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Suppression of EGFR Autophosphorylation by FKBP12

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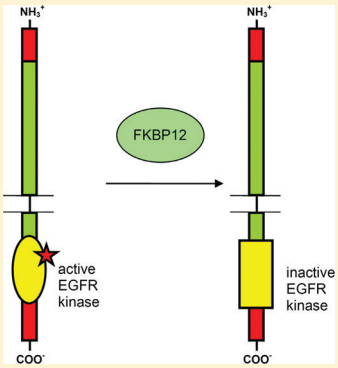
ABSTRACT: FK506 binding proteins (FKBPs) represent a subfamily of peptidyl prolyl *cis/trans* isomerases that can control receptor-mediated intracellular signaling. The prototypic PPIase FKBP12 functionally interacts with EGFR. FKBP12 was shown to inhibit EGF-induced EGFR autophosphorylation with all internal phosphorylation sites equally affected. The inhibition of EGFR catalytic activity is conducted by targeting the EGFR kinase domain. The change of intracellular FKBP12 levels resulted in a change of EGFR autophosphorylation level. Collectively, our results demonstrate that FKBP12 forms an endogenous inhibitor of EGFR phosphorylation directly involved in the control of cellular EGFR activity.



FK506 binding proteins (FKBPs) belong to the enzyme class of peptidyl prolyl *cis/trans* isomerases (PPIases) that are evolved to catalyze the *cis/trans* isomerization of prolyl bonds. This interconversion affects the spatial arrangement of backbone segments in the substrate proteins.[1]

Mainly FKBPs are found to be receptor accessory proteins. On the one hand, the hetero-oligomeric steroid receptor and the arylhydrocarbon receptor complexes contain multidomain PPIases in direct interaction with the chaperone Hsp90 via the TPR modules supplementing the FKBP domains of the respective folding helper enzymes.[2,3] On the other hand, the single-domain PPIase FKBP12 and FKBP12.6 active sites interact with the ryanodine receptor (RyR) calcium release channels and the receptor serine/threonine protein kinase TGFβR-I[4−6] as shown by FK506-mediated changes of the receptor response. FK506 represents a tight binding inhibitor of the PPIase activity of FKBPs.[7,8]

FKBP12 binding to skeletal muscle RyR1 and FKBP12.6 binding to cardiac muscle RyR2 play an important role in excitation−contraction coupling, and subconductance levels are thought to arise from FKBP depletion.[9−11] The FKBP12.6−RyR2 complex was shown to be an important candidate target for pharmacological prevention of ventricular tachycardia.[12] FKBP12 forms a regulator of TGFβR-I in that it prevents it from leaky signaling under suboptimal ligand concentrations.[13] Also, the epidermal growth factor receptor (EGFR) forms an FKBP target since FKBP12 was shown to inhibit EGFR autophosphorylation *in vitro*.[14]

EGFR is a receptor tyrosine kinase that belongs to the ErbB family of cell surface receptors and is involved in a variety of cellular processes.[15] The binding of the ligand epidermal growth factor (EGF) to EGFR induces an array of signaling events originating from activation of the receptor's intrinsic tyrosine kinase that phosphorylates specific tyrosine residues in the intracellular domain of the receptor dimers.[16−18] Numerous human carcinoma are promoted by uncontrolled EGFR activation and signaling. Besides EGFR stimulation through autocrine loops, it is well-known that elevated levels of EGFR in human tumors, including breast and ovarian tumors, correlate with earlier patient relapse and poor prognosis.[19−21]

A number of endogenous regulatory mechanisms are used by cells to circumvent oversignaling by ErbB receptors, including degradation of receptors mediated by receptor ubiquitination[22] or by modulator proteins interfering with ligand binding and receptor activation or suppressing signaling following receptor activation.[23,24] Whereas binding of the feedback inhibitors LRIG1, SOCS4, and SOCS5 promotes degradation of EGFR,[25,26] RALT/MIG6 mediates suppression of the EGFR tyrosine kinase catalytic activity.[27]

The results of the present study provide evidence that human EGFR functionally interacts with the human prototypic PPIase FKBP12. FKBP12 was shown to inhibit *in vitro* EGF-induced EGFR autophosphorylation with all internal phosphorylation sites equally affected. This inhibition of EGFR catalytic activity is conducted by targeting the EGFR kinase domain. The change of intracellular FKBP12 level by overexpression or RNA interference resulted in a change of the autophosphorylation level of EGFR.

Taken together, our results provide support for the hypothesis that the prototypic FKBP12 forms an endogenous inhibitor of EGFR phosphorylation directly involved in the control of cellular EGFR activity.

EXPERIMENTAL PROCEDURES

Materials. Anti-EGFR antibody and anti-Phospho-Tyr antibody were purchased from Sigma. Phosphorylation site specific anti-Phospho-Tyr antibodies were purchased from Cell Signaling Technology, Epitomics, and Antibodies-online.

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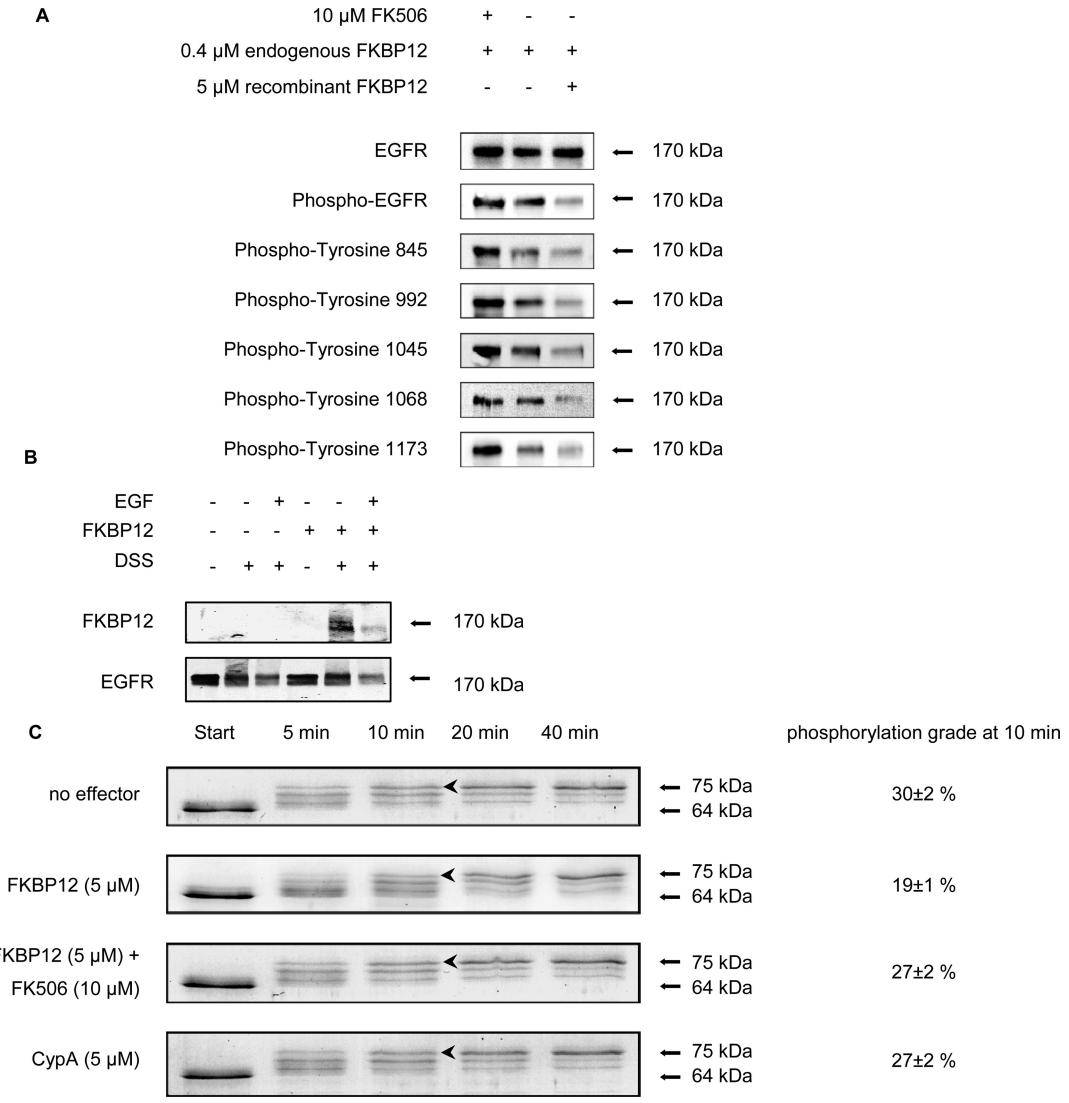

Figure 1. FKBP12 decelerates the autophosphorylation of EGFR. (A) A431 cell lysates were preincubated in the presence of FK506 or FKBP12, respectively. After 60 min reaction in the presence of ATP, samples were analyzed by immunoblotting. (B) A431 cell lysates were incubated in the presence of FKBP12. After 60 min cross-linking by DSS, samples were analyzed by immunoblotting. (C) Recombinant $EGFR_{645-1186}$ was preincubated in presence of FKBP12, FKBP12/FK506, or CypA. Sample analysis after autophosphorylation in the presence of ATP was performed by 10% SDS-PAGE and Coomassie staining. From the densitometric quantification of the unphosphorylated $EGFR_{645-1186}$ (start) and the fully phosphorylated $EGFR_{645-1186}$ (arrowheads), the percentage of fully phosphorylated $EGFR_{645-1186}$ at 10 min was calculated. The experiments were performed in triplicate.

Peptides were synthesized by Fmoc chemistry according to standard protocols.

Overexpression and Purification of Proteins. Recombinant FKBP12 was expressed and purified as previously described.[28] $EGFR_{645-998}$ and $EGFR_{645-1186}$ with an additional N-terminal Strep-tag II were expressed by baculovirus in Sf9 insect cells. The soluble proteins were purified from the cell lysate by streptactin affinity chromatography according to the supplier's instructions followed by gel filtration yielding in >95% pure protein.

Cell Culture and Transfection. A431 and HeLa cells were cultured according to the supplier's instructions. To generate the plasmid pEGFP-FKPB12, the FKBP12 *Hin*dIII/*Eco*RI fragment was inserted into the *Hin*dIII-*Eco*RI site of pEGFP-C1 (Clontech). For confocal microscopy, exponentially growing A431 cells were transfected with the plasmid pEGFP-FKPB12 and cultured in μ-Slide 8-well plates (ibidi). The cells were visualized with a Nikon Confocal C1 microscope. The FKBP12 siRNA sense sequences used in this study were 5′-GCACAAGUGGUAGGUUAAC-3′ (S1) (MWG Biotech) and 5′-CCCUUUAAGUUUAUGCUAGG-CAAGC-3′ (S2) (OriGene). The siRNAs were transfected into HeLa cells using Nanofectin siRNA reagent (PAA).

EGFR Kinase Assays. Prior to the phosphorylation reactions, the reaction mixtures were preincubated for 30 min in the presence of the indicated PPIases. All reactions were carried out in 50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and 2 mM DTT. Phosphorylation started after the addition of ATP (2 mM).

A431 cell lysate containing full length EGFR was complemented with EGF (250 nM). Autophosphorylation (0 °C) was stopped by the addition of SDS sample buffer and incubation at 95 °C. Western blot analysis using anti-EGFR antibody and anti-Phospho-Tyr antibody revealed the EGFR's

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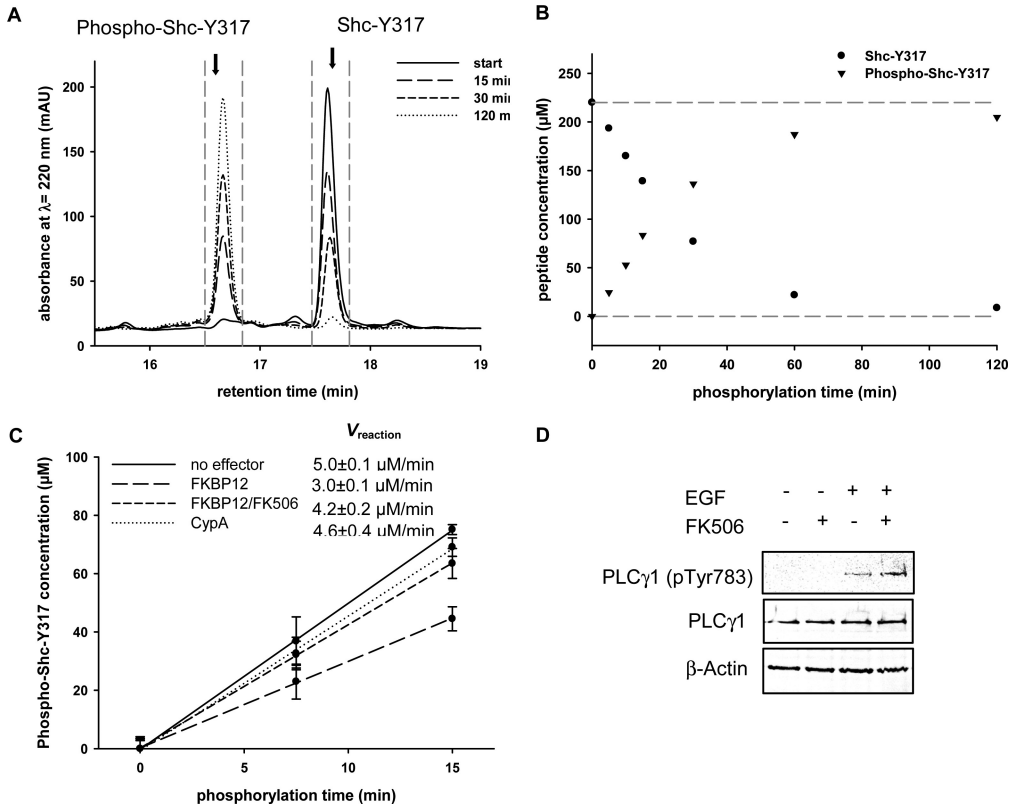

Figure 2. FKBP12 decreases the kinase activity of $EGFR_{645-998}$. (A) HPLC profiles of the peptide Shc-Y317 previously incubated in presence of $EGFR_{645-998}$ (0.25 μM) and ATP. (B) Time course of the decrease of ShcY317 concentration (circles) and the increase of Phospho-ShcY317 concentration (triangles) during the phosphorylation reaction as calculated from peptide peak areas in the HPLC profiles. (C) Phosphorylation rates of the controls or in the presence of FKBP12 (long dashed line). Each data point was measured in triplicate. (D) FK506 increases EGF-induced PLCγ1 tyrosine phosphorylation. HeLa cells were incubated with or without FK506 and EGF. Samples were analyzed by SDS-PAGE and immunoblotting using the respective antibodies. The experiments were performed in triplicate.

phosphorylation status. The quantification analysis of Western blotting results was performed by using the aida software. The autophosphorylation of 0.7 μM $EGFR_{645-1186}$ (25 °C) was monitored by SDS-PAGE, Coomassie staining, and densitometric analysis of the appropriate bands. The phosphorylation of the peptide substrate Shc-Y317 (220 μM) by 0.25 μM $EGFR_{645-998}$ (25 °C) was stopped by the addition of 50 mM EDTA. Reaction mixtures were separated by RP-HPLC (Vydac 218TP C18 column) using a 30 min 10−40% water/acetonitrile (0.1% TFA) gradient. The identity of the phosphorylated and unphosphorylated peptide Shc-Y317 was verified by ESI mass spectrometry (Esquire-LC MS, Bruker).

Chemical Cross-Linking. Cross-linking was carried out as described in the literature.[29] A431 cell membranes were preincubated with and without 4 μM FKBP12 for 30 min in PBS, followed by treatment with and without 0.6 μM EGF, and then incubated with 1 μM DSS. A431 cells cultured in serum-free medium were grown in the presence and absence of 4 μM FK506 or 5.5 μM external FKBP12 followed by treatment with and without 0.6 μM EGF. After washing with PBS 3 times they were subjected to DTSSP.

RESULTS

FKBP12 Inhibits the Autophosphorylation of EGFR *in Vitro*. EGFR harbors several autophosphorylation sites with different signaling functions. Therefore, analysis of FKBP12-mediated inhibition of phosphorylation of specific Tyr residues of the EGFR was undertaken. Autophosphorylation of EGFR in A431 cell lysates was performed in the additional presence of either the tight binding FKBP inhibitor FK506 or externally supplied FKBP12. EGF-induced receptor phosphorylation was examined using various phosphorylation site specific anti-Phospho-EGFR antibodies and an anti-EGFR antibody (Figure 1A). The phosphorylation status of all Tyr residues examined was decreased by FKBP12 to an extent similar to the overall autophosphorylation level when compared to the EGFR autophosphorylation without additives. In the latter case, 0.4 μM intrinsic FKBP12 is present which originates from the A431 cell lysate used as determined densitometrically by Western blot analysis. To avoid confounding effects of the intrinsic FKBP12, the EGFR autophosphorylation reaction was performed in the presence of the FKBP inhibitor FK506. Consistently, in the presence of FK506, all Tyr residues exhibited stronger ligand-induced phosphorylation when compared to EGFR in the absence of FK506.

In order to capture the transient interaction between FKBP12 and EGFR, we performed chemical cross-linking (Figure 1B). As detected by immunoblotting, both proteins form a complex of ca. 170 kDa whose presence is dependent on stabilization by cross-linking with DSS.

Since FKBP12 is a predominantly cytoplasmic protein which interacts with several receptors at their cytoplasmic domains, we analyzed how FKBP12 affects the autophosphorylation of the cytoplasmic part of EGFR ($EGFR_{645-1186}$). The $EGFR_{645-1186}$ protein was expressed and purified as Step-tag II fusion protein from Sf9 cells. Progression of autophosphor-

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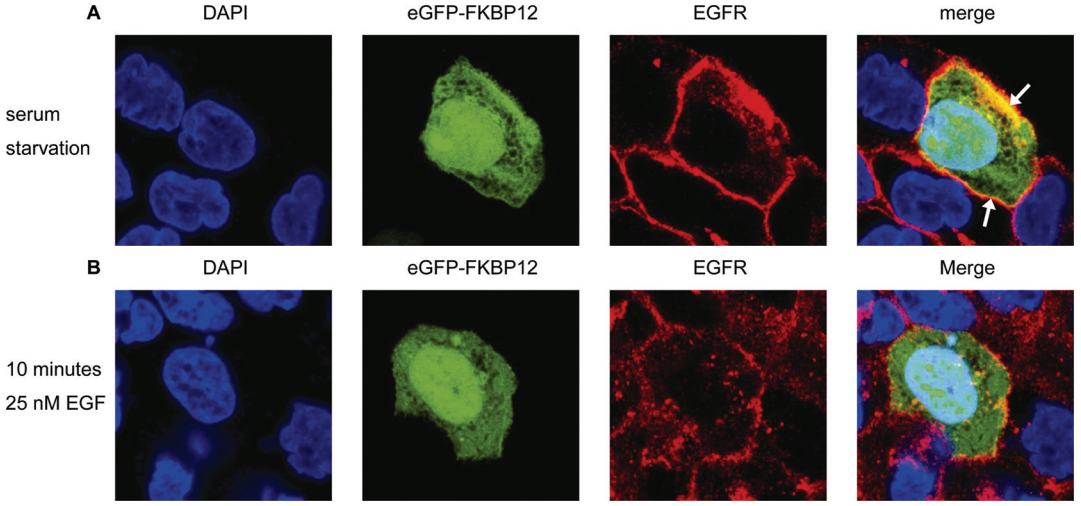

Figure 3. EGFR and eGFP-FKBP12 colocalize in unstimulated A431 cells (A) but not in EGF-stimulated A431 cells (B). DNA was stained with DAPI (blue), eGFP-FKBP12 is shown in green, and EGFR is shown in red. The preparations were examined by confocal microscopy.

ylation of $EGFR_{645-1186}$ was examined by SDS-PAGE at different time points (Figure 1C). A shift of electrophoretic mobility from 64 to 75 kDa indicated complete phosphorylation under the conditions studied. To verify this, the upper band isolated by SDS-PAGE was in-gel digested by trypsin and the peptide mixture was analyzed by MALDI-TOF mass spectrometry (Ultraflex II MS, Bruker). Thus, phosphorylation of $EGFR_{645-1186}$ on eight Tyr residues (Tyr845, Tyr974, Tyr992, Tyr1045, Tyr1068, Tyr1086, Tyr1148, and Tyr1173) was revealed. Minor protein bands between 64 and 75 kDa could not be assigned to a distinct phosphorylation pattern of $EGFR_{645-1186}$.

We performed the autophosphorylation assay in the absence of any effector, in the presence of FKBP12, in the presence of FKBP12/FK506, and, as an additional control, in the presence of CypA. CypA is an alternate PPIase that does not interact with EGFR. In the presence of FKBP12, the initial velocity of formation of the fully phosphorylated $EGFR_{645-1186}$ was decelerated in comparison to the controls (Figure 1C, arrowheads), whereas the final yield of fully phosphorylated $EGFR_{645-1186}$ at the end of the reaction was unaffected. Because no proteins additional to the two purified recombinant proteins FKBP12 and $EGFR_{645-1186}$ were present in the assay, we conclude that FKBP12 directly targets the cytoplasmic part of EGFR without any interaction-mediating protein.

Next, we analyzed if FKBP12 influences the EGFR tyrosine kinase activity toward an external peptide substrate. To exclude an influence of EGFR autophosphorylation, an EGFR kinase domain ($EGFR_{645-998}$) that lacked the C-terminal regulatory region was expressed and purified as Step-tag II fusion protein from Sf9 cells. As substrate, we used the synthetic 17mer peptide Shc-Y317 that corresponds to the sequence of the principal phosphorylation site of the signaling protein Shc1 (amino acids 309−325).[30]

The Shc-Y317 peptide phosphorylation was monitored at different time points by rpHPLC where the phosphorylated peptide derivative can be resolved from the nonphosphorylated peptide, thus allowing quantification (Figure 2A). The identity of the peptides was verified by ESI-MS. The interconversion of the Shc-Y317 substrate peptide to the phosphorylated product by $EGFR_{645-998}$ is linear over the first 15 min (Figure 2B) as calculated by monitoring the decrease in the substrate peak as well as the increase of the product peak area. This allowed us to compare the rates of Shc-Y317 phosphorylation by $EGFR_{645-998}$ in the presence or absence of FKBP12. The rate in the presence of 5 μM FKBP12 was reduced to 60% of the value for the reaction in the absence of effector. The restored kinase activity of $EGFR_{645-998}$ in the presence of FK506 additional to FKBP12 underlines the importance of the PPIase activity of FKBP12. Inside HeLa cells, phosphorylation of a downstream substrate by EGFR is affected by FKBP12. We analyzed the phosphorylation status of PLCγ1 at Tyr783 in HeLa cells in the presence and absence of FK506. Consistently, an increase in the phosphorylation of PLCγ1 upon FKBP12 inhibition in HeLa cells activated with EGF was observed.

Colocalization of eGFP-FKBP12 and EGFR. In order to study a possible colocalization of FKBP12 and the EGFR in the cell, A431 cells were transfected transiently with the plasmid pEGFP-FKBP12 expressing the fusion protein eGFP-FKBP12. EGFR was visualized by using anti-EGFR antibody in combination with its rhodamine-conjugated secondary antibody (Figure 3A). As already described before, eGFP-FKBP12 locates predominantly in the cytoplasm and the nucleus while the EGFR is localized mainly at the plasma membrane. However, there is a partial colocalization of the two proteins at the plasma membrane (Figure 3A, white arrows) indicating the spatial and temporal potential of an interaction of both proteins in the cell. EGF-mediated stimulation of EGFR resulted in EGFR internalization, and no obvious eGFP-FKBP12/EGFR colocalization was observed (Figure 3B).

FKBP12 Influences EGFR Autophosphorylation in HeLa Cells. We next investigated the influence of different FKBP12 levels on EGFR autophosphorylation in HeLa cells. First, effects of eGFP-FKBP12 overexpression were analyzed. HeLa cells were transfected with plasmids that encoded eGFP-FKBP12 or eGFP as a control (Figure 4A). Expression of eGFP-FKBP12 additionally to endogenous FKBP12 resulted in an overall FKBP12 concentration of about 5 μM in HeLa cells. Phosphorylation of EGFR was slightly reduced in HeLa cells expressing eGFP-FKBP12 additionally to endogenous FKBP12.

Additionally, we analyzed whether endogenous FKBP12 inhibits EGFR autophosphorylation by suppressing FKBP12 protein levels using RNA interference. In order to exclude off-target silencing effects of the FKBP12 siRNA, we employed two

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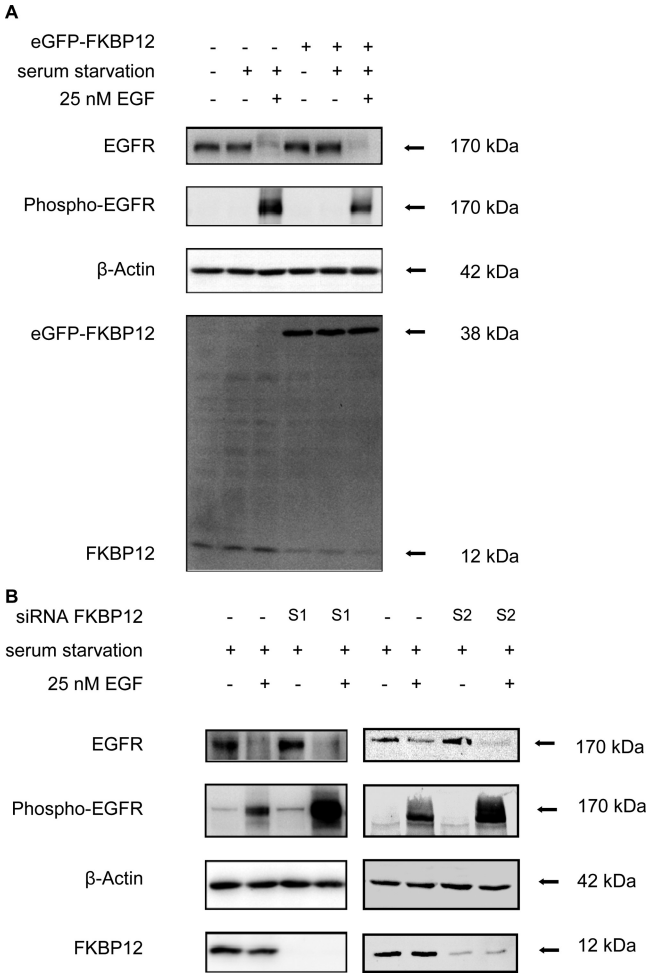

Figure 4. EGF-induced EGFR autophosphorylation in HeLa cells is dependent on intracellular FKBP12 concentration. (A) Comparison of cells expressing eGFP-FKBP12 to the vector control. The increased FKBP12 concentration led to a reduction in phosphorylated EGFR. Signal intensity for phosphorylated EGFR of cells with increased FKBP12 level was significantly reduced to about 60 ± 20% of the signal intensity of the control. (B) Comparison of cells transfected with siRNA sequence 1 (S1) and sequence 2 (S2) against FKBP12 or control siRNA. The decreased FKBP12 concentration led to an increased amount of phosphorylated EGFR. Signal intensity for phosphorylated EGFR of cells with strongly decreased FKBP12 level (S1) was significantly increased to about 210 ± 70% of the signal intensity of the control. All samples were separated by SDS-PAGE and immunoblotted using the respective antibodies. Experiments were performed in triplicate ($p < 0.05$).

independent FKBP12 siRNAs, sequence 1 (S1) and sequence 2 (S2), and a control siRNA. HeLa cells were transfected and the levels of FKBP12 were analyzed by immunoblotting. Both FKBP12 siRNAs, but not control siRNA, specifically knocked down endogenous FKBP12 72 h post-transfection with siRNA S1 mediating the stronger reduction in the FKBP12 protein level (Figure 4B). Endogenous actin levels were unaffected. Depletion of endogenous FKBP12 from HeLa cells resulted in a strong increase in EGF-induced phosphorylation of EGFR of about 210 ± 70% of the signal intensity of the control containing endogenous FKBP12 as quantified for the efficient knock down by siRNA S1.

FKBP12 Suppresses EGFR Dimer Formation. To examine the influence of FKBP12 on the oligomeric state of EGFR, we undertook cross-linking analyses with the homobifunctional reagent DSS. Membrane preparations of A431 cells were preincubated in the presence or absence of FKBP12 with or without EGF and treated with the cross-linker. As already described, EGFR is able to form ligand-independent dimers additional to the ligand-induced dimers.[29] The formation of ligand-induced dimers was slightly reduced in the presence of FKBP12, whereas the formation of ligand-independent dimers was prevented completely (Figure 5A).

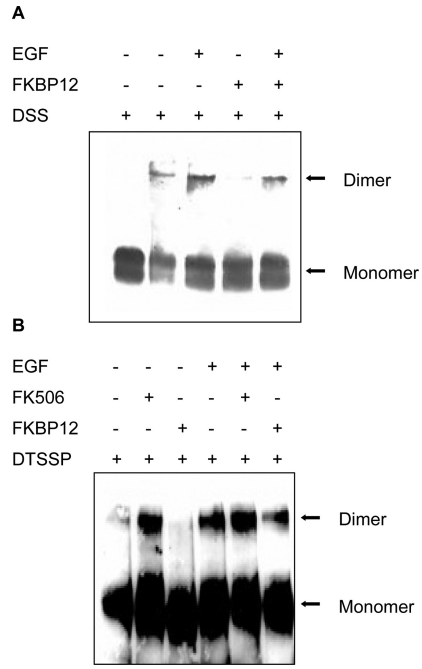

Figure 5. FKBP12 suppresses the ligand-induced and the ligand-independent EGFR dimer formation. (A) A431 cell membranes were preincubated with or without FKBP12 for 30 min, followed by treatment with or without EGF and incubation with DSS. (B) A431 cells cultured in serum-free medium were grown in the presence and absence of FK506 and external FKBP12 followed by treatment with and without EGF and then subjected to DTSSP. The samples were separated by 5% SDS-PAGE in the absence of reducing agents and immunoblotted using anti-EGFR antibody.

Next we analyzed the oligomeric state of EGFR in the plasma membrane of intact A431 cells. A431 cells cultured in serum-free medium were preincubated with and without EGF and treated with the membrane-impermeable reagent DTSSP (Figure 5B). In intact cells, EGFR formed a small amount of ligand-independent dimers additional to the ligand-induced dimerization. Both in the absence and in the presence of EGF, the addition of FK506 clearly increased the dimer formation. External application of FKBP12 to A431 cells did not influence dimer formation, indicating the intracellular action of the enzyme.

DISCUSSION

Signaling by EGFR is known to require a counterbalance by negative signaling events to regulate proper thresholds of receptor signals. It has been shown that negative regulation of the EGFR is performed by several proteins expressed in response to EGFR activation by a feedback inhibition mechanism. Whereas the transcriptionally controlled feedback inhibitors LRIG1, SOCS4, and SOCS5 result in enhanced

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degradation of EGFR,[25,26] RALT/MIG6 mediates suppression of the EGFR tyrosine kinase activity.[27]

Suppression of the EGFR activity by FKBP12 shown herein represents an alternate negative signaling event in EGFR signal transduction. The inhibition of autophosphorylation of EGFR by FKBP12 is probably caused by targeting the tyrosine kinase of EGFR. Similar to the TGFβR-I/FKBP12 and RyR2/FKBP12.6 interactions,[9,13] the importance of the PPIase activity of FKBP12 for suppression of EGFR phosphorylation is underlined by its abrogation in the additional presence of FK506. FK506 is known to bind tightly to the active site of FKBP12, thus inhibiting its PPIase activity.[7,8] Thus, a common feature of single-domain FKBPs in receptor protein kinase and calcium release channel heterocomplexes is the influence of the PPIase active site on signaling in a manner dependent on enzymatic catalysis of prolyl bond isomerization.[6]

Consistently, reduction of endogenous FKBP12 protein levels by RNAi resulted in a significant increase in EGF-induced phosphorylation of EGFR. The fact that depletion of FKBP12 even increased ligand-independent phosphorylation of EGFR in HeLa cells (Figure 4B) suggests that FKBP12 might be involved in preventing leaky signaling. Expression of eGFP-FKBP12 additionally to endogenous FKBP12 resulted only in a slight reduction of EGF-induced EGFR phosphorylation, probably because the intracellular concentration of endogenous FKBP12 is rather high.

There is a growing body of literature about the diversity of conformations that EGFR can assume. Unligated and inactive EGFR spontaneously exists in preformed dimeric or oligomeric forms in principle sufficient for EGF-independent activation. The fact that FKBP12 significantly reduced ligand-independent formation of EGFR dimers (Figure 5) suggests that FKBP12 binding induces an EGFR conformation unable to form dimers. Alternatively, stoichiometric FKBP12 binding could sterically hinder dimer formation. Consistently, application of FK506, which releases bound FKBP12 from the FKBP12/EGFR complex, increased the formation of EGFR dimers significantly in nonstimulated A431 cells and to a minor extent in EGF-stimulated A431 cells.

The significant consequences of EGFR activation on cell life imply the necessity of multiple levels of control that minimize aberrant or mistimed EGFR activation. Possibly, the existence of multiple active and inactive conformations of EGFR[31] generates EGFR molecules exhibiting different affinities toward FKBP12. Data from the analysis of the interaction of FKBPs with RyRs or TGFβR-I suggest that FKBPs do not act as simple on/off switches but are involved in the subtle modulation of these signaling pathways. Our observations also show that FKBP12 elicits a gradual change in EGFR activity. We hypothesize that FKBP12 may act to form an activation threshold in which the activation of EGFR is achieved only above a certain level of ligand. Possibly, FKBP12 may be involved in suppression of transactivation under normal conditions. Alteration of the interaction between EGFR and FKBP12 in order to modulate signaling processes may arise from the occurrence of alternate FKBP12 substrates in the cell, change of receptor affinities, for example, by post-translational modifications or the change of the intracellular FKBP12 level. Downregulation of the FKBP12/12.6 level was already shown after aldosterone exposure.[32] This may result in shifting the equilibrium of basal autophosphorylation and dephosphorylation of EGFR toward the activated state. As FKBP12 is involved in the regulation of TGFβ signaling, calcium influx, and EGFR phosphorylation, influencing FKBP12 level or activity may result in rather complex changes of the signaling network.

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ABBREVIATIONS

EGFR, epidermal growth factor receptor; FKBP, FK506 binding protein; PPIase, peptidyl prolyl *cis/trans* isomerase.

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