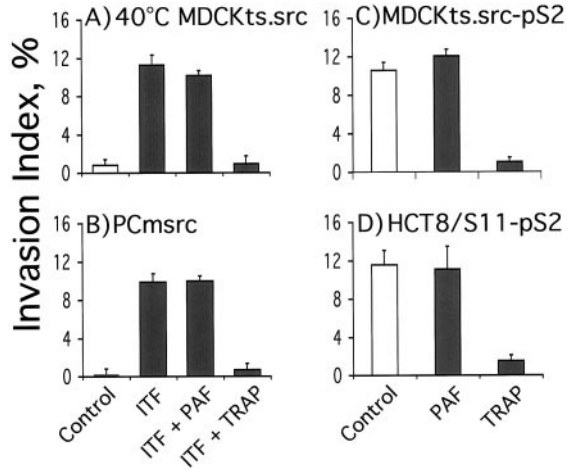

Fig. 4. Differential signaling of PAF-R and PAR-1 thrombin receptors toward cellular invasion pathways induced by trefoil peptides in kidney and colonic epithelial cells. The effect of PAF (0.1 μM) and the PAR-1 agonist TRAP (10 μM) was tested on invasion of collagen gels induced by ITF (100 nM) in kidney MDCKts.src and colonic PCmsrc epithelial cells (A and B). Results were compared with the effects of PAF and TRAP on constitutive cellular invasion induced by overexpression of the pS2 cDNA in kidney MDCKts.src-pS2 and human colonic HCT/8S11-pS2 epithelial cells (C and D). The percentage of invasive cells was determined as indicated under *Materials and Methods*. Data are means ± S.E. of three separate experiments.

specific isoforms of adenylate cyclase (Hamm, 1998), G-protein-coupled receptor kinases GRK1 to -3 involved in receptor desensitization (Pitcher et al., 1992; Carman et al., 2000), membrane-targeting of Gα subunits and binding to pleckstrin homology domains (Touhara et al., 1994), Gβγ exchange between Gαi-coupled and Gαq-coupled receptors (Quitterer and Lohse, 1999), src- and ras-dependent activation of mitogen-activated protein kinases, Gβγ-sensitive PI3′-Kγ and Jun kinases, and phospholipase C-β are concerned with Gβγ signaling (Luttrell et al., 1996; Lopez-Ilasaca et al., 1998; Rickert et al., 2000). Recently, release of Gβγ from activated insulin-like growth factor I receptor (Hallak et al., 2000) and direct interactions between Gβγ and the Rho family of GTPases, Rho and Rac, have been documented (Harhammer et al., 1996; Ueda et al., 2000). The role of Gβγ signaling in either cellular invasion or adhesion is unknown. Accordingly, we have established MDCKts.src cell lines stably expressing the Gβγ sequestrant vector comprising the CD8 receptor and ct-βARK.

As shown in Fig. 6, immunoblot analysis identified the ectopic expression of the myc epitope-tagged CD8–ct-βARK protein in MDCKts.src–ct-βARK cells (clones 3–5). The signal was not detected by immunoblotting in parental MDCKts.src cells and their transfected counterparts, the MDCKts.src–ct-βARK cells (clones 1 and 2). Strong expression of the Gβγ subunit inhibitor was confirmed by indirect immunofluorescence in MDCKts.src–ct-βARK clone 3, whereas a much weaker signal was present in MDCKts.src–ct-βARK clone 1 cells, at barely detectable levels. We therefore examined the invasive properties of the low- and high-expressing MDCKts.src–ct-βARK cells (clones 1 and 3). In the MDCKts.src–ct-βARK clone 1 expressing very low levels of CD8–ct-βARK, cellular invasion induced by HGF was reduced by 50% (Fig. 7A) compared with nontransfected parental MDCKts.src cells. This residual activity was still sensitive to PAF inhibition, whereas src-induced cellular invasion was unaffected in MDCKts.src–ct-βARK clone 1 (Fig. 7B). In contrast, high expression of the Gβγ scavenger leads to total inhibition of the invasive phenotype controlled by HGF and/or src in the MDCKts.src–ct-βARK clone 3 (Fig. 7, A and B). These findings suggest that the Gβγ subunits are required as potential links between G-protein-coupled recep-

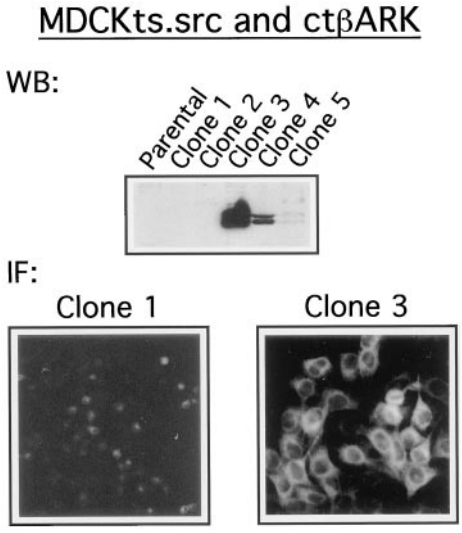

Fig. 6. Western blot and immunofluorescence analysis of the Gβγ sequestrant peptide ct-βARK in MDCKts.src cells stably transfected the CD8–ct-βARK expression vector. Top, expression of the myc-tagged β-adrenergic receptor kinase (ct-βARK) peptide in MDCKts.src cells transfected by the CD8–ct-βARK vector (clones 1–5). Immunoblotting of total cell lysates was performed using the c-myc mAb and revealed by the ECL Western detection system. Bottom, stable expression of the Gβγ sequestrant was analyzed by indirect immunofluorescence in MDCKts.src–ct-βARK cells (clones 1 and 3). Cells were processed with the mAb against the c-myc epitope, followed by fluorescein-conjugated goat anti-mouse IgG as secondary antibody, as described under *Materials and Methods*.

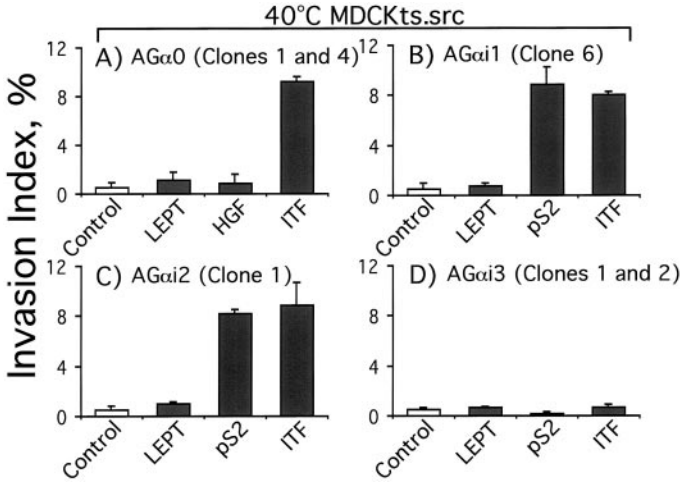

Fig. 5. Differential signaling of activated AGαo/i1–3 subunits toward cellular invasion pathways induced by leptin, HGF, and trefoil factors in kidney and colonic epithelial cells. Cellular invasion induced by leptin (100 ng/ml), HGF (10 units/ml), and the trefoil factors ITF or pS2 (100 nM) was measured at 40°C in MDCKts.src cells before (parental cell line) and after stable expression of the constitutively activated forms of Gαo (clones 1 and 4), Gαi1 (clone 6), Gαi2 (clone 1), Gαi3 (clones 1 and 2). The percentage of invasive cells was determined as indicated under *Materials and Methods*. Data are means ± S.E. of three or four separate experiments.

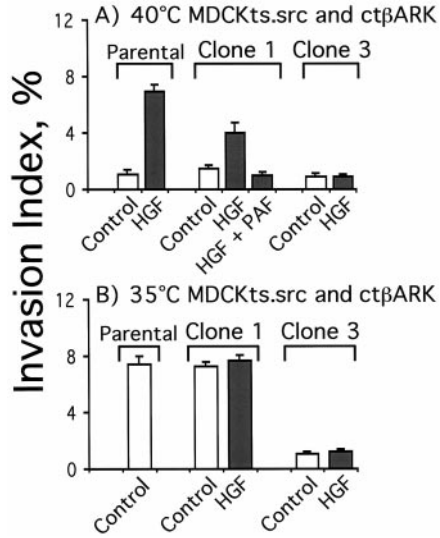

Fig. 7. Negative control of src- and HGF-induced invasion of collagen gels by the Gβγ sequestrant peptide ct-βARK in MDCKts.src cells stably transfected with the CD8–ct-βARK expression vector. Cellular invasion index was determined in parental and ct-βARK-transfected MDCKts.src cells (clones 1 and 3) incubated at the nonpermissive temperature 40°C for src activation (A) or at the permissive temperature 35°C (B). MDCKts.src–ct-βARK cells were treated with 10 units/ml HGF alone or with 0.1 μM PAF and compared with their respective control cells (no effector). The percentage of invasive cells was determined as described under *Materials and Methods*. Data are means ± S.E. of three separate experiments.

tors and positive cellular invasion pathways induced by the src and Met oncogenes. Similarly, the G$\beta\gamma$ inhibitor CD8–ct-βARK abolished leptin-, pS2- and ITF-induced cellular invasion in MDCKts.src cells (not shown). Over the last few years, a number of interesting connections between invasion pathways and cellular adhesion to extracellular matrix proteins have emerged. Increasing evidence shows multiple collaborations between cell-cell and cell-substratum interactions, assembly of the actin cytoskeleton, and signal transduction pathways involved in invasion and metastasis. These interactions, together with recent reports suggesting activation by G$\beta\gamma$ subunits of the Rho family GTPase including Ras, Rho, and Rac (Harhammer et al., 1996; Ueda et al., 2000), prompted us to examine the adhesive properties of MDCKts.src cells transfected with the G$\beta\gamma$ sequestrant CD8–ct-βARK. The interdependence of these pathways is further illustrated by the sequential role of Rac and Rho in the initiation of new adhesion sites and their maturation into focal adhesions.

As shown in Fig. 8, A and B, sequestration of the G-protein $\beta\gamma$ subunits impaired cell adhesion and spreading to collagen gels and filopodia formation in MDCKts.src–ct-βARK clone 3 activated by the HGF/Met and src oncogenes compared with the low-expressing ct-βARK clone 1. Cell adhesion to extracellular matrix proteins is responsible for cell spreading through integrin receptors, which promotes integrin clustering and cytoskeletal reorganization and induces cells to spread. Disruption of the gene encoding the G-protein β-subunit impaired the regulation of the actin cytoskeleton at cell-yeast particle adhesion sites during phagocytosis and abrogated chemotaxis in the *Dictyostelium discoideum* amebae (Peracino et al., 1998), suggesting that Gβ is intimately involved in signal transduction networks linking cytoskeletal responses to chemoattractants. The G-proteins $\beta\gamma$ may also be associated with adhesion and/or morphological changes (Hansen et al., 1994). In the present study, inhibition of G$\beta\gamma$ signaling effectively induced cell rounding and alterations of spreading in MDCKts.src–ct-βARK clone 3 that was refractory to activation of src and Met (Fig. 8B).

As shown in Fig. 9, overexpression of the G$\beta\gamma$ sequestrant vector Gαt transducin also prevented cellular invasion induced by HGF, leptin, and pS2, either alone (A) or combined with activation of the src oncogene (B) in MDCKts.src-Gαt cells (clone 5). Similar results were obtained in MDCKts.src-Gαt cells (clones 3 and 4) established from transfected MDCKts.src cells expressing the human Gαt transducin protein by immunoblot (data not shown) and RT-PCR (Fig. 9, inset). Because G$\beta\gamma$ subunits have been implicated in the regulation of Rho GTPases that are dynamic regulators of the actin cytoskeleton (Harhammer et al., 1996; Hall, 1998; Ueda et

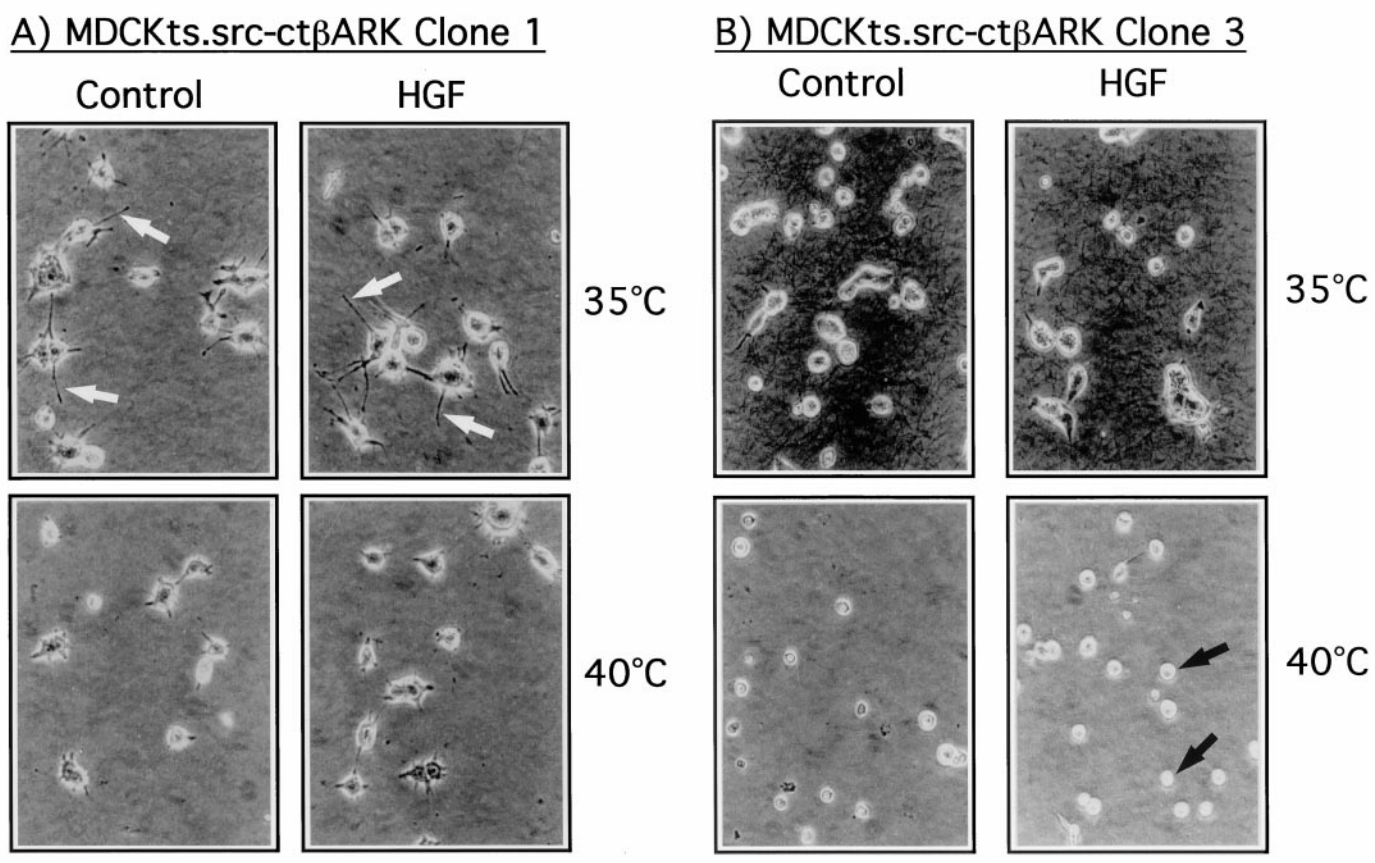

Fig. 8. Effect of overexpressed G$\beta\gamma$ sequestrant peptide CD8–ct-βARK on adhesive properties and spreading of MDCKts.src cells on collagen gels. Adhesion to collagen type I gels was compared in MDCKts.src–ct-βARK cells (clones 1 and 3 in A and B, respectively) incubated for 24 h at 35°C or 40°C in the presence or absence of HGF (10 units/ml). Cell were seeded at the density of 10^5 cells/ml. The white arrows in A underline the src-dependent filopodia formation in MDCKts.src–ct-βARK clone 1 incubated in the presence or absence of HGF. In contrast, the MDCKts.src–ct-βARK clone 3 showed a remarkable reduction of filopodia protrusions and cellular spreading induced by src (B). Most interestingly, ct-βARK caused shape change and cellular rounding in MDCKts.src–ct-βARK clone 3 incubated at the nonpermissive temperature for src activation (B), leading to a loss of cell adhesion, even in the presence of the Met activator HGF (black arrows).

al., 2000), including the Rho/Rac interplay, our data on the βγ sequestering peptides ct-βARK and Gαt transducin are consistent with the direct implication of Gβγ subunits in several signaling pathways involved in cellular invasion and switch between cellular rounding/retraction and spreading/cellular adhesion.

Activation of Cellular Invasion by Coexpression of Gβγ Subunits. Our data on the Gβγ sequestering proteins ct-βARK and Gαt support the hypothesis that free Gβγ endogenous dimers released from activated PTx-sensitive G-proteins are required for initiation and activation of positive cellular invasion pathways controlled by oncogenes and epigenetic factors. To explore further such a possibility, we next examined the effect of ectopic overexpression of Gβ1 alone or combined with Gγ2 in MDCKts.src cells. First, we stably transfected MDCKts.src cells with Gβ1 and selected four G418-resistant colonies (Fig. 10A). Immunoblot analysis identified two MDCKts.src-Gβ1 cells (clones 1 and 3) overexpressing the Gβ1 protein (36 kDa). As shown in Fig. 10B, HGF-induced invasion of collagen gels was completely blocked by overexpression of Gβ1 in clone 3, whereas src-induced activation was unaffected (Fig. 10C). Thus, we examined the Gβ1-dependence of the negative control exerted by PAF-R and PAR-1 on src-induced invasion. As a consequence of Gβ1 overexpression, there was a substantial attenuation of PAF-R-mediated inhibition of cellular invasion (Fig. 10C), but no significant effect on the negative control exerted by PAR-1 was found. Our data are therefore consistent with the possibility that overexpression of Gβ1 alone exerts a selective inhibition of the PAF-R and HGF/Met signaling pathways. This possibility is consistent with the recent finding that molecular complexes between Gβ subunits and several regulators of G-protein signaling (RGS) have been identified, because structural domains in RGS proteins exhibit striking homologies to Gγ subunits (Snow et al., 1998; Levay et al., 1999; Sowa et al., 2000). Most importantly, some Gβ/RGS heterodimers behave as GTPase-activating proteins for certain Gα subunits, such as Gαo (Snow et al., 1998). The RGS activity and selectivity for PTx-sensitive or -insensitive Gα subunits can be directly or indirectly determined through their interactions with different Gβ subunits, G-protein-coupled receptors, or other binding partners. For example, RGS1, RGS3, RGS4, and GAIP stimulate the GTPase activity of Gαi family members but are ineffective against Gαs. Thus, overexpression of Gβ1 can selectively mimic, at least in part,

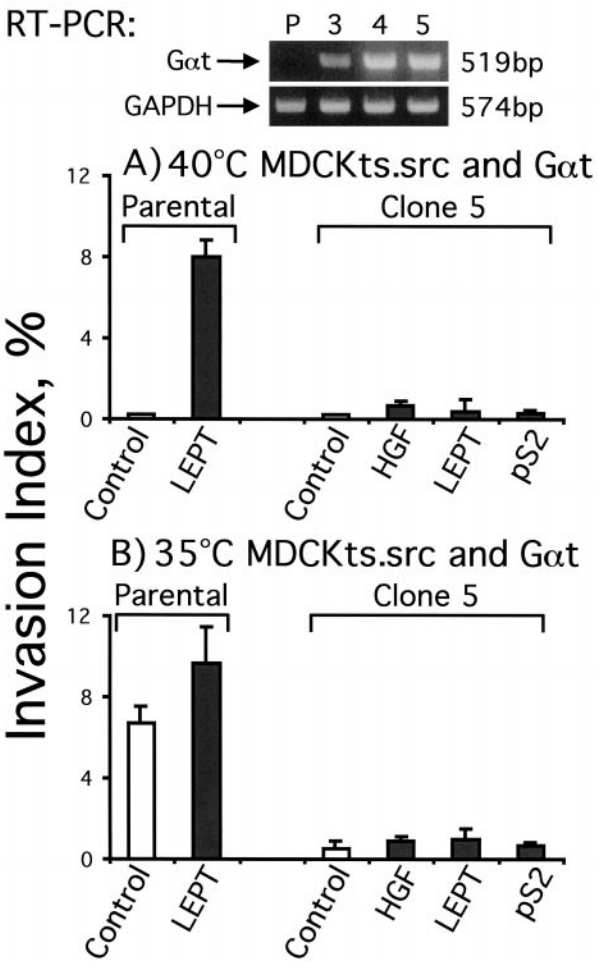

Fig. 9. Negative control of src- and HGF-induced invasion of collagen gels by the Gβγ sequestrant peptide Gαt in MDCKts.src cells stably transfected by the Gαt transducin expression vector. Cellular invasion index was determined in parental and Gαt-transfected MDCKts.src cells cultured at 40°C (A) or at the permissive temperature 35°C for src activation (B). Stably transfected MDCKts.src-Gαt cells (clone 5) were treated with either 10 units/ml HGF, 100 ng/ml leptin, or 100 nM pS2 and compared with their respective control cells (no effector) and parental MDCKts.src cells incubated in the presence or absence (Control) of 100 ng/ml leptin. The percentage of invasive cells was determined as described under *Materials and Methods*. Data are means ± S.E. of three separate experiments performed in clone 5 and are representative of another experiment performed in clones 3 and 4.

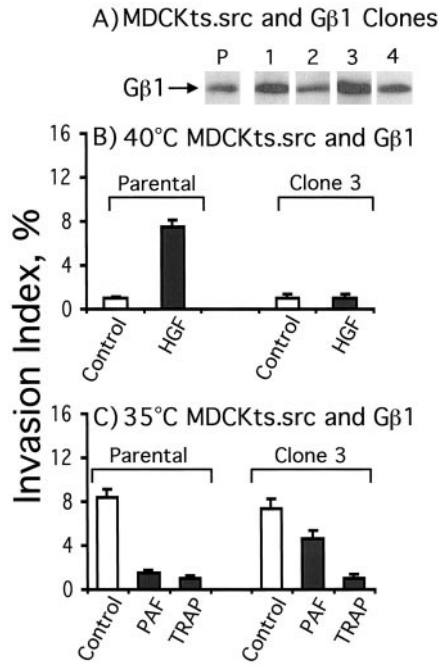

Fig. 10. Effect of overexpressed Gβ1 subunit on positive and negative invasion pathways in stably transfected MDCKts.src-Gβ1 cells. A, expression of the Gβ1 subunit was analyzed by Western blot in parental MDCKts.src cells (P) and transfected MDCKts.src-Gβ1 cells (G418-resistant clones 1–4), using the Gβ1 pAb. The Gβ1 subunit migrates at 36 kDa (arrow). Cellular invasion was then quantified in the MDCKts.src–Gβ1-positive clone 3 incubated at 40°C (B) or 35°C (C). Parental MDCKts.src and MDCKts.src-Gβ1 cells were treated with either HGF (10 units/ml), PAF (0.1 μm), or TRAP (10 μm) and compared with their respective control cells (no effector). The percentage of invasive cells was determined, as described under *Materials and Methods*. Data are means ± S.E. of three separate experiments.

PTx-induced deactivation of the PAF-R and Met signaling pathways involved in cellular invasion, as shown in Fig. 1.

Individual Gβ or Gγ subunits exert divergent signaling functions, depending on the functional status and relative distribution of the resident Gα and Gβγ subunits and dimers interacting with a given signal transduction system. In the Gβγ complexes, the Gβ subunits consist of seven subtypes with high amino acid sequence identity from 80 to 90% for Gβ1- to -4 and 52% identical for Gβ5 (Watson et al., 1994). Thus, Gγ2 subunit was expressed together with Gβ1 in MDCKts.src cells after cotransfection of MDCKts.src-Gβ1 cells (clone 3) with the Gγ2 (3 μg) and pcDNA3.1/Hygro (0.3 μg) expression vectors and selection for 2 weeks in the presence of hygromycin (0.5 mg/ml). As shown in Fig. 11, immunoblot analysis identified two MDCKts.src-Gγ2-positive cells (clones 4 and 11) expressing the ectopic Gγ2 protein (7 kDa) after subsequent transformation of MDCKts.src-Gβ1 cells by the Gγ2 vector. The Gγ2 subunit protein was not detected in MDCKts.src cells. Coexpression of both Gβ1 and Gγ2 subunits in MDCKts.src cells resulted in a remarkable induction of cellular invasion in MDCKts.src-Gβ1γ2 cells (Fig. 11B; clones 4 and 11) and potentiation of invasiveness induced by HGF (Fig. 11, B and C), and src (Fig. 11C). Furthermore, invasion induced by overexpressed Gβ1γ2 proteins in MDCKts.src cells (invasion index, 5.2% and 4.8% in clones 4 and 11, respectively) is abrogated by activation of the PAF-R and PAR-1. Our data therefore indicate that formation of the Gβ1γ2 complex in MDCKts.src-Gβγ cells is associated with induction and potentiation of cellular invasion pathways. This is in agreement with our demonstration that cellular invasion induced by src, Met, and epigenetic factors was abrogated by sequestration of free Gβγ subunits by ct-βARK and Gαt. Thus, PTx neutralizes HGF-induced invasion (Fig. 1) and reverses inhibition of cellular invasion controlled by activated PAF-R and PAR-1, suggesting that PTx abolishes 1) Gβγ-signaling pathways involved in the induction of cellular invasion by HGF, and 2) Gαo/i-mediated inhibition of cellular invasion induced by PAF/TRAP. Alternatively, it should be stressed that Gαi subunits are also activators of invasion pathways controlled by src and PI3′-Kγ, for example.

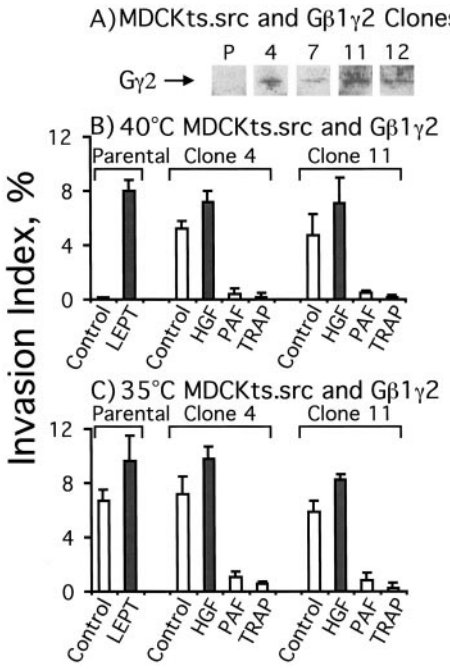

Fig. 11. Positive control of cellular invasion after ectopic coexpression of Gβ1 and Gγ2 subunits in stably transfected MDCKts.src-Gβ1γ2 cells. Reversion by PAF-R and PAR-1 activation. A, expression of the Gγ2 subunit in MDCKts.src-Gβ1 cells (P) subsequently transfected by the Gγ2 vector. Immunoblotting of total cell lysates was performed using the affinity-purified rabbit pAb Gγ2 (A-16) and revealed two positive MDCKts.src-Gβ1γ2 cells (clones 4 and 11) by the ECL Western detection system. B and C, cellular invasion index was determined in parental MDCKts.src cells incubated in the presence or absence of 100 ng/ml leptin and in MDCKts.src-Gβ1γ2 cells (clones 4 and 11) incubated at 40°C (B) or at the permissive temperature 35°C for src activation (C). Stably transfected MDCKts.src-Gβ1γ2 cells and parental MDCKts.src cells were treated with either 10 units/ml HGF, PAF (0.1 μM), or the PAR-1 agonist TRAP (10 μM) and compared with their respective control cells (no effector). The percentage of invasive cells was determined as described under *Materials and Methods*. Data are means ± S.E. of three separate experiments.

In conclusion, our data indicate that the redistribution of heterotrimeric G-proteins from activated PAF and PAR-1 receptors lead to a negative control of cellular invasion via the activation of Gαo/i subunits in response to multiple invasion pathways induced by oncogenes and epigenetic factors (Fig. 12). Thus, the Gαi/o subunits exert a dominant invasion-suppressor role in our assay. In contrast, Gβγ subunits that are liberated from PTx-sensitive Gα subunits are critical mediators and links between positive cellular invasion and adhesion pathways. In this scenario, Gβγ dimers can induce activation of multiple signaling pathways that are critical components of cellular invasion, namely Rho-like small G-proteins, the β/γ isotypes of PI3′-K, and the phospholipase C/protein kinase C cascade (Rickert et al., 2000). The individual functions of the paired Gβγ proteins and the PTx-insensitive Gα subunits associated with PAF-R and PAR-1 may provide an alternative pathway by which these heterotrimeric G-proteins may exert opposing effects on cellular invasion (i.e., positive and negative signals). Precisely how the balance between these positive and negative signals is regulated in the integration of the cellular responses remains to be elucidated. Disruption of this equilibrium might have important biological consequences, because we have shown that PAF and PAR-1 receptors exert a dominant negative function on cellular invasion. Deactivation of Gαo/i subunits by PTx in the present study can be physiologically induced and mimicked by the RGS proteins that negatively control these PTx-sensitive G-proteins and Gαz, Gα12/13 subunits, and PAF-R phosphorylation as well. In contrast, we found that overexpressing Gβγ subunits was sufficient to induce invasiveness. Thus, RGS signals might abolish the dominant negative control exerted by Gαo/i subunits on cellular invasion and trigger a permissive action on positive invasion pathways governed by Gβγ. These results are analogous to the negative cross-talk exerted by Gαo on the Gαq pathway and its downstream effectors (Hajdu-Cronin et al., 1999). Abnormalities in the expression, protein structure, and constitutive activation of G-protein subunits (Gβ, Gαi2, Gαi3) have been reported in human pathologies, including tumors of the ovary, neuroendocrine tumors, and experimental models of colonic neoplasms induced by azoxymethane in rats (Lyons et al., 1990; Bolt et al., 1998; Farfel et al., 1999). Recently, mutants of the exchange factor cdc24 for the Rho-like GTPase Cdc42 defective in binding to the G-protein Gβ subunit have been described (Nern and Arkowitz, 1998) in

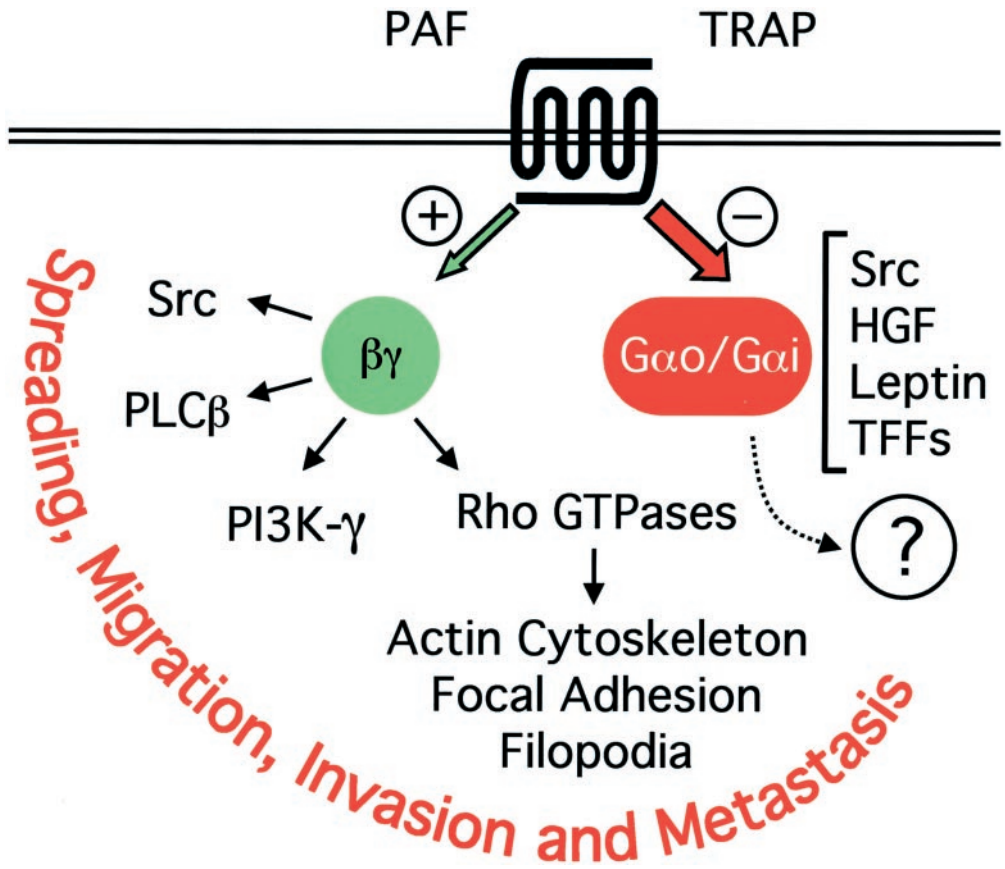

Fig. 12. Model depicting the negative and positive invasion pathways induced by activated PAF-R and PAR-1 in kidney and colonic epithelial cells. Activation of Gαo/i subunits by serpentine PAF-R and PAR-1 exerts a dominant negative control (−) on cellular invasion pathways governed by src, HGF/Met, the epigenetic factors leptin and TFFs, and the Gβγ subunits (+) that are released from these PTx-sensitive G-proteins. The Gβγ subunits, on the other hand, are invasion promoters, according to their connections with src, phospholipase C-β, PI3′-kinases, and the Rho-like GTPases that induce the formation of filopodia and lamellipodia (Cdc42 and Rac), focal adhesions, and stress fibers (Rho). The Gαo/Gαi downstream targets remains to be identified (?) in the context of the integration of the positive and negative signaling pathways implicated in cellular spreading, migration, local invasion, and distant metastasis.

the context of the cytoskeletal reorientation. Therefore, activation or inactivation of the molecular components of the PTx-sensitive and -insensitive trimeric G-proteins in familial and sporadic tumors (Rodrigues et al., 2001) should be considered in view of their differential localization and function in basolateral and cytoskeletal domains, including focal adhesion and stress fibers in polarized epithelial cells in kidney and intestine (Hansen et al., 1994; Bolt et al., 1998; Saha et al., 1998). Further studies will shed more light for understanding the relative contribution and functioning of heterotrimeric G-proteins Gα and Gβγ in positive and negative signaling pathways involved in cellular adhesion and invasion in the context of neoplasia and metastasis.

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