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Crosstalk between the extracellular domain of the ErbB2 receptor and IGF-1 receptor signaling☆

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Abstract

Insulin-like growth factor 1 receptor (IGF-1R) plays an important role in cell growth and malignant transformation. To investigate IGF-1R-dependent signaling events and its effects on apoptosis induction and cellular proliferation, we generated a constitutively active, ligand-independent IGF-1R variant. We fused the cytoplasmic domain of the IGF-1R to the extracellular and transmembrane domains of the oncogenic ErbB2 receptor (ErbB2$^{V\rightarrow E}$/IGF-1). A fusion protein in which the wild-type sequence of the ErbB2 receptor was used, served as a control (ErbB2^{V}/IGF-1R). ErbB2^{V}/IGF-1R, ErbB2$^{V\rightarrow E}$/IGF-1R and IGF-1R were stably transfected into interleukin 3 (IL-3)-dependent BaF/3 cells. ErbB2$^{V\rightarrow E}$/IGF-1R expressing cells exhibited ligand-independent, constitutive tyrosine phosphorylation of the receptor fusion protein. Constitutively, activated ErbB2$^{V\rightarrow E}$/IGF-1R conferred IL-3 independence for growth and survival to the transfected BaF/3 cells. Constitutive activation of the IGF-1R results in cellular growth and protection against apoptosis upon IL-3 withdrawal in BaF/3 cells.

Keywords: Insulin-like growth factor receptor (IGF-1R); ErbB2 receptor; Receptor fusion protein; Tyrosine phosphorylation; Apoptosis; Cell proliferation

1. Introduction

The insulin-like growth factor-1 receptor (IGF-1R) consists of two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bonds. The type II hetero-tetrameric tyrosine kinase receptor is closely related in structure and function to the insulin receptor [1,2]. Binding of IGF-1 to the extracellular α-subunits causes a conformation of the intracellular domains of the β-subunits which results in autophosphorylation. The activation of the tyrosine kinase activity and the formation of phosphorylated tyrosine residues allow recruitment of the intermediate docking proteins Shc, PI3 kinase, Grb2, Grb10, PLCχ1 and IRS-1 [3–6]. These proteins cause the activation of the Ras/Raf/mitogen-activated protein kinase cascade and the phosphatidylinositol-3 kinase pathway [7–9].

Depending on the cellular context, IGF-1 R activation leads to diverse phenotypes including proliferation, differentiation and inhibition of apoptosis [5,10] The IGF-1R activation also plays an important role in the malignant transformation of cells [11–15]. Enhanced IGF-1 receptor expression can cause ligand-dependent, neoplastic transformation and tumorigenesis [16,17]. IGF-1 receptor function also seems to be required for the transformation of cells through activated oncogenes [18–20]. The transformed phenotypes of human and rat glioblastoma cells [21], human mammary carcinoma cells [22] and human small cell lung carcinomas [23] as well as human and mouse melanomas [24] can be influenced in their growth properties by manipulation of the level and activation of the IGF-1 receptor [25,26].

The effects on tumor formation are mainly due to the ability of the IGF-1 receptor to stimulate cell proliferation [27–29] and to protect cells against pro-apoptotic signals [9,23,30–32]. To study the signaling pathways which confer these cellular phenotypes, receptor variants have been of great value in the past and have led to important insights into the IGF-1R mediated signaling events [33]. These studies have shown that the activation of the Akt/protein kinase B pathway and bad phosphorylation by Akt is a major

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Abbreviations: IGF-1, insulin-like growth factor 1; IGF-1R, the receptor for IGF-1; IRS1/2, insulin receptor substrate-1/2; PI3 kinase, phosphoinositide-3-OH kinase; IL-3, interleukin 3; ErbB2$^{V\rightarrow E}$/IGF-1R, fusion of ErbB2 with IGF-1R in which Val659 of the transmembrane domain of ErbB2 is mutated to Glu; ErbB2^{V}/IGF-1R, fusion as ErbB2$^{V\rightarrow E}$/IGF-1R but Val659 is not mutated; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Phospho-Tyr, phosphotyrosine; MAPK, mitogen-activated protein kinase; MEK, MAP kinase; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; EGF, epidermal growth factor; EGFR, the receptor for EGF.

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contributor to the anti-apoptotic function of IGF-1. The mutant receptor variants used mostly relied on the substitution of individual amino acids which are known to interact with signaling components and which resulted in a loss of function [34–36].

We have used a different strategy and generated a ligand-independent, constitutively active variant of the IGF-1R. Mutations of growth factor receptors in the intracellular domain have been described which lead to constitutively activation of the intrinsic tyrosine kinase activities [37–40], others are activated through mutations outside of this domain. The transforming potential of the ErbB2 receptor, for example, a member of the EGF receptor family, is dependent upon an amino acid substitution at position 659, valine to glutamic acid (ErbB2$^{V\rightarrow E}$). This position localizes to the transmembrane domain and the mutation results in the constitutive activation of intrinsic protein tyrosine kinase activity [37,41,42].

We have constructed a constitutively activated, ligand-independent variant of the IGF-1R by fusing the extracellular and transmembrane domains of the oncogenic ErbB2$^{V\rightarrow E}$ and the cytoplasmic part of the IGF-1R, to derive the ErbB2$^{V\rightarrow E}$/IGF-1R chimera. The extracellular and transmembrane domains of ErbB2$^{V\rightarrow E}$ confer constitutive activation to the intracellular domain of the IGF-1R. Upon introduction into BaF/3 cells, the chimeric receptor is persistently autophosphorylated, promotes proliferation and protects the cells against the pro-apoptotic effects of IL-3 withdrawal. The ErbB2$^{V\rightarrow E}$/IGF-1R chimera activates the Ras/MAP kinase cascade and the phosphatidylinsolitol-3 kinase pathways to an extent comparable to that of the wild-type IGF-1R in the presence of IGF-1. The chimeric ErbB2$^{V\rightarrow E}$/IGF-1R might become a useful tool to understand the signaling events triggered by the IGF-1 receptor and complement the information gained with the loss of function mutants.

2. Experimental procedures

2.1. Materials

Recombinant murine IL-3 was purchased form Sigma. Recombinant human IGF-1 and G418 were obtained from Life Technologies, Inc. Both PI3 kinase-specific inhibitor Ly294002 and MEK1/2-specific inhibitor U0126 were form Cell Signaling Technology, Inc. Stock solutions of pharmacological inhibitors such as Ly294002 and U0126 were prepared in DMSO at a concentration of 1000-fold.

The antibodies used for Western blotting were purchased from Santa Cruz Biotechnology: IGF-1R β chain (C-20), Raf-1 (C-12), MEK1 (C-18) polyclonal antibodies and phospho-tyrosine (PY99) monoclonal antibody; from Cell Signaling Technology, Inc.: phospho-Raf (Ser259), phospho-MEK1/2 (Ser217/221), phospho-MAPK (Erk1/2) (Thr202/Tyr2204), phospho-p90RSK (Ser381), phospho-GSK3 (Ser21/9), as well as Akt and p44/42 MAP kinase polyclonal antibodies and the anti-rabbit IgG secondary antibody (horseradish peroxidase-conjugated). ErbB2-specific FRP5 monoclonal antibody [43] was kindly provided by Dr. N. Hynes, Basel.

2.2. Generation of chimeric receptors

The chimeric ErbB2^{V}/IGF-1 receptor constructs comprises the extracellular and transmembrane domains of the human ErbB2 receptor (up to and including amino acid 682) and the cytoplasmic tail of the human IGF-1R (amino

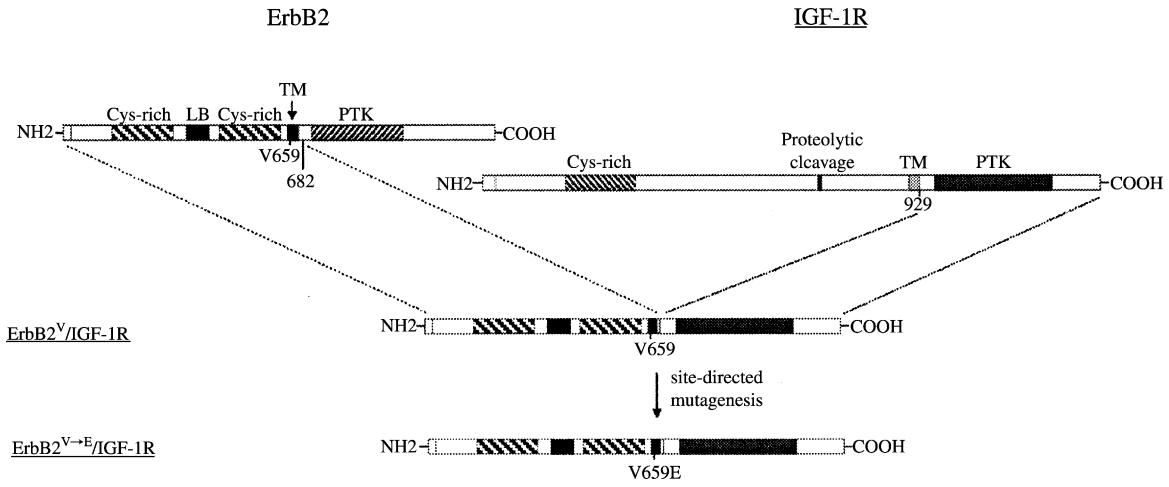

Fig. 1. Construction of ErbB2-IGF-1chimeric receptors. The full-length ErbB2 and IGF-1 receptors are shown in the top line. The fragments which were used to derive ErbB2^{V}/IGF-1R and ErbB2$^{V\rightarrow E}$/IGF-1R are indicated. The cDNA sequence encoding the cytoplasmic domain of the IGF-1R β-subunit (amino acid positions 929–1337) was fused to the extracellular and transmembrane domains of the ErbB2 receptor (amino acid positions 1–682) followed by site-directed mutagenesis to generate the mutation V659E in the transmembrane domain of the chimeric construct; LB: ligand binding domain; TM: transmembrane domain; PTK: protein tyrosine kinase domain.

acids 929–1337). Cloning was performed by polymerase chain reaction (PCR) as illustrated in Fig. 1. To generate DNA for the extracellular and transmembrane domains of wild-type ErbB2, pSV2/ErbB2 (V) [34] was used as a template for 30 cycles of PCR: 94 °C (1 min), 55 °C (1 min) and 72 °C (1,5 min) using Taq polymerase with a primer (P1) containing a *Hin*dIII site and ErbB2 5′ sequences: 5′-GGAAGCTTATGGAGCTGGCGGCCT-3′ (the *Hin*dIII site is underlined), and a primer (P2) containing a *Nhe*I site and ErbB2 3′ sequences: 5′-GAGCTAGCAATCTTCTGCTGCCGT-3′ (the *Nhe*I site is underlined). Full-length IGF-1R cDNA was cloned into pcDNA3 plasmid (Invitrogen) with a modification in the multiple cloning site (pcDNA3 + 2). To generate the cytoplasmic domain of IGF-1R, pcDNA3 + 2/IGF-1R was amplified by PCR for 30 cycles: 94 °C (1 min), 55 °C (1 min) and 72 °C (1.5 min) with a 5′ primer (P3) containing a *Nhe*I site and IGF-1R 5′ sequences: 5′-CGGCTAGCAGAAAGAGAAATAAC-3′ (the *Nhe*I site is underlined) and a 3′ primer (P4) downstream of IGF-1R coding sequence: 5′ ATTTAGGTGACACTATAG-3′. The resulting ErbB2 and IGF-1R PCR product were cleaved with *Hin*dIII and *Nhe*I (ErbB2) and with *Nhe*I and *Eco*RI (IGF-1R) and ligated to the *Hin*dIII and *Eco*RI digested pcDNA3 + 2 expression vector. The sequence of the entire chimeric ErbB2^{V}/IGF-1R was confirmed by automatic sequencing. To generate the chimeric ErbB2$^{V\rightarrow E}$/IGF-1R site-directed mutagenesis (Stratagene) was used to introduce the V659E point mutation in the transmembrane domain of the chimeric ErbB2^{V}/IGF-1R, using the following oligo primers: (E1): 5′-CTCTGCGGTG**GAA**GGCATTCTGCTG-3′ and (E2): 5′-CAGCAGAATGCC**TTC**CACCGCAGAG-3′(mutations are in bold face). All constructs were resequenced to verify the presence of the desired point mutation and absence of spurious mutations resulting from PCR amplification.

2.3. Cell culture and stable transfection of BaF/3 cells

The bone marrow-derived IL-3-dependent BaF/3 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and supplemented with 1 ng/ml recombinant murine IL-3.

Twenty micrograms of pcDNA3 + 2/IGF-1R, ErbB2^{V}/IGF-1R, ErbB2$^{V\rightarrow E}$/IGF-1R or empty vector were electroporated in BaF/3 cells (350 V, 960 μF). Cells were expanded for 24 h, then the growth medium was changed and incubation continued for another 24 h before the G418 selection (1 mg/ml) was started. Pools of G418 resistant cells were cloned by limited dilution.

2.4. Immunoprecipitation and western blotting

Cells were starved at a density of (0.5–1.0) × 10^6 cells/ml for 16 h in the absence of IL-3. Induction was performed by the addition of 50 ng/ml of recombinant human IGF-1. Cells were washed in cold PBS and pellets were lysed for 10 min at 4 °C in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 10 μg/ml aprotinin, 2 μg/ml pepstatin, 100 μM pefablock, 1 μg/ml leupeptin, 1 mM DTT, and 1 mM sodium orthovanadate). The lysates were centrifuged for 10 min at 4 °C to eliminate cellular debris. Protein concentration was determined by the Bradford procedure using the Bio-Rad protein assay. For immunoprecipitation, 1 μg anti-IGF-1Rβ antibody was coupled to protein A-coated magnetic Dynabeads (Dynal Biotech) following manufacturer's instructions, and incubated with 0.5–1 mg of total cell lysate at 4 °C for 2 h. After washing the beads three times in lysis buffer the immunoprecipitates or 100 μg total protein were separated on 12.5, 10, or 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with the antibodies indicated. Bound antibodies were detected with horseradish peroxidase-coupled antibodies and the chemiluminescence detection system (Amersham). Membranes were stripped and rehybridized with specific antibodies as indicated.

2.5. Cell viability assays

Cells were washed free of residual IL-3 and cultured at a density of 5 × 10^5 ml^{-1} in growth medium supplemented with 1 ng/ml of IL-3, 10 μM of PI3-K inhibitor Ly294002, 10 μM of ERK1/2 inhibitor U0126, or a combination of both inhibitors, for 1 h prior to addition of 50 ng/ml of IGF-1 (BaF/3-IGF-1R cells) or left untreated. At daily intervals, aliquots were transferred to 96-well plates (5 × 10^4 cells per well). Then 50 μl of XTT/PMS (Sigma) in a ratio of 50:1 was added and incubation continued for 4 h at 37 °C. The absorbance of each sample at 492 nm (reference wavelength 690 nm) was determined in a microplate reader as a measure of the relative amount of viable cells in comparison to the number of seeded cells.

2.6. Measurements of apoptosis

Apoptotic cells were detected using the In Situ Cell Death Detection Kit (TUNEL-FITC) according to manufacturer's instructions (Roche). Briefly, 5 × 10^5 cells/ml were incubated in RPMI 10% FBS in the presence or absence of 1 ng/ml of IL-3 (Sigma) or 50 ng/ml of IGF-1 (Life Technologies, Inc.). After the indicated time points, 1 × 10^6 cells were collected and washed twice in PBS. Cell pellets were then resuspended in 100 μl of PBS, fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton in 0.1% sodium citrate for 2 min on ice and labeled with TUNEL reaction mixture for 1 h at 37 °C. Apoptosis was determined by flow cytometry. For each sample, 10,000 cells were counted on a FACS Calibur using CellQuest Software (Becton-Dickinson).

2.7. Akt-Kinase assay

The Akt kinase assay was performed using the Akt Kinase Assay Kit (Cell Signaling Technology, Inc.). Cells

were cultured for 16 h in the absence of IL-3. Stimulations were performed with 50 ng/ml of IGF-1. Cells were washed in cold PBS and pellets were lysed for 10 min at 4 °C in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin). The lysates were cleared by centrifugation for 10 min at 4 °C. Akt kinase protein was immunoprecipitated by incubation of 2 mg of cell lysate with 20 μl of immobilized Akt antibody (coupled to agarose beads) for 2 h at 4 °C. Beads were washed twice with 500 μl of lysis buffer. Then pellets were suspended in 40 μl kinase buffer (25 mM Tris, pH 7.5, 5 mM glycerolphosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$) supplemented with 200 μM ATP and 1 μg of GSK-3 fusion protein and incubated for 30 min at 30 °C. The reaction was terminated with 20 μl three times SDS sample buffer and boiled for 5 min. Samples were analyzed by Western blotting using phospho-GSK antibody (Ser21/9). To determine the amount of Akt in the lysate, 100 μg of lysate protein was subjected to immunoblotting.

3. Results

3.1. Design of the chimeric receptors

To study the phenotypic consequences of persistent IGF-1 receptor activation, we have generated a constitutively active, ligand-independent variant of the IGF-1R. For this purpose, we have employed the extracellular domain and the transmembrane domain of the ErbB2 receptor. This oncogenic variant of the ErbB2 receptor is distinguished from the wild-type by an amino acid exchange at position 659 (valine to glutamic acid). The oncogenic ErbB2 receptor is constitutively active and thought to dimerize in the absence of ligand or co-receptor. We tested the possibility that this activation principle could be conferred to a heterologous intracellular domain, that of the IGF-1R.

The extracellular domain and transmembrane domain of the ErbB2 receptor was fused to the intracellular domain of the IGF-1R. The contributions to the chimeric receptors are shown in Fig. 1. In a first step, the extracellular and transmembrane domains of the wild-type ErbB2 receptor ($ErbB2^{V}$), amino acid positions 1–682, were fused to intracellular domain of the IGF-1R, amino acid positions 929–1337, to obtain the $ErbB2^{V}$/IGF-1R gene. The amino acid valine at position 659, located in the transmembrane domain of the chimera, was changed to a glutamic acid by site-directed mutation, to obtain the $ErbB2^{V\rightarrow E}$/IGF-1R gene.

3.2. Expression and activation of the chimeric receptors in BaF/3 cells

We stably transfected BaF/3 cells, a bone marrow-derived lymphoid cell line, with the IGF-1 receptor, $ErbB2^{V\rightarrow E}$/IGF-1R and $ErbB2^{V}$/IGF-1R. BaF/3 cells are suited for the our experiments, because they do not endogeneously express members of the ErbB family, only very low levels of the IGF-1R and are strictly dependent on IL-3 for survival and proliferation. We analyzed the expression and tyrosine phosphorylation of the transfected receptor proteins in these cells. To visualize the response of IGF-1R transfected cells (BaF/3-IGF-1R) to IGF-1 the cultures were deprived of serum and IL-3 for 16 h and induced with 50 ng/ml IGF-1 for 10 min or for 16 h. Cell lysates were prepared and proteins immunoprecipitated with an antibody specific for the β-subunit of the IGF-1R followed by immunoblot analysis with antibodies specific for phosphotyrosine residues (Fig. 2, panel A), IGF-1Rβ (panel B) or the extracellular domain of the ErbB2 receptor (panel C).

Tyrosine phosphorylation of wild-type IGF-1R is strongly induced by addition of IGF-1 to the transfected cells (panel A, lanes 3 and 4). The extent of tyrosine phosphorylation is similar in cells treated with IGF-1 for 10 min or for 16 h. Long-term treatment with IGF-1 does not seem to cause the downregulation of receptor expression (panel, lane 4). The $ErbB2^{V\rightarrow E}$/IGF-1R fusion protein, with an activating mutation in the transmembrane domain, is phosphorylated on tyrosine in the absence of ligand stimulation (panel A, lane 6). The $ErbB2^{V}$/IGF-1R variant, without the activating mutation in the transmembrane region, is not tyrosine phosphorylated (panel A, lane 5). This is not different when the cells are cultured in 10% serum (data not shown).

The levels of tyrosine phosphorylation of $ErbB2^{V\rightarrow E}$/IGF-1R and IGF-1R in ligand stimulated cells is similar. Since BaF/3 cells do endogenously express members of the EGFR family, cross-activation of the fusion protein by ligands for ErbB1, three or four receptors and receptor hetero-dimerization is most unlikely. These results indicate that $ErbB2^{V\rightarrow E}$/IGF-1R is a ligand-independent, constitutively active receptor variant.

3.3. Consequences of constitutively activated $ErbB2^{V\rightarrow E}$/IGF-1R expression for cell proliferation and anti-apoptosis

The IGF-1R, activated by its ligands, plays an important role in the growth of cells, in cellular transformation and in the protection of cells from apoptotic stimuli. We investigated if the constitutively activated $ErbB2^{V\rightarrow E}$/IGF-1R would confer similar effects on the transfected BaF/-3 cells. Cell proliferation assays were performed with BaF/3 cells transfected with $ErbB2^{V}$/IGF-1R, $ErbB2^{V\rightarrow E}$/IGF-1R, wild-type IGF-1R genes or with the empty gene expression vector. Cultures were grown in the presence or absence of 1 ng/ml IL-3 for 3 days and viable cell numbers were determined from triplicate cultures. All BaF/3 derived transfectants grew at a similar rate in the presence of IL-3 (Fig. 3A), i.e. the IL-3 responsiveness is not affected by the introduction of the chimeric receptor constructs.

Withdrawal of IL-3 had different effects on the transfected cell populations. Cells transfected with the expression

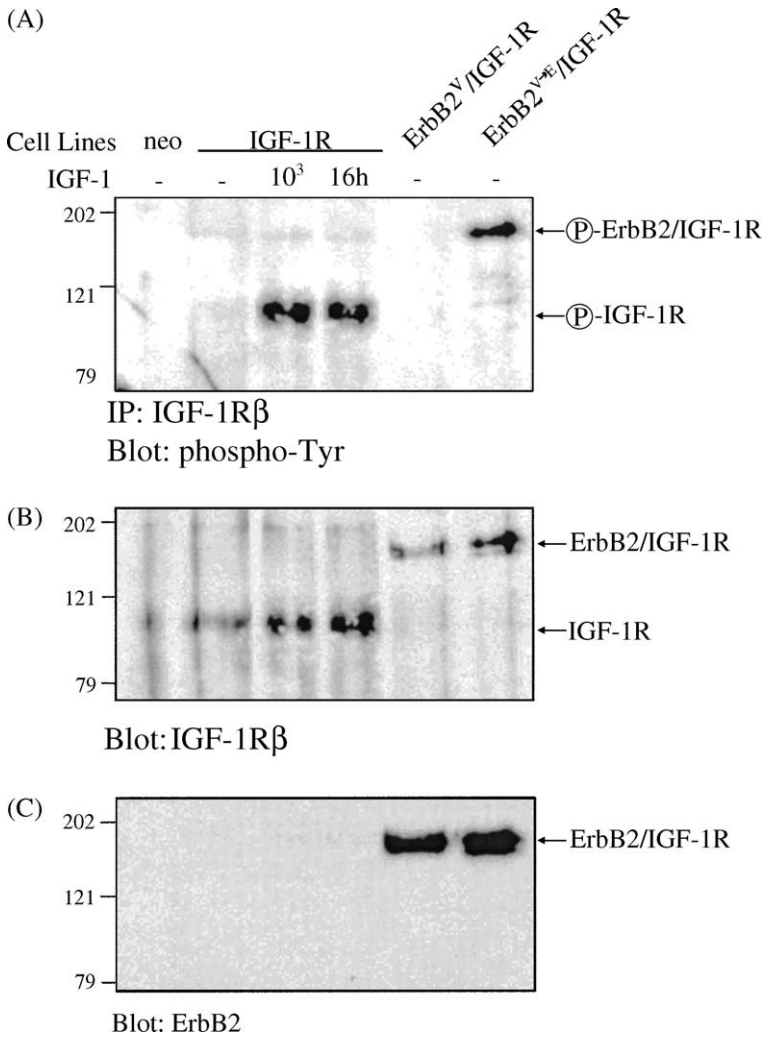

Fig. 2. Expression and activation state of ErbB2$^{V\rightarrow E}$/IGF-1R in transfected BaF/3 cells. BaF/3 cells were stably transfected with ErbB2^{V}/IGF-1R, ErbB2$^{V\rightarrow E}$/IGF-1R, IGF-1R or the empty cDNA expression vector (neo). The cells were grown in media without serum and IL-3 for 16 h. BaF/3 cells expressing the IGF-1R were treated with 50 ng/ml IGF-1 for 10 min or left untreated. Cells were lysed and extracts were immunoprecipitated with an antibody specific for the β chain IGF-1R. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-phosphotyrosine antibody (A). The blot was stripped and reprobed with the IGF-1R β chain (B) or ErbB2 (C) specific antibodies.

vector (Fig. 3B, lane 1), wild-type IGF-1R (lane 2) or ErbB2^{V}/IGF-1R (lane 4) did not proliferate and all cells died within 4 days in the absence of IL-3 even when the growth medium was supplemented with 10% FBS. BaF/3 cells expressing ErbB2$^{V\rightarrow E}$/IGF-1R survived and proliferated in the absence of IL-3 (lane 5). Similarly, cells transfected with IGF-1R and supplemented with 50 ng/ml IGF-1 proliferated in the absence of IL-3. The growth rates of both lines were reduced when compared to those observed in the presence of IL-3. These results demonstrate that constitutively activated ErbB2$^{V\rightarrow E}$/IGF-1R and ligand stimulated IGF-1R are able to overcome the IL-3 dependence of BaF/3 cells and induce a proliferative response. Both cell lines have been maintained in culture in the absence of IL-3 for several passages.

To analyze the involvement of distinct signaling pathways in the conferal of viability and proliferative capacity by the ligand stimulated and constitutively active IGF-1 receptor, cells were treated with specific kinase inhibitors. Viability and proliferation of the cells was determined after growth in the presence of the PI3 kinase inhibitor Ly294002, the MEK1/2 inhibitor U0126 or a combination of both inhibitors and IL-3 withdrawal (Fig. 3C). The number of viable cells transfected with ErbB2$^{V\rightarrow E}$/IGF-1R was strongly decreased by exposure to 10 μM Ly294002 or 10 μM U0126, an effect further accentuated by the simultaneous presence of both inhibitors. The inhibitors also had detrimental effects on IGF-1R transfected cells grown in the presence of IGF-1. BaF/3 cells expressing only the expression vector or ErbB2^{V}/IGF-1R were affected either by IL-3 withdrawal or by exposure to the inhibitors in a similar fashion. We conclude that the constitutively activated ErbB2$^{V\rightarrow E}$/IGF-1R generates a signal in BaF/3 cells which induces proliferation, is dependent on PI3 kinase and MEK1/2 activity and

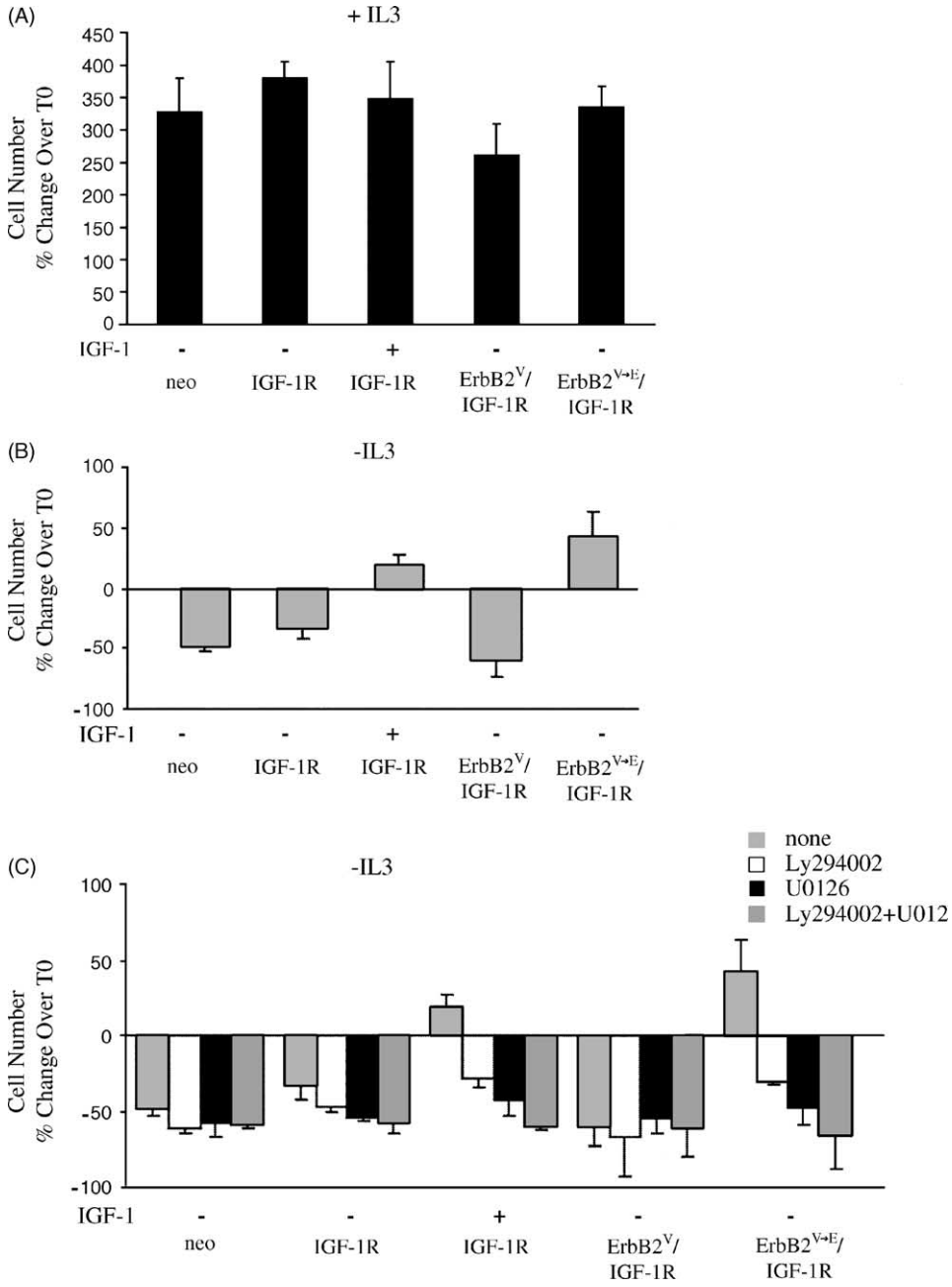

Fig. 3. Constitutive activation of ErbB2$^{V\rightarrow E}$/IGF-1R confers IL-3 independence to BaF/3 cell growth. BaF/3-neo, BaF/3-ErbB2^{V}/IGF-1R, BaF/3-ErbB2$^{V\rightarrow E}$/IGF-1R and BaF/3-IGF-1R cells were seeded at 5×10^4 cells per plate in 10% FBS in the presence (A) or absence (B) of 1 ng/ml IL-3. Cells expressing IGF-1R were supplemented with 50 ng/ml IGF-1. Cells were also incubated with 10 μM of the PI3K inhibitor Ly294002, 10 μM of MEK1/2 inhibitor U0126 or a combination of both inhibitors (C). Viable cells were determined by addition of XTT labeling mixture as described under Section 2 from triplicate cultures at daily intervals. Results are means ± S.D. of three independent experiments, at day 3, expressed as percentage change over seeded cells.

seems to be closely related to the proliferation signals emenating from IL-3R or IGF-1R activation.

We quantitated the anti-apoptotic potential of the constitutively activated ErbB2$^{V\rightarrow E}$/IGF-1R in BaF/3 cells upon IL-3 withdrawal. Cells were cultured in the absence or presence of 1 ng/ml IL-3 and the percentage of apoptotic cells were estimated by TUNEL staining and flow cytometry at different time points. Representative results are shown in Fig. 4. Cells were grown in the presence of IL-3 (left panel) or in the absence of IL-3 for 40 h (middle panel) and 72 h (right panel). The BaF/3 cells and its transfected derivatives were resistant to apoptosis when IL-3 was present in the medium. Apoptosis can be observed in the parental and in the transfected cells 40 h after IL-3 withdrawal and increases further at 72 h after withdrawal. 40.65% of the control BaF/3 cells and 35.57% ErbB2^{V}/IGF-1R expressing

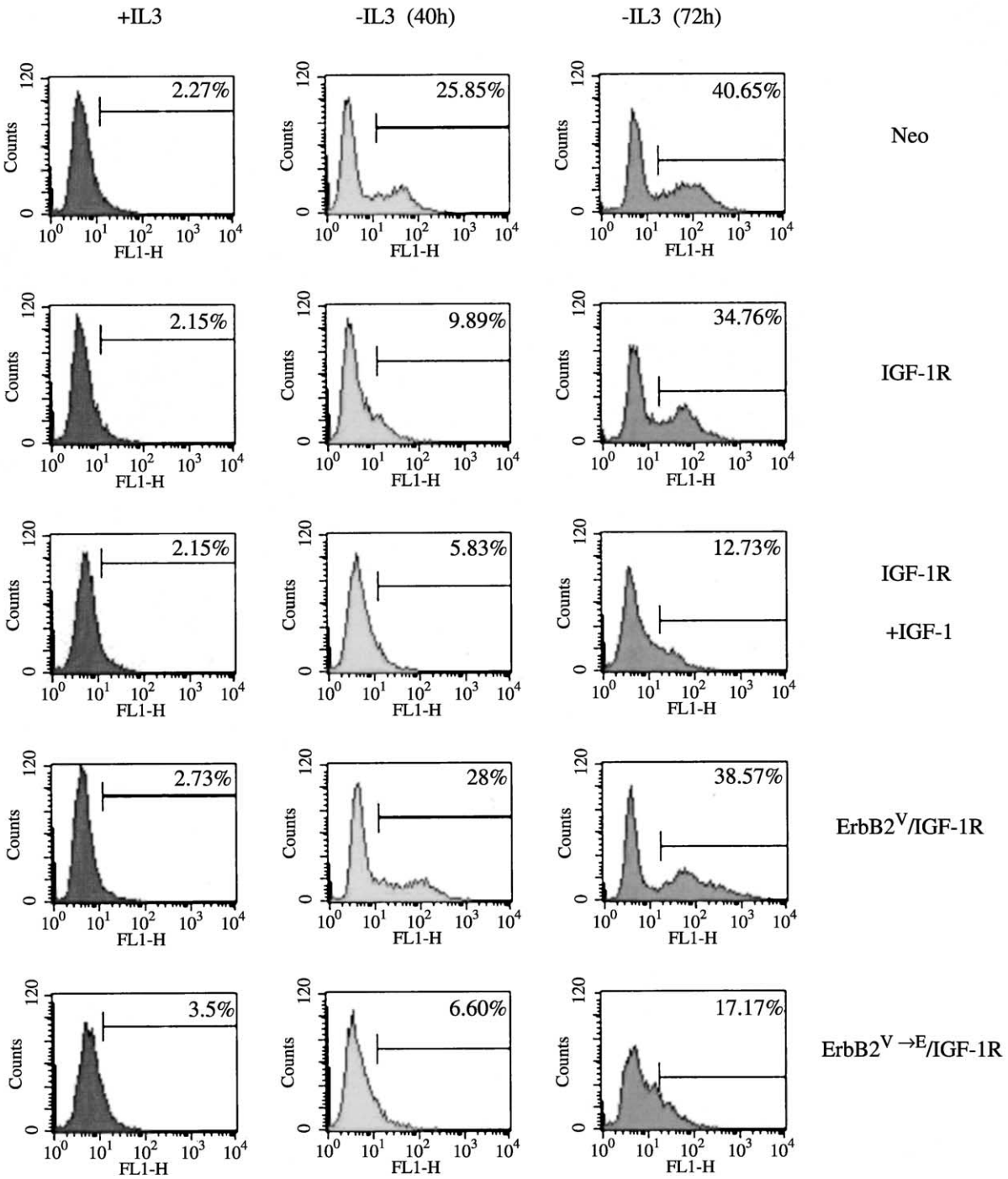

Fig. 4. ErbB2$^{V\rightarrow E}$/IGF-1R expression protects BaF/3 cells from apoptosis after IL-3 withdrawal. BaF/3 cells stably transfected with empty vector (neo), IGF-1R, ErbB2^{V}/IGF-1R or ErbB2$^{V\rightarrow E}$/IGF-1R were washed free of IL-3 and cultured with or without 1 ng/ml IL-3, as indicated. BaF/3-IGF-1R cells were supplemented with 50 ng/ml IGF-1. Cells were harvested at the indicated time points and stained with TUNEL followed by flow cytometry analysis. The percentage numbers shown correspond to percent apoptotic cells.

cells appear apoptotic 72 h after IL-3 withdrawal. Expression of ErbB2$^{V\rightarrow E}$/IGF-1R caused a strong reduction in the appearance of apoptotic cells (17.17%). IGF-1 treatment caused a reduction in apoptosis of IGF-1R transfected cells from 34.76 to 12.73%.

3.4. Consequences of ErbB2$^{V\rightarrow E}$/IGF-1R expression for Raf, Mek1,2, Erk1/2 and Akt signaling

Two important signaling cascades activated through IGF-1R are the MAP kinase (Erk1/2) via Ras, Raf and MEK

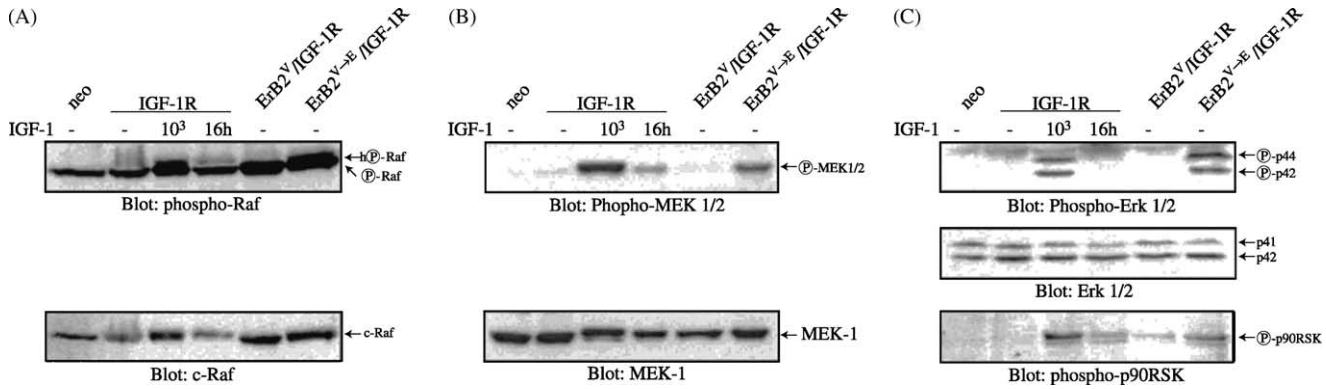

Fig. 5. Activation of Ras/MAP kinase cascade by ErbB2$^{V\rightarrow E}$/IGF-1R. BaF/3 cells stably transfected with ErbB2$^{V\rightarrow E}$/IGF-1R, ErbB2^{V}/IGF-1R, IGF-1R or empty vector (neo) were cultured in the absence of IL-3 and treated with or without 50 ng/ml IGF-1. Cell lysates (100 μg) from each of the stable transfectants were separated by SDS-PAGE and immunoblotted with activation state specific antibodies: anti-phospho-Raf (A, top panel), anti-phospho-MEK1/2 (B, top panel), anti-phospho-p42/44 ERK or anti-phospho-p90RSK (C, top and bottom panels). The blots were stripped and blotted with anti-Raf-1 (A, bottom panel), anti-MEK1 (B, bottom panel) or anti-p42/44 ERK (C, middle panel).

and the PI3K/Akt pathways. We utilized specific antibodies that recognizes only the phosphorylated, active forms of Raf, MEK1/2, Erk1/2 and the Erk1/2 substrate p90RSK to detect activated signaling components in BaF/3 cells stably transfected with ErbB2$^{V\rightarrow E}$/IGF-1R, ErbB2^{V}/IGF-1R, IGF-1R or the cDNA expression vector. The cells were serum-deprived for 16 h and cultured in serum-free medium in the absence of IL-3.

The level of activation of components of the MAP kinase cascade is shown in Fig. 5. ErbB2$^{V\rightarrow E}$/IGF-1R causes activation of c-Raf to a comparable degree as wild-type IGF-1R stimulated with IGF-1 for 10 min (panel A, lanes 3 and 6). Two phosphorylated bands of c-Raf can be detected. ErbB2$^{V\rightarrow E}$/IGF-1R also caused the activation of MEK (Fig. 5B), Erk1/2 and p90RSK (Fig. 5C).

Activation of PI3 kinase leads to the generation of 3′-phosphorylated phosphatidylinositodes which in turn are important for the activation of Akt kinase [37]. The activation state of Akt kinase was assessed in immunoabsorption experiments in which Akt kinase was bound to an immobilized antibody and the phosphorylation of the Akt kinase substrate, GSK-3, was measured. No kinase activity was detected in BaF/3 cells expressing ErbB2^{V}/IGF-1R, IGF-1R or the empty expression vector following 16 h of culture in the absence of serum and IL-3. In contrast, ErbB2$^{V\rightarrow E}$/IGF-1R constitutively activated Akt kinase to a level comparable to that of IGF-1R stimulated with IGF-1 (Fig. 6, upper panel, lanes 3, 4 and 6). Similar levels of Akt protein were present in the extracts used (lower panel). We conclude that ErbB2$^{V\rightarrow E}$/IGF-1R activates the MAP kinase signaling cascade and the Akt kinase in a very similar fashion as the ligand induced IGF-1R.

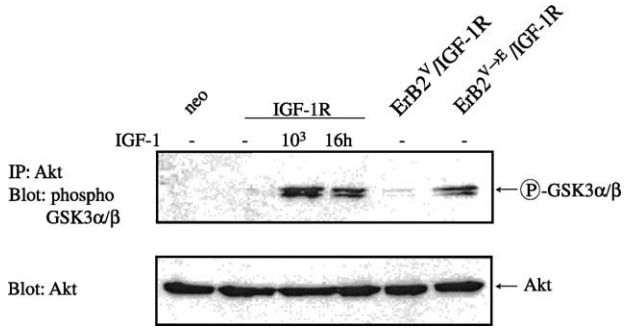

Fig. 6. Akt kinase is activated by ErbB2$^{V\rightarrow E}$/IGF-1R. BaF/3 transfectants ErbB2$^{V\rightarrow E}$/IGF-1R, ErbB2^{V}/IGF-1R, IGF-1R or empty vector (neo) were grown in medium without serum and IL-3 for 16 h and treated with or without 50 ng/ml IGF-1. Akt kinase was immunoprecipitated with immobilized 1G1 antibody from cell extracts. The precipitates were incubated with GSK-3 substrate and phosphorylation of GSK-3 was detected by immunoblot using activation state specific anti-phospho-GSK-3 antibody (top panel). Cell extracts were blotted with anti-Akt to compare the amounts of Akt in each lysate (bottom panel).

4. Discussion

Our experiments document that the replacement of the α-subunit and the transmembrane domains of the IGF-1R with the extracellular and transmembrane domains of the oncogenic c-ErbB2 results in a constitutively active receptor variant. Upon introduction into BaF/3 cells, the chimeric receptor exhibits properties which are nearly indistinguishable from those of the ligand induced IGF-1R. It results in the constitutive activation of the MAP kinase and PI3 kinase pathways, protects BaF/3 cells from apoptosis after IL-3 withdrawal and promotes IL-3-independent proliferation. Both phenotypes are sensitive to PI3 kinase and MAP kinase inhibitors.

The experiments emphasize the extraordinarily potent effect which the valine to glutamic acid mutation in the transmembrane domain of the ErbB2 receptor can exert. The mutation was originally discovered in the rat homologue of the ErbB2 receptor, the neu oncogene, and was the result of chemical mutagenesis. The mutation did not spontaneously arise in human tumor cells. The in vitro alteration of the valine at the equivalent position (664 in the rat gene,

659 in the human gene) to a glutamic acid, however, converted the human ErbB2 gene into a constitutively active molecule and into a potent oncogene. Extensive studies have addressed the contribution of the transmembrane domains in the dimerization and activation of members of the erbB family [45]. Although the precise role still remains to be fully established, transmembrane domains might promote self-associations and these interactions might contribute to the formation of latent oligomers. The presence of the receptors, in a non-activated, but already dimerized state, may facilitate and accelerate the process of ligand binding. Ligand binding in turn might stabilize the active conformation of the dimeric state [44].

Studies in which the self-association of the ErbB2 transmembrane domain was measured in cell membranes showed, contrary to expectation, that the activating mutation does not enhance dimerization. Mendrola et al. found that it causes a 50% reduction in dimerization when compared to the wild-type sequence [45] and propose that the transforming consequences of the mutation might not be due to an enhanced dimerization propensity, but to structural alterations in the dimerization interface and the resulting conformation of the interaction partners. Our experiments show that this active conformation can be conferred to a heterologous intracellular domain, i.e. that there must be common structural principles in the activation of the intracellular tyrosine kinase activity of the ErbB2 and IGF-1 receptors which are independent from their sequence divergence.

Other molecular constellations have been investigated which resulted in the activation of IGF-1R in the absence of ligand. The first description of an activated variant of the IGF-1 receptor was based on the fusion of the β-subunit (intracellular domain, transmembrane domain and 36 amino acids of the extracellular domain) to the 5′ portion of the gag gene of the avian sarcoma virus UR2 [46,47]. This chimeric protein was activated in chicken embryo fibroblasts, but was not tumorigenic. Elimination of the residual extracellular portion of the molecule resulted in a tumorigenic molecule, suggesting an inhibitory function for this domain. Subsequent studies showed that the Gag-IGF-1 fusion protein, NM1, induced molecular signals qualitatively indistinguishable from those of ligand activated IGF-1 receptor [48]. Quantitative differences, however, were observed. The tyrosine phosphorylation was of the recombinant protein was three-fold higher than that of the wild-type IGF-1R β-subunit [47–49].

The truncation of the extracellular domain of the IGF-1R and replacement with viral Gag sequences resulted in constitutive activation of kinase activity, suggesting that this domain exerts an inhibitory influence over the intracellular kinase domain. Relief of inhibition and assumption of an active conformation could be the result of truncation of ligand binding. These results were corroborated by the use of deletion mutants. Deletion of 36 amino acids (positions 870–905) immediately N-terminal to the transmembrane domain [50] in the context of the wild-type receptor sequence resulted in a ligand-independent, constitutive receptor variant able to stimulate the PI3 kinase signaling pathway, but unable to promote cell proliferation or activation of the ras/MAP kinase pathway in the absence of IGF-1.

The replacement of valine 922 by a glutamic acid residue [40] resulted in a receptor with constitutive tyrosine kinase activity and stimulation of IRS-1 associated PI3 kinase activity in the absence of ligand. Ras-mitogen-activated protein kinase was not activated under the same conditions.

The effects of ErbB2$^{V\rightarrow E}$/IGF-1R on, e.g. the phosphorylation of p42/44 Erk kinase and the Akt GSK-3 substrate were equivalent to those observed when IGF-1R was induced with its natural ligand. The signaling events induced by the ErbB2$^{V\rightarrow E}$/IGF-1R fusion protein resemble most closely those induced by the Gag-IGF-1 fusion protein, e.g. the activation of the PI3 kinase and MAP kinase pathways, and the effects on cell growth and suppression of apoptosis. The restriction to activation of PI3 kinase by a truncated IGF-1R [51], or a receptor with the transmembrane mutation V922E [40] and the lack of induction of a proliferative response indicate that in addition to a basic off/on switch, the mutations can influence individual intracellular signaling pathways in a subtle fashion. This could be explained by differences in conformation of the intracellular domain of the IGF-1R depending on the replacement of its α-subunit by, e.g. Gag or ErbB2 sequences or simple truncation.

Our results provide further support for the idea that disruptions of the primary structure of the α-subunit or the β-subunit of the IGF-1R can have distinct qualitative and quantitative consequences for intracellular signaling. The conformation of the intracellular domain induced by the oncogenic variant of the ErbB2 transmembrane region is probably very similar to the conformation resulting from IGF-1 binding to the wild-type receptor.

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