A role for chemokine receptor transactivation in growth factor signaling

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Complex cell responses require the integration of signals delivered through different pathways. We show that insulin-like growth factor (IGF)-I induces specific transactivation of the G_i-coupled chemokine receptor CCR5, triggering its tyrosine phosphorylation and Gα_i recruitment. This transactivation occurs via a mechanism involving transcriptional upregulation and secretion of RANTES, the natural CCR5 ligand. CCR5 transactivation is an essential downstream signal in IGF-I-induced cell chemotaxis, as abrogation of CCR5 function with a transdominant-negative KDELccr5Δ32 mutant abolishes IGF-I-induced migration. The relevance of this transactivation pathway was shown *in vivo*, as KDELccr5Δ32 overexpression prevents invasion by highly metastatic tumor cells; conversely, RANTES overexpression confers built-in invasive capacity on a non-invasive tumor cell line. Our results suggest that this extracellular growth factor-chemokine network represents a general mechanism connecting tumorigenesis and inflammation.

INTRODUCTION

To coordinate their functions, cells must communicate with one other and with the extracellular matrix through specific molecules that transduce extracellular stimuli in intracellular signaling cascades. Receptor pathways do not function in isolation; indeed, signals from multiple receptors and the underlying cytoskeleton influence one other substantially. This crosstalk is illustrated by the connections between structurally distinct classes of receptors, such as G protein-coupled receptor (GPCR) and tyrosine kinase receptor (RTK).

The convergence of GPCR and RTK signaling pathways is supported by observations that platelet-derived growth factor, epidermal growth factor (EGFR) and type-1 insulin-like growth

factor receptors (IGF-1R) are tyrosine phosphorylated after GPCR activation, and the mechanisms underlying this cross-communication have begun to be understood (Rao *et al.*, 1995; Prenzel *et al.*, 1999). Although transactivation of GPCR signaling by RTK is reported (Luttrell *et al.*, 1995; Kanzaki *et al.*, 1997), it is controversial (Siebler *et al.*, 1996), as the mechanism underlying this crosstalk remains unknown.

Here we show that IGF-I, a growth factor that signals through an RTK, induces specific transactivation of the CCR5 chemokine receptor, a type of GPCR, through a mechanism involving the extracellular secretion of its natural ligand, RANTES. Our results also establish that CCR5 transactivation is an important step in IGF-I-triggered signaling, since abrogation of CCR5 function with the dominant-negative KDELccr5Δ32 mutant abolishes IGF-I-induced cell chemotaxis. *In vivo* tumor cell invasion may be enhanced or prevented by switching this transactivation pathway on or off. Extension of this model to basic fibroblast growth factor (bFGF) suggests that this extracellular growth factor-chemokine network may represent a prevalent feature of growth factor-triggered chemotactic signaling in malignant and untransformed cell lines.

RESULTS AND DISCUSSION

To study GPCR transactivation by RTK, we analyzed IGF-I- and chemokine-induced chemotactic responses in MCF-7 cells. MCF-7 cells respond chemotactically to IGF-I and poorly to β chemokines (Figure 1A). Pretreatment of MCF-7 cells with pertussis toxin (Ptx), a drug that inactivates G_i by ADP ribosylation of the Gα subunit and hence inhibits β chemokine signaling, blocks chemotaxis induced by RANTES, a chemokine that binds the CCR5 chemokine receptor (Figure 1A). Ptx also inhibits

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Fig. 1. IGF-I induces specific CCR5 transactivation. (A) cells were untreated or Ptx-pretreated before assay for chemotaxis by the indicated stimuli. Ptx does not affect MCF-7 cell viability in these conditions (not shown). The results show the number of cells per field (200×) of Ptx-treated (open bar) or untreated (solid bar) MCF-7 cells stimulated by IGF-I or RANTES ($n = 2$; * $p < 0.01$, two-tailed t -test). Dashed line indicates chemotaxis in the absence of stimulus. Serum-starved cells were stimulated with IGF-I, RANTES (B), or SDF-1 α (C), and IGF-1R, CCR5 or CXCR4 selectively precipitated (i.p.). Filters were probed sequentially (w.b.) with anti-phosphotyrosine (PY) mAb, -IGF-1R, -CCR5, -G α_i or anti-CXCR4 mAb.

IGF-I-induced MCF-7 migration, suggesting that IGF-I activity requires downstream activation of a Ptx-sensitive GPCR.

We thus analyzed whether IGF-I stimulation of MCF-7 IGF-I promoted specific signaling events mediated by the chemokine receptors expressed by these cells. Chemokine receptors are tyrosine phosphorylated immediately after activation (Vila-Coro *et al.*, 1999) and MCF-7 stimulation with RANTES- or SDF-1 α (stromal cell-derived factor-1) induces time-dependent tyrosine phosphorylation of CCR5 (Figure 1B) or CXCR4 (Figure 1C), respectively. IGF-I stimulation induces tyrosine phosphorylation of CCR5 (Figure 1B), but not of other chemokine receptors such as CXCR4 (Figure 1C), indicating that IGF-I specifically transactivates CCR5. Compared to the rapid CCR5 phosphorylation that follows RANTES stimulation, IGF-I induces biphasic CCR5 activation, with a first phosphorylation peak at 15 min post-stimulation and a second peak after 120 min (Figure 1B). This lag in IGF-I-mediated CCR5 transactivation is not due to a general delay in IGF-I-mediated responses, since the type-1 IGF receptor (IGF-1R) is activated by 1 min of IGF-I stimulation (Figure 1B). IGF-I stimulation also induces G α_i recruitment to the activated

Fig. 2. IGF-I-induced CCR5 transactivation is dependent on RANTES secretion. MCF-7 cells were stimulated with IGF-I alone or in the presence of anti-RANTES mAb (A), or preincubated with monensin before IGF-I stimulation (B). IGF-1R or CCR5 were selectively precipitated; G α_i recruitment to CCR5, or IGF-1R autophosphorylation (PY) were analyzed by western blotting. Membranes were re-probed with anti-CCR5 and -IGF-1R antibodies. For monensin treatment, western blot data are represented as the densitometry intensity ratio for G α_i and CCR5, or that calculated for tyrosine-phosphorylated and total IGF-1R β -subunit (** $p < 0.001$, two-tailed t -test). (C) IGF-I-induced MCF-7 chemotaxis was assayed in the presence of neutralizing anti-chemokine mAb. An isotype-matched non-specific (non-sp) and a blocking anti-IGF-I mAb were included as negative and positive controls, respectively. Results are expressed as a percentage, and indicate inhibition of chemotaxis in the presence of each antibody ($n = 3$; ** $p < 0.001$, two-tailed t -test). (D) RANTES levels of cell-free supernatants from serum-depleted (-) and IGF-I (10 nM; +)-stimulated cells.

CCR5 (Figure 1B), but G α_i association to the IGF-1R was not observed (not shown). These data show that IGF-I stimulation transactivates CCR5 in a manner similar to activation by RANTES.

To characterize the molecular mechanisms by which IGF-I triggers specific CCR5 transactivation, we studied the effect of neutralizing anti-RANTES monoclonal antibodies (mAb) in this process. IGF-I-induced early CCR5 transactivation is prevented by adding anti-RANTES mAb (Figure 2A) or by blocking protein secretion with monensin (Figure 2B); these treatments do not affect early IGF-1R signals such as receptor autophosphorylation (Figure 2A and B). The requirement for extracellular RANTES secretion in IGF-I-induced signaling was confirmed, as both neutralizing anti-RANTES and anti-IGF-I mAb inhibit IGF-I-mediated chemotaxis equally well (Figure 2C). Conversely, antagonist mAb against other chemokines such as MCP-1

Fig. 3. IGF-I regulates RANTES expression. (A) Equal amounts of lysates from unstimulated (–) or IGF-I-stimulated (+) cells were assayed for RANTES in ELISA ($n = 3$). (B) RANTES or GAPDH mRNA were stimulated in unstimulated (–) or stimulated with IGF-I alone (solid bars) or in the presence of actinomycin D (open bars). The graph represents the RANTES/GAPDH mRNA ratio expressed in arbitrary units ($n = 3$; $**p < 0.001$, two-tailed t -test). (C) MCF-7 cells transfected with a luciferase reporter gene under transcriptional control of RANTES (solid bars) or MIP-1 β (open bars) promoters, unstimulated (–) or IGF-I-stimulated (+) for 5 or 24 h ($**p < 0.001$, two-tailed t -test).

(monocyte chemotactic protein-1), MIP-1 α/β (macrophage inflammatory proteins-1 α/β) or SDF-1 α do not affect IGF-I activity (Figure 2C). This suggests that IGF-I-induced CCR5 transactivation is mediated by extracellular RANTES release. Concurring with this, IGF-I stimulation specifically increases RANTES levels (Figure 2D), but not those of MIP-1 α/β , growth-related peptide (Gro α), or MCP-1 (not shown) in MCF-7-conditioned medium.

To gain insight into IGF-I-mediated RANTES production, we analyzed its intracellular concentration. IGF-I induces a biphasic increase in intracellular RANTES, with the same kinetics as IGF-I-induced CCR5 transactivation (Figure 3A). The intracellular RANTES protein concentration correlates with RANTES-encoding mRNA levels (Figure 3B). IGF-I increases the cellular content of RANTES mRNA by both a transcription-dependent and -independent effect, as actinomycin D does not affect the RANTES mRNA peak at 15 min, but abolishes the IGF-I-mediated increase at longer stimulation times (Figure 3B). Accordingly, IGF-I treatment increases RANTES promoter-driven luciferase activity, whereas the MIP-1 β promoter is unaffected (Figure 3C). The IGF-I-induced increase in the RANTES transcription rate was significant as early as 5 h, but augmented further at longer stimulation times. These results suggest that IGF-I-induced CCR5 transactivation is mediated by extracellular secretion of RANTES, which in turn binds to and activates CCR5. IGF-I regulates RANTES expression in MCF-7 cells in two discrete stages. First, IGF-I induces RANTES production by stabilizing RANTES mRNA or increasing its translation rate; the

Fig. 4. Chemokine receptor signals are required for growth factor-induced cell polarization and chemotaxis. (A) CCR2, CCR5 and CXCR4 levels in mock- (dotted line) and KDELccr5 Δ 32- (solid line) transduced cells were estimated by flow cytometry. Background fluorescence of an isotype-matched non-specific mAb is shown in gray. (B) IGF-I (solid bars), IGF-I+RANTES (open bars) or IGF-I+SDF-1 α (gray bars)-induced chemotaxis of mock- and KDELccr5 Δ 32-transduced cells. The number of migrating mock-transduced cells using IGF-I as chemoattractant was considered 100% ($n = 3$). (C) Paxillin staining (red) of mock- and KDELccr5 Δ 32-expressing MCF-7 cells untreated (none), or stimulated with IGF-I, RANTES or SDF-1 α ; transduced cells co-express GFP (green). Bar, 10 μ m. (D) Quantification of random fields from (C) (three independent experiments; $**p < 0.001$, two-tailed t -test).

second phase involves specific upregulation of RANTES gene transcription by a direct or indirect IGF-I-mediated mechanism.

To study the functional relevance of IGF-I-induced CCR5 transactivation, cells were transduced with a CCR5 deletion mutant engineered to be retained in endoplasmic reticulum (KDELccr5 Δ 32), which is phenotypically similar to the natural ccr5 Δ 32 mutant (Liu *et al.*, 1996). Overexpression of KDELccr5 Δ 32 in MCF-7 cells specifically sequestered endogenous CCR5 (Figure 4A), probably by forming heterocomplexes as reported for the ccr5 Δ 32 form (Benkirane *et al.*, 1997), thus acting as a CCR5 dominant-negative mutant. KDELccr5 Δ 32 overexpression drastically decreases IGF-I-induced chemotaxis (Figure 4B). IGF-I stimulation of mock-transduced cells induces acquisition of a polarized phenotype, shown here as focal

Fig. 5. Chemokine receptor signals modulate tumor invasiveness *in vivo*. **(A)** RANTES levels measured by ELISA in cell-free supernatants from DU-145. **(B)** Mock and KDELccr5Δ32-DU-145 (10^6 cells) were inoculated onto chick embryo CAM, and intravasated cells estimated (three independent experiments, $n = 16$ for each cell line). **(C)** Mock- and RANTES-MCF-7 (2×10^6 cells) were inoculated onto the CAM as in (B). Three independent experiments, $n = 14$ for each cell line.

contact accumulation at the leading edge (Figure 4C and D), which is essential for IGF-I-induced MCF-7 chemotaxis (Mañes *et al.*, 1999b). Nonetheless, KDELccr5Δ32 overexpression incapacitates cell acquisition of this IGF-I-induced motile phenotype (Figure 4C and D). SDF-1α stimulation rescues both the enhanced chemotaxis observed with SDF-1α plus IGF-I (Figure 4B) and acquisition of the polarized phenotype in KDELccr5Δ32-expressing cells (Figure 4C and D). KDELccr5Δ32 thus inhibits IGF-I chemotaxis by specific CCR5 sequestration, rendering the cell unable to respond to the RANTES secreted; indeed, RANTES-induced MCF-7 polarization is lost in KDELccr5Δ32-expressing cells. These results establish that CCR5 transactivation is a downstream target activated by auto-crine RANTES, required for IGF-I-induced cell polarization and chemotaxis. We also observed that bFGF-induced BAEC chemotaxis is dependent on CCR2 transactivation (see Supplementary material Online), suggesting that growth factor-induced chemokine secretion may be a prevalent feature of RTK-triggered chemotactic signaling.

We tested whether abrogation of CCR5 function prevents *in vivo* tumor invasion of IGF-I-dependent cell lines. We over-expressed KDELccr5Δ32 in DU-145 cells, a highly invasive prostate carcinoma with a well-characterized IGF-I autocrine loop (Mañes *et al.*, 1999a). In addition to IGF-I, DU-145 cells secrete high RANTES levels without exogenous stimulation (Figure 5A), and IGF-1R and CCR5 are constitutively activated in DU-145 (Mañes *et al.*, 1999a). KDELccr5Δ32 overexpression reduces IGF-I- and RANTES-induced DU-145 chemotaxis *in vitro* (not shown), indicating that this mutant sequesters CCR5 in DU-145, as it does in MCF-7.

To assay the role of CCR5 in tumor invasion, we seeded mock- and KDELccr5Δ32-transduced DU-145 cells onto chick embryo chorioallantoic membrane (CAM). The mean number of KDELccr5Δ32-DU-145 cells recovered in the lower CAM was up to 80% lower than that of mock-transduced cells, indicating that KDELccr5Δ32 overexpression prevents DU-145 cell invasiveness in the CAM model (Figure 5B; $p < 0.05$). To study whether CCR5 signals modulate tumor invasiveness, mock-transduced and MCF-7 cells overexpressing RANTES were analyzed in the CAM assay. MCF-7 cells were non-invasive (Kim *et al.*, 1998) and secreted no detectable RANTES in unstimulated conditions (Figure 2D); indeed, very few mock-transduced MCF-7 cells reach the lower CAM (Figure 5C). RANTES over-expression increases the number of MCF-7 cells recovered from the lower CAM by >10-fold (Figure 5C; $p < 0.05$).

Our findings distinguish extracellular chemokine secretion as a pivotal RTK signal, and extend understanding of the pathways leading to many growth factor-regulated biological processes. RTK-induced chemokine secretion is identified in tumor and untransformed cells, as both cell types invade tissues using identical molecular strategies. This leads us to propose that this growth factor-chemokine network represents a general mechanism of broad physiological significance.

Previous data suggested that RTK-induced GPCR transactivation was mediated exclusively through intracellular signals (Hallak *et al.*, 2000). Although constitutive $G\alpha_i$ binding to the IGF-1R has been reported, this association does not explain the mechanism by which RTK transactivates GPCR. Further, $G\alpha_i$ is not a substrate for IGF-1R kinase activity. Our data indicate that IGF-I-induced GPCR transactivation involves two transmembrane signaling events: first, growth factor-induced RTK activation triggers extracellular release of a GPCR ligand and secondly, the released ligand binds to and activates the GPCR in an autocrine/paracrine manner. A similar mechanism was recently proposed to explain RTK transactivation by GPCR receptors. GPCR-induced EGFR transactivation involves EGF precursor release from the membrane, which in turn binds to and activates the EGFR (Prenzel *et al.*, 1999). Taken together, these data indicate that crosstalk between RTK and GPCR uses an extracellular crosscommunication mechanism that may operate in both directions.

SPECULATION

The growth factor-induced CCR5 transactivation described here reveals a mechanism that links chronic inflammation and tumorigenesis. Our results in the CAM model suggest that CCR5 signals modulate the *in vivo* invasiveness of tumor cells. In addition, the upregulation of RANTES gene expression has been detected in invasive human breast carcinomas (Sgroi *et al.*, 1999). As constitutive growth factor secretion is observed in invasive tumor cells, it is tempting to speculate that this may increase proinflammatory chemokine concentration in the environment, providing the cells with a built-in invasive capacity. Comparative study of DU-145 and MCF-7 cells suggests a link between autocrine production of growth factors (IGF-I) and chemokines (RANTES), which correlates to their invasive capacity. Detailed study of this coordinated signaling circuit may thus provide insights into the onset of tumor invasion, as well as potential tools for clinical intervention.

METHODS

Cell culture. MCF-7 human breast carcinoma cells (ATCC, HTB-22) were maintained in MEM with 10% FCS, antibiotics and non-essential amino acids. Bovine aortic endothelial cells (BAEC), kindly provided by M. Grandi (Pharmacia, Nerviano, Italy), and DU-145 human prostate adenocarcinoma (ATCC, HTB-81) were cultured in DMEM with 10% FCS and antibiotics.

Immunoprecipitation, western blot and immunofluorescence. Serum-starved MCF-7 cells were stimulated with IGF-I (50 ng/ml, Pharmacia), RANTES or SDF-1 α (both 10 ng/ml; R&D Systems), lysed and CCR5, CXCR4 and IGF-1R were precipitated with specific mAb followed by agarose-anti-mouse IgG (Sigma). Tyrosine phosphorylated proteins were detected with 4G10 mAb (UBI), G α_i with C20, and the IGF-1R β -subunit with C20 (both from Santa Cruz). In some cases, anti-RANTES antagonist mAb (10 μ g/ml; R&D Systems) was added simultaneously with IGF-I. To block protein secretion, MCF-7 cells were incubated with monensin (0.5 μ M, 30 min, 37°C; Sigma), cells washed with serum-free medium, then IGF-I stimulated. Immunofluorescence analyses were as reported (Mañes *et al.*, 1999b) on cells stimulated with IGF-I (10 ng/ml) or SDF-1 α (10 ng/ml) (20 min, 37°C).

Estimation of RANTES protein levels. Chemokine levels in conditioned media or in RIPA cell lysates (50 μ g) from IGF-I- or serum-starved MCF-7 cells were assessed by specific ELISA (R&D Systems; BioSource).

Measurement of RANTES mRNA levels and transcription assays. MCF-7 cells were incubated with IGF-I (10 nM) alone or with actinomycin D (5 μ g/ml). mRNA coding for RANTES was specifically measured by semiquantitative PCR in a LightCycler System (Roche Diagnostics), RANTES mRNA levels were normalized with those for GAPDH mRNA.

For the IGF-I effect on the RANTES transcription rate, MCF-7 cells were transiently transfected with RANTES and MIP-1 β promoters (from W.J. Leonard, NIH, Bethesda, MD) and promoter-less (pEFBOS-luc from D. Wotton; University of Virginia, Charlottesville, VA) luciferase reporter gene constructs, and the activity measured using luciferase assay reagent (Promega) in a luminometer.

Cloning and viral transduction. The bicistronic retroviral vector pLZIRES-gfp was derived from pLZR-CMV-gfp (R. Delgado, Hospital 12 Octubre, Madrid, Spain). The KDELccr Δ 32 mutant, obtained from an hCCR5 cDNA clone, was subcloned in pLZIRES-gfp. Human RANTES was similarly cloned to obtain pLZ-RANTES-IRES-gfp. For viral transduction, MCF-7 or DU-145 (10⁶ cells) were incubated with retroviral pLZ-RANTES-IRES-gfp, pLZ-KDELccr Δ 32-IRES-gfp or pLZIRES-gfp (mock) supernatants in protamine sulphate (5 μ g/ml, 14 h, 37°C), and cells expressing GFP were selected 72 h after infection by fluorescence-activated cell sorting. Chemokine receptor levels in KDELccr Δ 32-transduced cells were evaluated by flow cytometry with anti-CCR2, -CCR5 (from M. Mellado and J.M. Rodríguez-Frade, DIO/CNB, Madrid, Spain) and -CXCR4 (R&D Systems).

Cell migration assays. MCF-7 and BAEC chemotaxis was performed as described (Mañes *et al.*, 1999b) using IGF-I (1 ng/ml), bFGF (50 ng/ml), RANTES (1–40 ng/ml), SDF-1 α (10–100 ng/ml), or combinations of these as chemoattractants. In some cases, cells were preincubated with neutralizing anti-RANTES, -SDF-1 α ,

-MCP-1, or -MIP-1 α/β mAb (all from R&D Systems) for 30 min before transwell seeding, or pretreated with Ptx (1 μ g/ml, 8 h, 37°C), which was removed before the chemotaxis assay.

Intravasation experiments were as described (Kim *et al.*, 1998) and human DNA present in the chicken CAM estimated by PCR in a LightCycler System with human Alu sequence-specific primers. A standard curve of serial dilutions of human DNA mixed with chicken DNA was obtained in each determination. The relative value of human DNA in CAM samples was calculated by the quantification software provided with the apparatus. The number of human cells in each DNA sample (30 ng) was calculated assuming 6 pg/cell of DNA.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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REFERENCES

- Benkirane, M., Jin, D., Chun, R., Koup, R. and Jeang, K. (1997) Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5 Δ 32. *J. Biol. Chem.*, **272**, 30603–30606.
- Hallak, H., Seiler, A., Green, J., Ross, B. and Rubin, R. (2000) Association of heterotrimeric G γ with the insulin-like growth factor-I receptor. Release of G $\beta\gamma$ subunits upon receptor activation. *J. Biol. Chem.*, **275**, 2255–2258.
- Kanzaki, M., Nie, L., Shibata, H. and Kojima, I. (1997) Activation of a calcium-permeable cation channel CD20 expressed in Balb/c 3T3 cells by insulin-like growth factor-I. *J. Biol. Chem.*, **272**, 4964–4969.
- Kim, J., Yu, W., Kovalski, K. and Ossowski, L. (1998) Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell*, **94**, 353–362.
- Liu, R. *et al.* (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell*, **86**, 367–377.
- Luttrell, L., van Biesen, T., Hawes, B., Koch, W., Touhara, K. and Lefkowitz, R. (1995) G $\beta\gamma$ subunits mediate mitogen-activated protein kinase activation by the tyrosine kinase insulin-like growth factor 1 receptor. *J. Biol. Chem.*, **270**, 16495–16498.
- Mañes, S., Llorente, M., Lacalle, R., Gómez-Moutón, C., Kremer, L., Mira, E. and Martínez-A., C. (1999a) The matrix metalloprotease-9 regulates the insulin-like growth factor-triggered autocrine response in DU-145 carcinoma cells. *J. Biol. Chem.*, **274**, 6935–6945.
- Mañes, S., Mira, E., Gómez-Moutón, C., Lacalle, R., Keller, P., Labrador, J. and Martínez-A., C. (1999b) Membrane raft microdomains mediate front-rear polarity in migrating cells. *EMBO J.*, **18**, 6211–6220.
- Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*, **402**, 884–888.
- Rao, G., Delafontaine, P. and Runge, M. (1995) Thrombin stimulates phosphorylation of insulin-like growth factor-1 receptor, insulin receptor

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- substrate-1 and phospholipase C- γ 2 in rat aortic smooth muscle cells. *J. Biol. Chem.*, **270**, 27871–27875.
- Sgroi, D., Teng, S., Robinson, G., LeVangie, R., Hudson, J. and Elkahouloun, A. (1999) *In vivo* gene expression profile analysis of human breast cancer progression. *Cancer Res.*, **59**, 5656–5661.
- Siebler, T., Kiess, W., Linder, B., Kessler, U., Schwarz, H. and Nissley, S. (1996) Pertussis toxin sensitive G-proteins are not involved in the mitogenic signaling pathway of insulin-like growth factor-I in normal rat kidney epithelial (NRKE) cells. *Regul. Pept.*, **62**, 65–71.
- Vila-Coro, A., Mellado, M., Martín de Ana, A., Martínez-A., C. and Rodríguez-Frade, J. (1999) Characterization of RANTES- and (AOP)-RANTES-triggered desensitization signals reveals differences in recruitment of the GPCR complex. *J. Immunol.*, **163**, 3037–3044.

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