Formation of Signal Transfer Complexes between Stem Cell and Platelet-Derived Growth Factor Receptors and SH2 Domain Proteins *in Vitro*†

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ABSTRACT: Cellular growth and differentiation signals are generated and defined by the interaction of specific phosphotyrosine residues of activated receptor tyrosine kinases (RTKs) and src homology-2 (SH2) domain-containing intracellular signal transducers. This appears to involve for both the p145$^{c\text{-}kit}$ and β platelet-derived growth factor receptor (PDGF-R) cytoplasmic domains the formation of multiprotein signal transfer complexes, which include combinations of noncatalytic and enzymatically active subunits of phosphatidylinositol 3′-kinase (PI3′-K), phospholipase C-γ (PLCγ), and guanosine trisphosphatase activating protein (GAP). In vitro association experiments indicate that PLCγ and PI3′-K bind the βPDGF-R simultaneously, while these two SH2 proteins compete for association to p145$^{c\text{-}kit}$ binding sites, with p85/PI3′-K exhibiting higher affinity. Interestingly, GAP and p85/PI3′-K binding to distinct p145$^{c\text{-}kit}$ phosphotyrosines is cooperative, enhancing formation of a heterotetrameric signaling complex, which may include different combinations of p85α and p85β with p110, p112, and p116 by interaction with the same tyrosine 721 docking site. The diversity of molecular interactions observed for PDGF-R and p145$^{c\text{-}kit}$ suggests a new mode of signal definition and modulation.

Regulation of cell growth and differentiation involves the cell surface interaction of peptide growth factors with a family of transmembrane receptors that are characterized by their intrinsic tyrosine kinase activity [reviewed in Ullrich and Schlessinger (1990)]. Binding of a ligand to the extracellular domain of its cognate receptor tyrosine kinase (RTK) leads to dimerization of receptor molecules, activation of the kinase, and phosphorylation of the receptor cytoplasmic domains on specific tyrosine residues (Yarden & Ullrich, 1988; Ullrich & Schlessinger, 1990). Transmission of the mitogenic stimulus from the cell membrane to the nucleus is accomplished by a signaling cascade that is triggered by the interaction of the activated, autophosphorylated RTK with a specific subset of cellular proteins (Cantley et al., 1991), which as a consequence may become phosphorylated on tyrosines.

A number of such substrates have been identified. They include the guanosine trisphosphatase activating protein (GAP) and two GAP-associated proteins, p190 and p62 (Molloy et al., 1989; Ellis et al., 1990; Kaplan et al., 1990; Kazlauskas & Cooper, 1990; Kazlauskas et al., 1990; Reedijk et al., 1990; Heidaran et al., 1991; Moran et al., 1991), phospholipase Cγ (PLCγ) (Wahl et al., 1988, 1989; Margolis et al., 1989; Meisenhelder et al., 1989; Nishibe et al., 1989; Rhee et al., 1989), the proto-oncogene product *vav* (Margolis et al., 1992), p85, the noncatalytic subunit of phosphatidylinositol 3′-kinase (PI3′-K) (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991; Herbst et al., 1992), GRB-2, the mammalian homologue of *Caenorhabditis elegans* Sem-5, which links RTKs to the ras signaling pathway (Clark et al., 1992; Lowenstein et al., 1992; Matuoko et al., 1992; Skolnik et al., 1993a,b), as well as the adaptor protein Nck (Lehmann et al., 1990; Li et al., 1992; Meisenhelder & Hunter, 1992; Park & Rhee, 1992). All of these proteins are characterized by a noncatalytic domain of ~100 amino acids, known as the src homology 2 (SH2) domain (Koch et al., 1991). The association of these proteins with RTKs is mediated by binding of the substrate SH2 domain(s) to phosphorylated tyrosine residues within the intracellular domain of the receptor (Kazlauskas & Cooper, 1990; Anderson et al., 1990; Moran et al., 1990; Matsuda et al., 1991; Hu et al., 1992; Klippel et al., 1992; McGlade et al., 1992). Recently, it has been demonstrated that the SH2 domains of individual substrates bind to specific phosphotyrosine residues and the specificity of these interactions are defined by short flanking sequences (Downing et al., 1991; Yu et al., 1991; Kashishian et al., 1992; Reedijk et al., 1992; Rotin et al., 1992).

The proto-oncogene c-*kit* (Besmer et al., 1986; Yarden et al., 1987; Qiu et al., 1988), which maps to the murine Dominant White Spotting (*W*) locus (Chabot et al., 1988; Geissler et al., 1988; Nocka et al., 1989), encodes an RTK that is activated by the soluble or membrane-bound stem cell factor (SCF) (Anderson et al., 1990; Huang et al., 1990; Martin et al., 1990; Nocka et al., 1990; Williams et al., 1990; Zsebo et al., 1990a,b). Mutations of the *W* locus have pleiotropic effects on the differentiation of hematopoietic, melanocyte, and germ cell lineages (Russell, 1979; Geissler et al., 1981). The c-*kit* gene product, p145$^{c\text{-}kit}$, is structurally related to the α- and β-platelet-derived growth factor receptors (α-,β-PDGF-R) (Yarden et al., 1986; Claesson-Welsh et al., 1989) and the macrophage colony-stimulating factor receptor (CSF-1-R) (Sherr et al., 1985), which together

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constitute RTK subclass III (Yarden & Ullrich, 1988). The common features of these receptors are an immunoglobulin-like distribution of cysteine residues within the extracellular ligand binding domain and a hydrophobic insertion sequence (KI) within their intracellular tyrosine kinase domains [reviewed in Ullrich and Schlessinger (1990)]. The signaling pathways engaged by p145$^{c\text{-}kit}$ in different cell types are only now beginning to be elucidated.

Using transient expression systems (Herbst et al., 1991, 1992; Reith et al., 1991) as well as stable cell lines (Lev et al., 1991), activated p145$^{c\text{-}kit}$ was shown to interact with PLCγ, p85/PI3′-K, and Raf-1. In these systems both p85 and PLCγ were found to be phosphorylated on tyrosine residues in a ligand-dependent manner. Interaction of p145$^{c\text{-}kit}$ with PI3′-K and PLCγ and activation of p21^{ras} in response to SCF has been observed in various mast cell lines (Rottapel et al., 1991; Duronio et al., 1992). In comparison with PLCγ and p85, GAP appears to be a much weaker substrate of the p145$^{c\text{-}kit}$ RTK. Ligand-dependent phosphorylation of GAP and GAP-associated proteins on tyrosine residues was only observed when both receptor and substrate were coexpressed in a transient expression system (Herbst et al., 1991) and in the myeloid cell line MO7e (Miyazawa et al., 1991).

Recent studies in various cell systems have yielded divergent results regarding p145$^{c\text{-}kit}$ substrate association and phosphorylation (Herbst et al., 1991; Lev et al., 1991; Rottapel et al., 1991). While this may reflect, in part, differences in experimental parameters, it could reflect functionally relevant variations in substrate expression profiles of individual cell types, which may be the basis of distinct signals and cell type-specific responses mediated by the same ligand/receptor system.

To further characterize the parameters that are involved in the definition of cell type-specific p145$^{c\text{-}kit}$ signals, we investigated the influence of different substrates on each other's binding capacity to the activated kinase of p145$^{c\text{-}kit}$. We also addressed the question of whether two or even more proteins could bind simultaneously to the same receptor molecule, forming a signal transfer complex (Ullrich & Schlessinger, 1990). For this purpose we performed *in vitro* association experiments, using as a target for substrate binding the chimeric receptor EK-R, which is composed of the extracellular ligand binding domain of the human epidermal growth factor (EGF) receptor and the transmembrane and tyrosine kinase domains of human p145$^{c\text{-}kit}$ (Herbst et al., 1991, 1992; Shearman et al., 1993).

MATERIALS AND METHODS

Construction of Expression Plasmids. The chimeric receptor EK-R, consisting of the extracellular domain of the human EGF-R and the transmembrane and intracellular domains of the human p145$^{c\text{-}kit}$, was constructed as described previously (Herbst et al., 1991). For transient expression, the cDNAs coding for the human homologues of GAP, p85α, and PLCγ, as well as for the chimeric construct EK-R, were subcloned in the cytomegalovirus promoter-based expression plasmid pRK5 (Herbst et al., 1991, 1992).

To mutate codons for individual Tyr residues to Phe codons, an *ApaI*/*Bst*EII cut cDNA fragment of human c-*kit* was subcloned into the *ApaI*/*Sph*I cut phage vector M13 BM20 (Boehringer Mannheim), with the *Bst*EII and *Sph*I sites made blunt-ended with T4-DNA polymerase prior to ligation. Site-directed mutagenesis was carried out according to the protocol of Kunkel et al. (1987) with the following oligonucleotides: 5′-CTA AGT TGG AGA AAA TAT GAT TGG T-3′ to exchange the codon for Tyr 936 to Phe; 5′-TTC ATG TCC ATA AAC TCA TTA GTA-3′ to exchange the codon for Tyr 721 to Phe; 5′-TGG GAC AAC GAA AGA AAC TCC A-3′ to exchange the codon for Tyr 730 to Phe; and 5′-CTC TTT CTA TAA ATG AGC CTA TT-3′ to exchange the codon for Tyr 747 to Phe (positions of amino acid residues refers to the primary structure of p145$^{c\text{-}kit}$). After the mutations were verified by sequencing, the mutated fragments were cloned into the EK-R background.

Transient Expression. Human embryonic kidney fibroblasts (293; ATCC CRL 1573) were routinely cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 μg/mL penicillin, 50 mg/mL streptomycin (all Gibco BRL), and 0.45% glucose. One day prior to transfection, 293 cells were seeded into 10-cm dishes (1.0×10^6 cells per dish). Transfections were then carried out using the calcium phosphate coprecipitation technique, according to the protocol of Chen and Okayama (1987), with a total of 16 μg of CsCl gradient-purified plasmid DNA per dish. Eighteen hours after addition of precipitates, cells were washed once with medium, and then 5 mL fresh medium containing 0.5% FCS was added.

For metabolic labeling of proteins, transfected as well as untransfected 293 cells were grown overnight in methionine-free DMEM containing 1.0% dialyzed FCS and [^{35}S]-methionine (50 μCi/mL; Amersham).

Cell Lysis and Immunoprecipitation. EK-R-expressing 293 cells were stimulated with 150 ng/mL recombinant human EGF (Intergen) or vehicle for 8 min at 37 °C prior to lysis. Cells were lysed on ice with 0.3 mL "lysis" buffer (50 mM HEPES, pH 7.5, containing 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM *p*-nitrophenyl phosphate, 1 mM $MnCl_2$). Lysates were transferred to microfuge tubes, mixed vigorously for 10 s, incubated for 20 min on ice, and then centrifuged at 12500*g* for 15 min at 4 °C.

For immunoprecipitations, receptor and substrate containing lysates were combined, the appropriate antiserum and 20 μL of protein A-Sepharose (Pharmacia; prewashed in 20 mM HEPES, pH 7.5, 1 mg/10 μL) was added, and the samples were incubated for 4 h at 4 °C on a slowly rotating wheel. Unless otherwise indicated in the figure legends, the lysate of one confluent dish of receptor-expressing 293 cells was used per sample in standard association experiments. The relative amounts of substrates added in each experiment are given in the figures, where a relative amount of 1.0 corresponds to the lysate of one confluent 10-cm dish of transiently transfected 293 cells. For all experiments, the final volumes of the samples were made equal by addition of lysis buffer. Immunoprecipitates were washed with 3 × 1 mL of "washing" buffer (20 mM HEPES, pH 7.5, containing 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM EGTA, 1 mM sodium orthovanadate, and 0.5 mM *p*-nitrophenyl phosphate). Sample buffer containing SDS and 2-mercaptoethanol was added, and the samples were denatured by heating at 95 °C for 3 min.

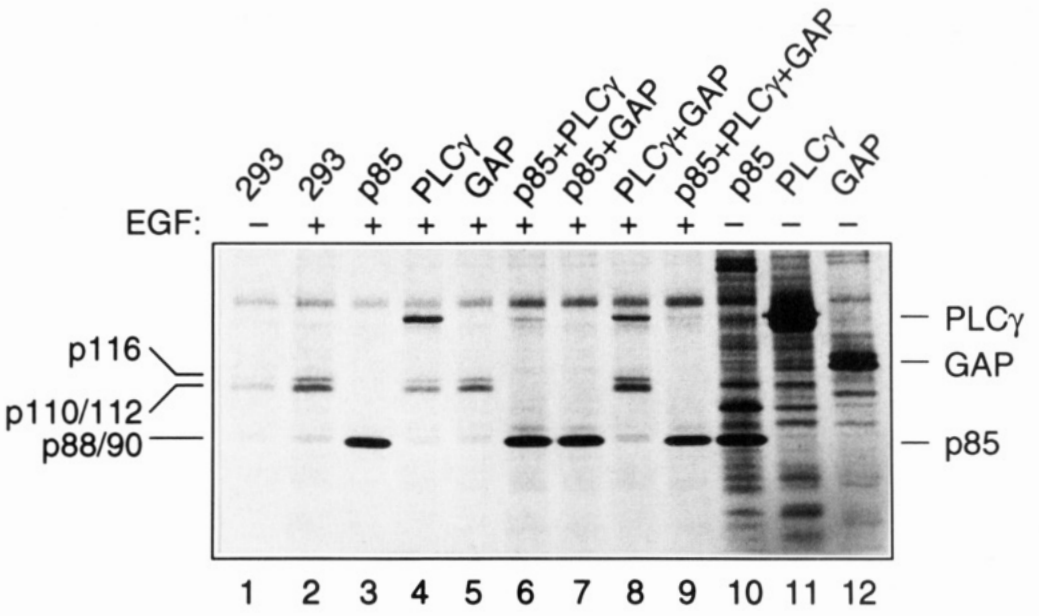

FIGURE 1: Ligand-stimulated association of metabolically radiolabeled cellular proteins and overexpressed substrates to EK-R. 293 cells expressing transiently EK-R were stimulated with EGF (150 ng/mL) or vehicle and then lysed. Receptor lysates were combined with lysates of metabolically radiolabeled cellular proteins from untransfected 293 cells (293) or from 293 cells transfected with p85, PLCγ, or GAP expression plasmids, respectively. Samples were immunoprecipitated with mAb 108.1 (αEGF-R; lanes 1−9). As controls, p85, PLCγ and GAP were directly immunoprecipitated from lysates prepared from 293 cells transiently overexpressing the individual proteins using the corresponding polyclonal antisera (CT-p85α, CT-PLCγ, CT-GAP; lanes 10−12) to quantitate the total amount of each of the substrates added to the receptor lysate. Samples were subjected to 7.0% SDS−PAGE, and the gel was dried and quantitated by autoradiography.

Autoradiography and Immunoblotting. Proteins were fractionated by 7.0% SDS−PAGE, and the gels were either processed for autoradiography or electrophoretically transferred to nitrocellulose filters. For autoradiographic analysis, gels were fixed in 30% methanol/10% acetic acid, stained with Coomassie G250 in 10% acetic acid, destained with 10% acetic acid, and dried before exposure to X-ray film. For immunoblotting analysis, nitrocellulose filters were first incubated with a 5% milk powder solution in TBST (20 mM Tris, pH 7.5, containing 150 mM NaCl and 0.02% Tween 20), incubated for 2 h at room temperature with the primary antibody, washed three times with TBST, and then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies. Immunoreactive proteins were visualized by using a horseradish peroxidase-catalyzed chemiluminescence reaction (ECL, Amersham).

Phosphatidylinositol 3′-Kinase Assay. The assay of PI3′-K activity was performed essentially as described by Whitman et al. (1985). To the immunoprecipitates (10 μL pellet of protein A-Sepharose beads) was added, in a final assay volume of 50 μL, 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM *p*-nitrophenyl phosphate, 0.2 mM adenosine, 0.5 mg/mL L-α-phosphatidylinositol, 0.5 mg/mL L-α-phosphatidylserine, and 50 μM [γ-^{32}P]ATP (800 cpm/pmol). Incubations were carried out at 30 °C for 15 min. The solvent-extracted phospholipids were either washed twice with a 1:1 mixture of methanol/1 N HCl and the radiolabel incorporated into phospholipids quantitated by Cerenkov counting, or applied to silica gel plates (Kieselgel 60W, E. Merck & Co., FRG) and developed in chloroform/methanol/acetone/glacial acetic acid/H_2O (ratio by vol. 40:13:15:12:7).

Antibodies. The following monoclonal antibodies and polyclonal antisera were used: 108.1 (Honegger et al., 1989), a mouse monoclonal antibody against the extracellular domain of the EGF-R; CT-p85α, rabbit polyclonal antiserum against a peptide corresponding to the C-terminal 15 amino acids of the human noncatalytic subunit of PI3-kinase, p85α (CNVTLAYPVYAQQRR); 51-4, rabbit polyclonal antiserum against a peptide corresponding to amino acid residues 513−531 of human p85α (Hu et al., 1992); CT-PLCγ, rabbit polyclonal antiserum against a peptide corresponding to amino acid residues 1255−1274 of human PLCγ (DFRISQEHLADHFDSRERR); CT-GAP, rabbit polyclonal antiserum against a peptide corresponding to the C-terminal 14 amino acid residues of human GAP (QQKQNQYTKTNDVR); N13, a mouse monoclonal antibody raised against bovine p85α; N15, a mouse monoclonal antibody raised against bovine p85β.

RESULTS

Ligand-Dependent Protein Association with EK-R. To analyze the association of endogenous and transfected substrates with the kinase domain of p145$^{c\text{-}kit}$, the chimeric receptor EK-R, consisting of the human EGF-R extracellular domain and the human p145$^{c\text{-}kit}$ transmembrane and intracellular domains, was transiently overexpressed in the embryonic kidney fibroblast 293 cell line. Cell lysates prepared from receptor-expressing cells were then combined with lysates of metabolically [^{35}S]methionine-radiolabeled 293 cells, which were either untransfected or transfected with expression plasmids for PLCγ, p85α, and GAP. The samples were subjected to immunoprecipitation with mAb 108.1 against the EGF-R extracellular domain and analyzed by SDS−PAGE and autoradiography.

As reported previously (Shearman et al., 1993), five proteins of apparent molecular masses of 88, 90, 110, 112, and 116 kDa were found to specifically associate with ligand-activated EK-R (Figure 1, lanes 1 and 2). When lysate containing overexpressed p85α was added to the receptor, p85α was bound almost quantitatively to EK-R (Figure 1, lanes 3 and 10). In comparison with p85α, the binding of

PLCγ was much less efficient but still clearly detectable (Figure 1, lanes 4 and 11), while GAP association with EK-R was either not detectable under our assay conditions or the GAP binding site was already occupied in the unstimulated EK-R (Figure 1, lanes 5 and 12). Simultaneous addition of PLCγ and p85α lysates led to a dramatic decrease in receptor-bound PLCγ (lane 6), whereas the amount of p85α bound to the receptor was unchanged. Addition of GAP further decreased PLCγ to barely detectable levels (lane 9). Interestingly, even though GAP association was not detected, combination of this substrate with PLCγ resulted in significantly reduced p145 PLCγ coprecipitation (Figure 1, lane 8). Furthermore, the binding of endogenous 293 cell proteins of 110, 112, and 116 kDa to EK-R was clearly influenced by addition of p85α-overexpressing cell lysates. All samples containing excess p85α exhibited a displacement of p110, p112, and p116 from the receptor. In a similar experiment, addition of unlabeled p85α displaced p88, p90, p110, p112, and p116 together with receptor-associated PI3′-K activity from activated EK-R (not shown).

p85α and p85β of PI3′-K both Interact with p145$^{c\text{-}kit}$ and βPDGF-R Cytoplasmic Domains. In addition to the endogenous proteins p110, p112, and p116, a protein doublet of 88 and 90 kDa was found to be associated with EK-R (Figure 1, lanes 2, 4, 5, and 8). While p88 was identified as the human homolog of bovine p85α (Shearman et al., 1993), we speculated that p90 could represent the human protein corresponding to bovine p85β, which, although somewhat smaller by calculated molecular mass, displays SDS−PAGE migration properties indicating an apparent MW of ~2000 higher than p85α (Ivan Gout, personal communication).

To examine whether both p85α and p85β can associate with the p145$^{c\text{-}kit}$ and βPDGF-R kinase domains, ligand-dependent binding of p85α and p85β to EK-R and EP-R, a chimeric receptor consisting of the human EGF-R extracellular domain and the human βPDGF-R kinase domain (Seedorf et al., 1992), was analyzed by immunoblotting (Figure 2). Upon ligand stimulation, both receptors were phosphorylated on tyrosine residues, resulting in comparable signals in an antiphosphotyrosine immunoblot (Figure 2, αPY). While endogenous PLCγ, which was monitored as a control, associated only with EP-R, p85α and p85β were found to associate with both receptors, as determined by immunoblotting with specific antibodies. p85α and p85β migrated at the expected apparent MWs of 88 000 and 90 000, respectively. Consistent with the results obtained with metabolically radiolabeled cell lysates (Shearman et al., 1993; and Figure 1), the signals obtained for the two proteins were of similar intensity.

p85α Displaces PLCγ from EK-R. The finding that under identical conditions EK-R bound only p85 from 293 cell lysates while EP-R associated with both p85 and PLCγ (Figure 2) suggested that each molecule of EK-R was only able to bind one of the two substrates at a time, whereas both proteins might simultaneously bind to the kinase of βPDGF-R. To test this possibility, an equilibrium binding experiment was performed in which both EK-R and EP-R were incubated with saturating concentrations of p85α and increasing concentrations of PLCγ (Figure 3). In the absence of PLCγ, both receptors bound exogenous p85α in a ligand-dependent manner (Figure 3, lanes 1, 2, 7, and 8). A 2-fold increase in the concentration of p85α in the reaction mixture did not result in an increase in p85α signal intensity (lanes 3 and 9), indicating that the binding sites of both receptors were saturated under these conditions. Addition of increasing amounts of exogenous PLCγ to the receptors (lanes 4−6 and 10−12), competed somewhat with p85α binding to EK-R (lanes 5 and 6). This led to a slight reduction in receptor-associated p85α when the maximal amount of PLCγ was added (see also lanes 13 and 14 for substrate controls). In contrast, under the same experimental conditions, binding of PLCγ to EP-R was approximately 10-fold more efficient without significantly affecting p85α association to the receptor.

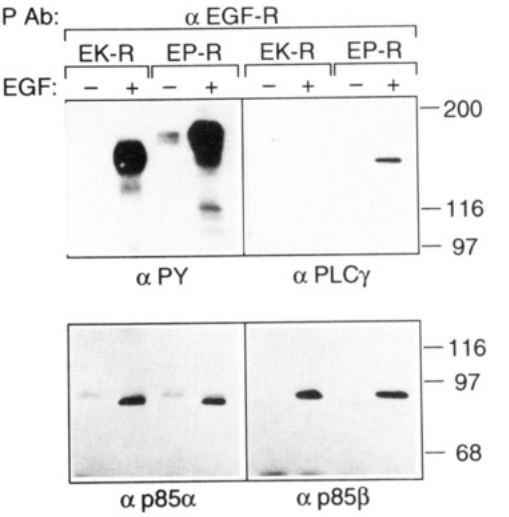

FIGURE 2: Ligand-stimulated binding of p85α and p85β to the kinase domains of p145$^{c\text{-}kit}$ and βPDGF-R. 293 cells expressing transiently EK-R or EP-R were stimulated with EGF (150 ng/mL) or vehicle and lysed. Receptor lysates were combined with lysate from untransfected 293 cells. For both receptors, four parallel samples for unstimulated and stimulated receptors were prepared. The samples were subjected to immunoprecipitation with mAb 108.1 (IP Ab.: αEGF-R), and precipitated proteins were separated by SDS−PAGE and electrophoretically transferred to nitrocellulose. The blot was cut into four strips and each strip individually probed with one of the following Abs: mAb 5E2 (αPY), CT-PLCγ (αPLCγ), mAb NI3 (αp85α), and mAb N15 (αp85β). Immunoreactive proteins were visualized by a horseradish peroxidase-catalyzed chemiluminescence reaction.

To investigate the interaction of PLCγ, p85α, and EK-R in more detail, we performed binding competition experiments by combining constant amounts of cold and [^{35}S]-methionine-labeled EK-R and PLCγ or p85α overexpressing 293 cell lysates, respectively, and increasing volumes of unlabeled cell lysates overexpressing either one of these substrates (Figure 4A,B). In the absence of overexpressed p85α, PLCγ specifically bound to the activated receptor (Figure 4A, lanes 1 and 2). The extent of PLCγ binding was dramatically reduced in a dose-dependent manner when lysate containing p85α was added, resulting in almost complete displacement of PLCγ. In contrast, PLCγ was not able to compete with the binding of p85α to the activated receptor, even when an excess of PLCγ was used (Figure 4B).

Simultaneous Association of GAP and p85α/PI3′-K with Activated EK-R. As shown in Figure 1, metabolic radiolabeling of proteins with [^{35}S]methionine was not sensitive enough to detect binding of GAP to activated EK-R. We therefore used a specific antiserum and immunoblotting analysis to detect GAP association (Figure 5). Furthermore, we determined if the binding of GAP is influenced by p85α.

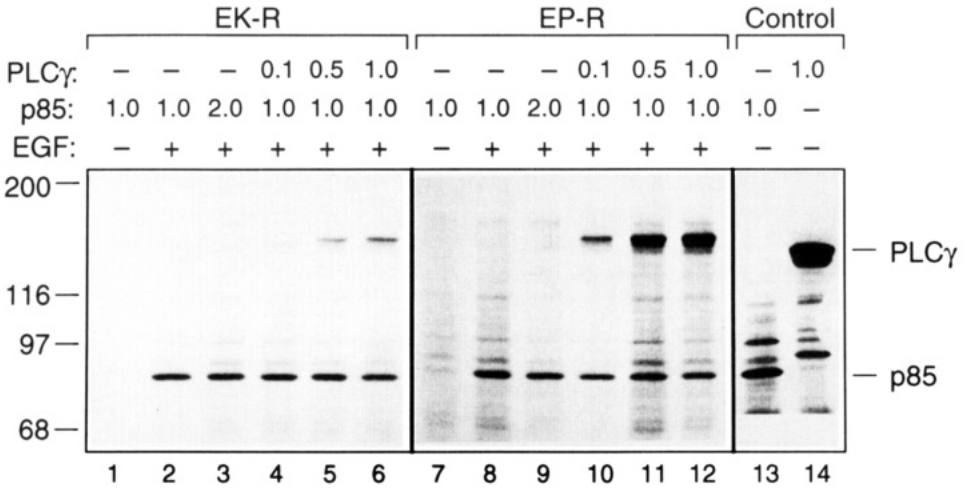

FIGURE 3: Competition binding of p85α and PLCγ to EK-R and EP-R. 293 cells expressing transiently EK-R or EP-R were stimulated with EGF (150 ng/mL) or vehicle and lysed. Receptor lysates were combined with lysates of metabolically radiolabeled proteins from 293 cells transiently expressing p85α or PLCγ. The relative amount of substrate lysate added to each sample is indicated. Samples were subjected to immunoprecipitation with mAb 108.1 (αEGF-R; lanes 1−12) and precipitated proteins separated by SDS−PAGE. As controls, p85α and PLCγ lysates were directly subjected to immunoprecipitation with the polyclonal antisera CT-p85α (lane 13) and CT-PLCγ (lane 14), respectively. The gel was dried and quantitated by autoradiography.

Lysates from ligand-stimulated or unstimulated cells overexpressing EK-R were combined with lysate from untransfected 293 cells (Figure 5, lanes 1 and 2) or from cells overexpressing GAP, either alone (Figure 5, lanes 3 and 4) or together with lysate from cells overexpressing p85α (Figure 5, lane 5). Samples were subjected to immunoprecipitation with mAb 108.1, and precipitated proteins were then separated by SDS−PAGE and electrophoretically transferred to nitrocellulose. The blot was first probed with the polyclonal antiserum CT-GAP (Figure 5, upper panel) and then reprobed with the polyclonal antiserum CT-p85α (Figure 5, lower panel). Immunoprecipitation of EK-R from samples containing overexpressed GAP revealed ligand-dependent association of GAP to the receptor (Figure 5, upper panel, lanes 3 and 4). When compared to the total amount of GAP present in the sample (lane 7), only a minor portion of GAP appeared to bind to activated EK-R, indicating a low affinity of this substrate for the p145$^{c\text{-}kit}$ cytoplasmic domain. However, the binding of GAP to the receptor was increased by approximately 2-fold when an aliquot of lysate containing overexpressed p85α was included in the sample (lane 5). Reprobing the blots with antiserum directed against p85α revealed binding of endogenous p85α to EK-R when lysate from untransfected cells (lane 2) or from cells overexpressing GAP (lane 4) were used, in which case p85α binding was approximately three times more efficient.

Taken together, our results show that GAP binds to EK-R in a ligand-dependent manner and that p85α does not displace GAP from the receptor. On the contrary, p85α and GAP binding to EK-R appears to be cooperative.

Cooperative and Competitive Interaction of Substrates with EK-R. To quantitate the influence of GAP, PLCγ, and p85α on the binding of PI3′-K to the chimeric receptor, PI3′-K assays were performed on the immunoprecipitated samples. Cell lysates containing ligand-activated EK-R were combined with lysate prepared from 293 cells transfected with PLCγ, p85α, and GAP expression plasmids. After immunoprecipitation with mAb 108.1, receptor-associated PI3′-K activity was quantitated as described in Materials and Methods. Lysates of substrate-expressing cells were added to the

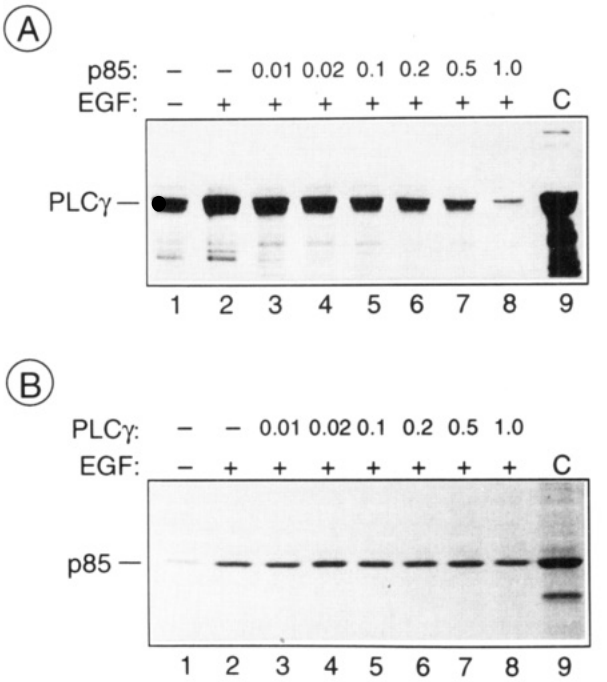

FIGURE 4: Competition binding of p85α and PLCγ to EK-R. 293 cells expressing transiently EK-R were stimulated with EGF (150 ng/mL) or vehicle and then lysed. Receptor lysates were added to a combination of metabolically radiolabeled and unlabeled lysates from 293 cells expressing transiently PLCγ or p85α. Samples were immunoprecipitated with mAb 108.1 (IP Ab: αEGF-R), subjected to SDS−PAGE, and the gel was dried and quantitated by autoradiography. **(A)** Dose-dependent displacement of PLCγ binding with p85α. Receptor lysates were combined with lysates of metabolically radiolabeled proteins from 293 cells transiently expressing PLCγ. The control (C; IP Ab: αPLCγ) shows the total amount of PLCγ added to each sample (relative amount = 1.0). To these samples either buffer alone or increasing volumes of unlabeled lysates from 293 cells transiently expressing p85α was added (relative amount = 0.01−1.0). **(B)** Dose-dependent displacement of p85α binding with PLCγ. Receptor lysates were combined with lysates of metabolically radiolabeled proteins from 293 cells transiently expressing p85α. The control (C; IP Ab: αp85) shows the total amount of p85α added to each sample (relative amount = 1.0). To these samples either buffer alone or increasing volumes of lysates from 293 cells transiently expressing PLCγ was added (relative amount = 0.01 to 1.0).

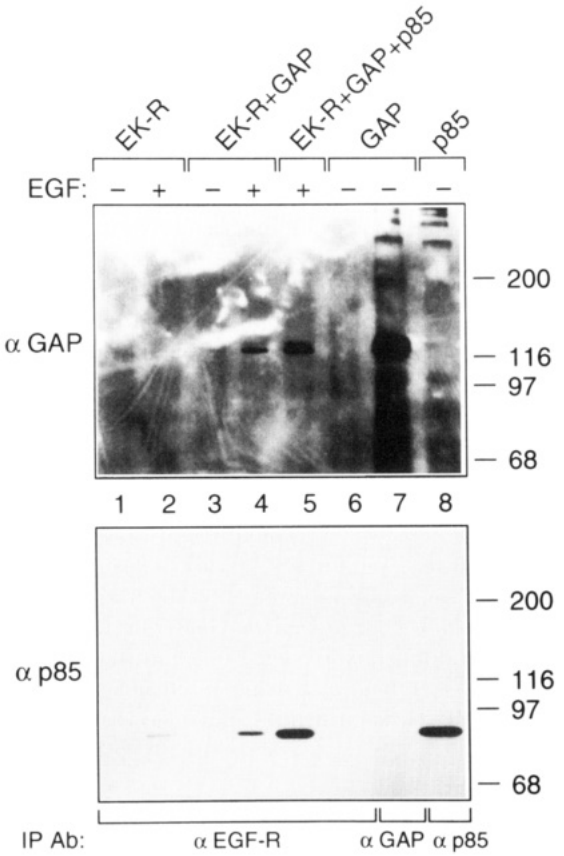

FIGURE 5: *In vitro* ligand-dependent association of GAP with EK-R. 293 cells expressing transiently EK-R were stimulated with EGF (150 ng/mL) or vehicle and then lysed. Receptor lysates were combined with lysate from untransfected 293 cells or from 293 cells transiently expressing GAP or p85α, as indicated. Samples were then subjected to immunoprecipitation with mAb 108.1 (IP Ab: αEGF-R, lanes 1−5). As controls, GAP and p85α were directly immunoprecipitated with the indicated antibodies from lysates of 293 cells transiently overexpressing the respective proteins (lanes 6−8). Immunoprecipitated proteins were subjected to SDS−PAGE and electrophoretically transferred to nitrocellulose. The blot was first probed with the polyclonal antiserum directed against GAP (CT-GAP; blot: αGAP) and reprobed with the polyclonal antiserum directed against p85α (CT-p85α; blot: αp85). Immunoreactive proteins were visualized by a horseradish peroxidase-catalyzed chemiluminescence reaction.

receptor lysate at two different amounts (i.e., 33 and 330 μg of total protein for each of the lysates) to monitor dose-dependent effects. Lysates from 293 cells transfected with the expression plasmid pRK5 were used as a control.

The binding of PI3′-K to EK-R is ligand-dependent (Figure 6). When compared to the stimulated receptor, unstimulated EK-R bound only 15% of PI3′-K activity. As expected, overexpressed p85α effectively competed with endogenous PI3′-K for the binding to the receptor. Low levels of p85α were sufficient to reduce receptor bound PI3′-K activity to 8% of the control (Figure 6). A 10-fold increase of p85α led to a further reduction of PI3′-K activity. Consistent with the results of the displacement experiment shown in Figure 4B, PLCγ had little effect on receptor-associated PI3′-K activity. In contrast to p85α, addition of GAP lysate resulted in a measurable increase in PI3′-K activity: 18% and 25% over control for 33 and 330 μg of total protein added, respectively (Figure 6).

Both p85α and p85β Bind to Tyr 721 of p145$^{c\text{-}kit}$. As shown in Figure 4, p85α efficiently blocked the binding of PLCγ to activated EK-R in an equilibrium binding assay.

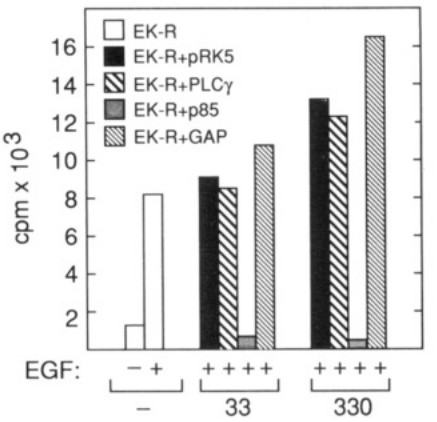

FIGURE 6: Ligand-stimulated association of phosphatidylinositol 3′-kinase activity with EK-R and its modulation by exogenous PLCγ, p85α, and GAP. 293 cells expressing transiently EK-R were stimulated with EGF (150 ng/mL) or vehicle and then lysed. For each sample, receptor lysate equivalent to 600 μg of total protein was combined with 600 μg of total protein of lysate from untransfected 293 cells. Lysate from 293 cells transiently expressing either PLCγ, p85α, or GAP or from pRK5-transfected control cells was added as indicated (33 or 330 μg of total protein for each of the lysates). Samples were subjected to immunoprecipitation with mAb 108.1 (αEGF-R) and receptor-associated PI3'-K activity was assayed as described in Materials and Methods. The radiolabel content of the phospholipids was quantitated by Cerenkov counting. Numbers given are mean values of duplicate samples. The data are representative of three individual experiments.

p85α and PLCγ may therefore either compete for the same binding site on the receptor, or the effect is a consequence of a steric hindrance in combination with a much higher affinity of p85α for the receptor. In order to identify the binding sites for p85 and PLCγ, the three Tyr residues in the kinase insert region of p145$^{c\text{-}kit}$ (Y721, Y730, Y747), as well as the C-terminal Tyr residue, Y936, were individually replaced with Phe residues by site-directed mutagenesis. All mutant receptors were constructed in the EK-R background.

In a first experiment, the pattern of metabolically radiolabeled proteins from 293 cells associating with EK-R and mutant receptors was compared. As shown in Figure 7, p88 (p85α) and p90 (p85β) as well as p110, p112, and p116 efficiently bound to ligand-activated EK-R and the mutant receptors EK-Y730F, EK-Y797F, and EK-Y936F. Only EK-Y721F failed to associate with p88 and p90. In addition, mutation of Y721 to Phe completely abolished the binding of p110, p112, and p116. We conclude that the human homologues of p85α and p85β, and the as yet unidentified proteins p110, p112, and p116 all bind via Y721 to p145$^{c\text{-}kit}$.

Since endogenous PLCγ does not bind to detectable levels to EK-R, overexpressed PLCγ was used to compare its binding to EK-R and the mutant receptors. Figure 8 shows the result of an association experiment in which receptor-associated PLCγ was detected by immunoblotting (upper panel, αPLCγ). PLCγ bound with comparable affinity to both EK-R and EK-Y721F. A slight reduction in receptor-associated PLCγ was detected for EK-Y730F and EK-Y747F, with the latter mutant exhibiting enhanced basal activation. The strongest effect was observed with EK-Y936F, where the binding of PLCγ was significantly reduced, although not completely abolished. Reprobing the blot with the polyclonal antiserum LJ11, directed against the C-terminus of p145$^{c\text{-}kit}$, confirmed that all receptors were expressed at comparable levels.

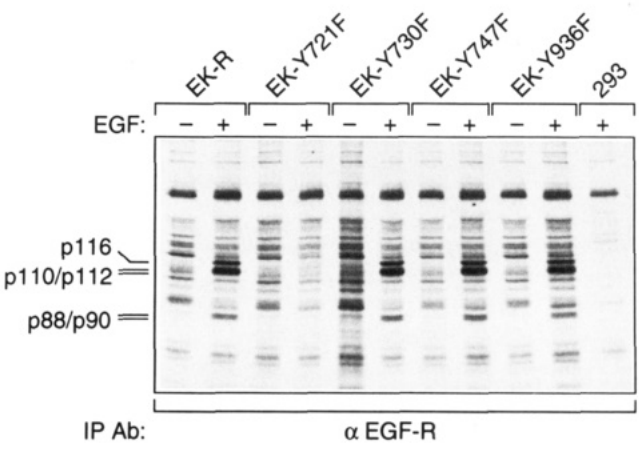

FIGURE 7: Association of metabolically radiolabeled cellular proteins with EK-R and tyrosine mutant receptors. 293 cells expressing transiently EK-R or tyrosine mutant receptors (EK-Y721F, EK-Y730F, EK-Y747F, EK-Y936F) were stimulated with EGF (150 ng/mL) or vehicle and lysed. Receptor lysates were combined with lysates of metabolically radiolabeled cellular proteins from untransfected 293 cells and subjected to immunoprecipitation with mAb 108.1 (IP Ab: αEGF-R). As a control, radiolabeled 293 cell lysate was directly immunoprecipitated with mAb 108.1 (293). Precipitated proteins were separated by SDS−PAGE, and the gel was dried and quantitated by autoradiography.

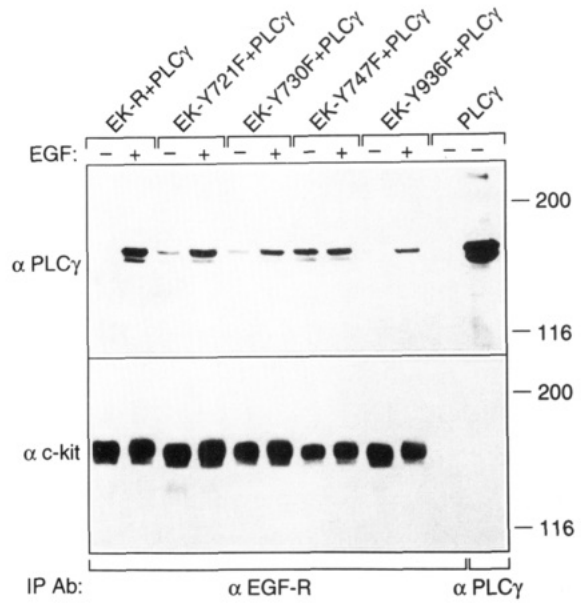

FIGURE 8: *In vitro* association of endogenous PLCγ with EK-R and tyrosine mutant receptors. 293 cells transiently expressing EK-R or tyrosine mutant receptors (EK-Y721F, EK-Y730F, EK-Y747F, EK-Y936F) were stimulated with EGF (150 ng/mL) or vehicle and lysed. Receptor lysates were combined with lysate from 293 cells transiently expressing PLCγ and subjected to immunoprecipitation with mAb 108.1 (IP Ab: αEGF-R). As controls, PLCγ lysate was directly immunoprecipitated with mAb 108.1 or with the polyclonal antiserum CT-PLCγ (PLCγ; IP Ab: αPLCγ). Precipitated proteins were separated by SDS−PAGE and electrophoretically transferred to nitrocellulose. The blot was first probed with the polyclonal antiserum CT-PLCγ (αPLCγ, upper panel) and then reprobed with the polyclonal antiserum LJ11 (αc-kit, lower panel). Immunoreactive proteins were visualized by a horseradish peroxidase-coupled chemiluminescence reaction.

DISCUSSION

Currently, little information is available regarding the intracellular events after RTK autophosphorylation and how sequential or simultaneous binding of cellular SH2/SH3 proteins may be coordinated to eventually yield the overall response of the cell. To address such aspects of RTK-mediated signal transduction, we investigated the interaction of three SH2 domain proteins with p145$^{c\text{-}kit}$ and PDGF-Rβ cytoplasmic domains. For these experiments we used transiently overexpressed proteins within a constant cell lysate background followed by biochemical analysis of protein−protein interactions in combined cell lysates, rather than purified TrpE- or GST-fusion proteins employed in similar studies (Hu et al., 1992; Rotin et al., 1992; Anderson et al., 1990; Margolis et al., 1990; Gout et al., 1993; Li et al., 1994). As with GST-fusion proteins, this *in vitro* approach allowed us to vary the concentration of each component but in addition eliminated problems related to the possibility that other, as yet unidentified, cellular components may be required for certain interactions or that fusion proteins produced in *Escherichia coli* may be incorrectly folded and therefore do not exhibit their normal binding characteristics. In several experimental systems, PLCγ, GAP, and p85/PI3′-K have been demonstrated to bind p145$^{c\text{-}kit}$ (Herbst et al., 1991, 1992; Reith et al., 1991; Lev et al., 1991; Rottapel et al., 1991; Miyazawa et al., 1991) and become phosphorylated on tyrosine residues by the activated kinase.

We have previously shown that in different cell types up to five different cellular proteins bind to p145$^{c\text{-}kit}$ with high affinity and that binding of these proteins correlates with association of PI3′-K activity (Shearman et al., 1993). In human 293 fibroblasts, these proteins are of 88, 90, 110, 112, and 116 kDa (Figures 1 and 7), similar to those binding the β-PDGF-R, which in addition associates with proteins of 74 and 145 kDa (Shearman et al., 1993). Of these proteins, we identified p88 as the human homolog of bovine p85α and now demonstrate that p90 represents the human homolog of p85β and that both p85 isoforms bind with approximately equal affinity to the kinase domains of p145$^{c\text{-}kit}$ and the βPDGF-R (Figure 2). Whereas *in vitro* binding of endogenous PLCγ to the overexpressed EP-R chimeric receptor was readily detectable, this was not true for EK-R under comparable conditions, indicating different affinities for this substrate (Figure 2). Only the addition of exogenous PLCγ, in the form of lysates from PLCγ cDNA-transfected 293 cells, to EK-R resulted in detectable binding of this substrate (Figure 1). Interestingly, increased PLCγ concentrations had no effect on the binding of the endogenous proteins or on EK-R-bound PI3′-K activity. These observations suggested a significantly higher affinity of p85 for p145$^{c\text{-}kit}$ cytoplasmic domain binding sites than PLCγ and prevention of simultaneous binding of the two substrates, presumably by steric hindrance or conformational changes induced by p85 interaction. Moreover, saturation binding of overexpressed p85α abolished the interaction of EK-R with five endogenous proteins, including p88 and p90, the human homologues of bovine p85α and β, respectively. These SH2 docking proteins may bind to the same phosphotyrosine target residue and mediate the interaction with the enzymatically active PI3′-K subunit. As our competition result with excess p85α demonstrates, the dimeric enzymes appear to exist in stoichiometric quantities in the cytoplasm as free preformed complexes, which upon receptor activation are translocated to autophosphorylated tyrosine target sites at the plasma membrane. Interestingly, in contrast to our observations with EK-R, PLCγ efficiently bound to EP-R

under p85α saturation conditions, demonstrating that the two closely related receptors, p145$^{c\text{-}kit}$ and PDGF-R, differ markedly in their ability to interact with substrate molecules. Our results strongly suggest that one PDGF-R molecule is able to simultaneously bind p85/PI3′-K and PLCγ, which creates a signaling complex consisting of four proteins with an approximate total MW of 485 000.

Prevention of PLCγ binding to EK-R by p85 was not caused by competition for the same binding site. Using site-directed mutagenesis to replace individual Tyr residues in the kinase insert region and within the C-terminal region of p145$^{c\text{-}kit}$ with Phe, we identified Tyr 721 as the binding site for p85, whose flanking sequences are homologous to Tyr 740 of the β-PDGF-R and Tyr 721 of the CSF-1-R, including the consensus sequence Y-X-X-M for p85/PI3′-K binding (Cantley et al., 1991). Since binding of p85/PI3′-K is dependent on kinase activity and autophosphorylation of p145$^{c\text{-}kit}$ (Shearman et al., 1993), we predict Tyr 721 as one of the *in vivo* p145$^{c\text{-}kit}$ autophosphorylation sites. Mutation of this residue to Phe had no effect on the binding of exogenous PLCγ to the receptor but resulted in a complete loss of affinity for p85α and β, and abolished the binding of p110, p112, and p116, which is further evidence for a preformed PI3′-K complex. Of all substrates tested, GAP appeared to have the weakest binding affinity to p145$^{c\text{-}kit}$, similar to βPDGF-R, which binds GAP with much lower affinity than p85/PI3′-K or PLCγ (Kashishian et al., 1992). Kashishian et al. proposed that only 4% of the βPDGF-R molecules in a cell are associated with GAP and explained this with differential phosphorylation efficiencies of βPDGF-R Tyr residues. In the case of p145$^{c\text{-}kit}$, a similar mechanism may exist, but we cannot exclude the possibility that high endogenous GAP levels in 293 cells may dilute the added radiolabeled protein to an extent that detection under our conditions failed.

This possibility is supported by the marked competitive effect that GAP-overexpressing cell lysate had on PLCγ association with EK-R, which was not observed under analogous conditions for p85α (Figure 1). In fact, saturating amounts of p85α enhanced GAP binding to EK-R about 3-fold, and GAP saturation binding had a similar effect on receptor-associated PI3′-K activity (Figure 6). These observations suggest either the existence of different activated conformations of the p145$^{c\text{-}kit}$ cytoplasmic domain, which may be stabilized by binding of GAP and p85, promoting the formation of a multimeric signaling complex, or the possibility of indirect interaction of GAP with the activated kinase of p145$^{c\text{-}kit}$ through as yet unidentified bindings sites on p85. With regard to the substrates PI3′-K, PLCγ, and GAP, ligand stimulation of p145$^{c\text{-}kit}$ in a given cell could lead to the formation of a variety of receptor−substrate complexes, in which the receptor is associated with both PI3′-K and GAP, and others which are composed only of the receptor and PI3′-K. At the same time, the composition of the PI3′-K multimer may differ in these complexes, since the two isoforms of the receptor binding subunit of PI3′-K, p85α, and p85β both utilize the same binding site within the cytoplasmic domain of p145$^{c\text{-}kit}$. Thus, with respect to PI3′-K, activation of the receptor in a given cell may lead to the formation of at least two distinct receptor/substrate complexes, in which the PI3′-K catalytic subunit is coupled to the receptor either by p85α or p85β.

Signal transfer complexes between RTKs and multiple SH2/SH3 domain proteins, which we demonstrate to be formed *in vitro*, may reflect an important mechanism necessary for fine tuning of the cellular signaling network. Moreover, the involvement of SH2/SH3 adaptor proteins such as p85, which may mediate the binding of diverse, functionally distinct enzymatic subunits, may allow the generation of specific physiological responses to multiple external stimuli.

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