Grb2 regulates Stat3 activation negatively in epidermal growth factor signalling

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EGF (epidermal growth factor) binding to its receptor (EGFR) induces dimerization and autophosphorylation of the receptor at multiple tyrosine residues, which serve as docking sites for recruitment of proteins with SH2 (Src homology 2) domains that activate multiple downstream signalling pathways. The adaptor protein Grb2 (growth factor receptor-binding protein 2) binds to EGFR, which leads to activation of Ras-MAPK (mitogen-activated protein kinase) cascade. The latent transcription factors, STAT (signal transduction and activator of transcription), can also be activated by EGF in certain cell types. Since Ras-MAPK and STAT pathways are simultaneously stimulated by EGF, and Tyr-1086 and Tyr-1068 of EGFR are reported to be the binding sites for both Grb2 and Stat3, we investigated the possible regulatory role of Grb2 in STAT activation. In the present study, we report that transient expression of Grb2 specifically down-regulates EGF-stimulated tyrosine phosphorylation of Stat3, which leads to a repression of Stat3 transcriptional activity. In contrast, depletion of Grb2 by RNA interference substantially increases Stat3 tyrosine phosphorylation induced by EGF. The inhibition is neither mediated by a direct interaction between Grb2 and Stat3 nor via activation of tyrosine phosphatases. However, the repression was abolished by a mutation in the SH2 domain, but not the SH3 domains of Grb2, suggesting that inhibition involves binding of the receptor. Indeed, Grb2 inhibits the interaction between Stat3 and EGFR by competitive binding to the EGFR. On the other hand, Grb2 does not interact with the same sites as Stat3 on the interleukin-6 receptor and, therefore, has no effect on interleukin-6-induced tyrosine phosphorylation of Stat3. Taken together, our results demonstrate that, in EGF signalling, Grb2 regulates Stat3 activation negatively at the receptor level.

Key words: epidermal growth factor, Grb2 (growth factor receptor-binding protein 2), receptor binding, SH2 domain (Src homology 2 domain), Stat3.

INTRODUCTION

Signalling by growth factor receptor tyrosine kinases (RTKs) elicits a wide range of cellular responses leading to proliferation, differentiation and cell survival in a variety of cell types. EGFR (epidermal growth factor receptor) was the first RTK to be discovered and characterized [1,2]. EGF (epidermal growth factor) binding induces receptor dimerization that triggers its intrinsic protein tyrosine kinase activity, which leads to autophosphorylation at multiple tyrosine residues in the cytoplasmic domain of the receptor. These phosphotyrosine motifs serve as docking sites for recruitment of the SH2 (Src homology 2)-domain- and phosphotyrosine-binding-domain-containing proteins. These include enzymes such as Src, phospholipase Cγ and phosphatidylinositol 3-kinase, which are activated by tyrosine phosphorylation, and adaptor proteins such as Grb2 (growth-factor-receptor-bound protein 2), Grb7 and Nck, which link RTKs to downstream signalling pathways [2].

Ras-MAPK (mitogen-activated protein kinase) is an important signalling pathway activated by EGF. The adaptor protein Grb2 plays a critical role in coupling signal from EGFR kinase with Ras. Grb2 contains a single SH2 domain flanked by two SH3 domains. The SH2 domain of Grb2 recognizes and binds to specific phosphotyrosine-containing motifs on EGFR, whereas the SH3 domains bind to the guanine nucleotide-releasing factor Son-of-sevenless, which catalyses the exchange of GDP to GTP on Ras, resulting in Ras activation [3,4]. Ras, in turn, activates the downstream kinases sequentially, which eventually leads to the activation of MAPKs/ERKs (extracellular-signal-regulated kinases). ERKs phosphorylate transcription factors that regulate gene transcription [5].

STAT (signal transduction and activator of transcription) represents a family of latent cytoplasmic transcription factors that were originally discovered in interferon signalling and subsequently found to be activated by various cytokines [6]. After cytokine stimulation, STAT proteins are recruited to the cytokine receptors and phosphorylated by the receptor-associated tyrosine kinases, Janus kinases, on a single tyrosine residue at the C-terminus. STAT proteins then form homo- or heterodimers by reciprocal interaction between the SH2 domains and phosphorylated tyrosine residues, translocate into the nucleus, bind to DNA and regulate their target gene expression [7]. In addition to cytokines, Stat proteins can also be activated by certain growth factors including EGF [8–11]. EGF induces the activation of Stat1, Stat3 and Stat5 in mouse liver and certain cell types such as cancer cell lines that overexpress EGFR [12,13]. Among them, Stat3 was demonstrated to play critical roles in EGF signalling in normal and tumour cells [14,15].

Therefore both Ras-MAPK cascade and Stat proteins can be simultaneously activated by EGF. Interestingly, it has been reported that Grb2 directly binds to the cytoplasmic domain of EGFR at phosphorylated residues Tyr-1068 and Tyr-1086 [4]

Abbreviations used: CAT, chloramphenicol acetyltransferase; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular-signal-regulated kinase; Grb2, growth-factor-receptor-bound protein 2; GST, glutathione S-transferase; HA, haemagglutinin; IL-6, interleukin-6; MAPK, mitogen-activated protein kinase; RTK, receptor tyrosine kinase; SH2, Src homology 2; siRNA, small interfering RNA; STAT, signal transduction and activator of transcription.

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to initiate the activation of the Ras-MAPK pathway. On the other hand, multiple tyrosine residues, including Tyr-1068 and Tyr-1086, were also reported to be required for receptor binding and activation of Stat3 in EGF signalling [16–18]. This raises questions as to how the receptor binding of these two proteins is co-ordinately regulated, and what may be the consequence of such regulation on the activation of Ras-MAPK and Stat pathways. In the present study, we investigated the effect of Grb2 on the activation of Stat3 by EGF. We found that overexpression of Grb2 resulted in the inhibition of Stat3 tyrosine phosphorylation and depletion of Grb2 leads to an increase in this phosphorylation. The inhibition was not due to a direct interaction between Grb2 and Stat proteins, but is mediated by a competitive binding of Grb2 to the EGFR. Our results identified Grb2 as a negative regulator of Stat3 in EGF signalling.

MATERIALS AND METHODS

Materials

EGF was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and IL-6 (interleukin-6) from PeproTech (London, U.K.). Antibodies against phospho-Tyr-701 of Stat1, phospho-Tyr-705 of Stat3 and pMAPK were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The antibody of EGFR was obtained from Transduction Laboratories (San José, CA, U.S.A.). The agarose-conjugate EGFR antibody and anti-HA (haemagglutinin) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-FLAG M2 antibody (where FLAG stands for DYKDDDDK) was purchased from Sigma (St. Louis, MO, U.S.A.). Anti-phosphotyrosine antibody, 4G10, and antibody against GST (glutathione S-transferase) were obtained from Upstate Biotechnology and Pharmingen (San Diego, CA, U.S.A.) respectively.

Construction of expression plasmids

Expression plasmids of mouse Stat3 (pRC/CMV-Stat3), human Stat1 (pMNC-91) and mouse Stat5a were obtained from Dr J. E. Darnell, Jr (Rockefeller University, New York, NY, U.S.A.) and Dr A. Miyajima (University of Tokyo, Tokyo, Japan). The full-length Stat1 and Stat5a were cloned into plasmid pXJ40-FLAG, and Stat3 was cloned into either plasmid pXJ40-FLAG or pXJ40-MYC as described previously [19,20]. The pEBG-Grb2 and its SH3 domain mutants were kindly provided by J. M. Masuda-Robens (University of Pennsylvania, Philadelphia, PA, U.S.A.) [21]. The wild-type and the SH3 mutants of Grb2 were fused to GST by cloning into the pXJ40-GST vector. The pXJ40-HA-Grb2 construct was obtained from D. H. Lao (Institute of Molecular and Cell Biology, Singapore). A point mutation in the SH2 domain of Grb2 was introduced by using primers containing the mutation and the full-length pXJ40-HA-Grb2 as templates. The long template PCR kit with *Pfu* DNA polymerase obtained from Promega (Madison, WI, U.S.A.) was used, followed by *Dpn*I digestion and subsequent transformation. The mutagenesis was confirmed by sequencing on a ABI PRISM 3700 DNA analyzer using BigDye (v3.0) obtained from Applied Biosystems (Foster City, CA, U.S.A.).

RNA interference

Two pairs of 21-nucleotide sense and antisense RNA oligonucleotides with two 3′ overhanged (2′-deoxy) thymidine residues (dT) were synthesized by Dharmacon Research (Lafayette, CO, U.S.A.). The oligonucleotides of Grb2 siRNA (small interfering RNA) were sense, 5′-CGAAGAAUGUGAUCAGAACdTdT-3′, and antisense, 5′-GUUCUGAUCACAUUCUUCGdTdT-3′, corresponding to the human Grb2 coding nucleotides 85–105. The non-specific oligonucleotides containing the following sequences were used as a negative control: sense, 5′-GCCAAUAGAUGCAAGAUCAdTdT-3′, and antisense, 5′-UGAUCUUGCAUCUAUUGGCdTdT-3′. Equal amounts of sense and antisense RNA oligonucleotides were mixed and annealed according to the manufacturer's instructions. A431 cells were transfected with 40 μl of 20 μM siRNA of Grb2 or the control siRNA duplex and 2 μg of Myc-Stat3 with LIPOFECTAMINE™ 2000 reagent for 12 h. Cells were changed to normal culture medium for 14 h and treated with EGF for 30 min before harvesting.

Cell culture and DNA transfection

COS-1, A431 and HepG2 cells were grown in Dulbecco's modified Eagle's medium with 10 % (v/v) foetal calf serum purchased from Gibco-Invitrogen (Carlsbad, CA, U.S.A.). Transient transfections were performed with LIPOFECTAMINE™ or LIPOFECTAMINE™ 2000 obtained from Gibco-Invitrogen or with Fugene 6 from Roche (Mannheim, Germany) according to the manufacturer's instructions.

Immunoprecipitation and Western-blot analysis

Transfected cells were washed with cold PBS, lysed in RIPA buffer [150 mM NaCl/50 mM Tris/HCl, pH 7.2/1 % (w/v) deoxycholic acid/1 % (v/v) Triton X-100/0.25 mM EDTA, pH 8.0/0.2 % (w/v) NaF/0.1 % (w/v) sodium orthovanadate] [protease inhibitor cocktail tablet (Roche) was added to the RIPA buffer according to the manufacturer's instructions] and subjected to immunoprecipitation as described previously [22]. The precipitates were washed twice with RIPA buffer and twice with PBS, separated by SDS/PAGE and transferred on to a PVDF membrane. The membrane was blocked with PBS containing 0.1 % Tween 20 and 1 % BSA before incubation with the appropriate primary and secondary antibodies. The bound proteins were visualized using ECL® solution from Amersham Biosciences (Uppsala, Sweden). For reblotting, membranes were incubated in stripping buffer [62.5 mM Tris/HCl, pH 6.8/2 % (w/v) SDS/100 mM 2-mercaptoethanol] for 30 min at 60 °C before washing, blocking and incubating with antibody.

CAT (chloramphenicol acetyltransferase) assay

The method employed was as described previously [23]. CAT assays were normalized with equivalent β-galactosidase activity. Acetylated and non-acetylated forms of [^{14}C]chloramphenicol were separated by TLC, followed by autoradiography and quantification using a Bio-Rad GS700 imaging densitometer (Bio-Rad, Hercules, CA, U.S.A.).

RESULTS

Grb2 inhibits Stat3 tyrosine phosphorylation stimulated by EGF

To investigate the effect of Grb2 on EGF-mediated Stat3 activation, COS-1 cells that express low levels of endogenous Stat3 and strongly respond to EGF stimulation were transfected with FLAG-tagged Stat3 and/or HA-tagged Grb2. The cells were either left untreated or treated with EGF, and the phosphorylation

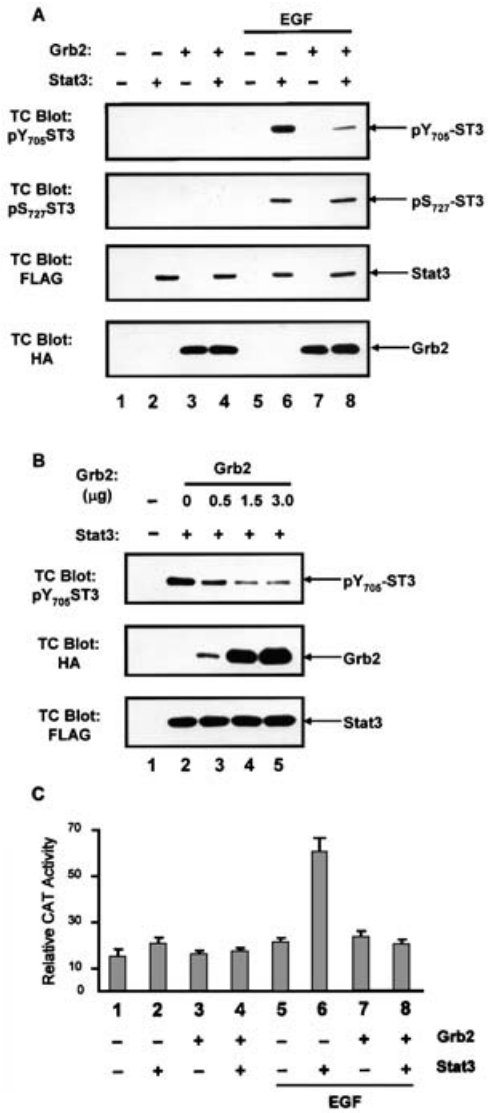

Figure 1 Repression of EGF-stimulated Stat3 tyrosine phosphorylation and transcriptional activity by Grb2

(**A**) COS-1 cells were transfected with FLAG-tagged Stat3 expression plasmid and/or HA-tagged Grb2 plasmid. The cells were either left untreated or treated with EGF (100 ng/ml) for 15 min. Total cell lysates (TC) were prepared and subjected to Western-blot analysis using antibody raised against phospho-Tyr-705-Stat3 ($pY_{705}ST3$) to detect the tyrosine-phosphorylated Stat3, as indicated in the top panel. The blot was stripped and reprobed with anti-phospho-Ser-727 (second panel), anti-FLAG (third panel) and anti-HA (bottom panel) antibodies respectively. (**B**) COS-1 cells were transfected with Stat3 and different amounts of the Grb2 expression plasmid and were stimulated with EGF for 15 min. Total cell lysates were subjected to Western-blot analysis as described in (**A**). (**C**) COS-1 cells were transfected with Stat3 and/or Grb2 expression plasmids, as labelled at the bottom of the Figure, together with pCMV-β-gal and reporter plasmid pSIE-CAT. Cell lysates were normalized by β-galactosidase assay and used for the CAT assay as described in the Materials and methods section. CAT activity was quantified using a Bio-Rad GS700 imaging densitometer and error bars denote S.D. from three independent transfection experiments.

status of Stat3 was examined by Western-blot analysis. In Stat3-transfected cells, a high level of Stat3 tyrosine phosphorylation was induced by EGF stimulation (Figure 1A, top panel, lane 6). However, it was inhibited when Grb2 was co-transfected (lane 8). On the other hand, EGF-induced serine phosphorylation of Stat3 was not affected by Grb2 (second panel), and the expression levels of Stat3 (third panel) and Grb2 (bottom panel) remained unchanged.

To confirm further the specificity of the negative effect of Grb2, various amounts of Grb2 were co-transfected with Stat3 and cells were treated with EGF. The inhibition of Stat3 tyrosine phosphorylation could be detected by as little as 0.5 μg of the Grb2 expression plasmid, and this inhibitory effect was increased in a dose-dependent manner (Figure 1B, lanes 2–5).

Grb2 represses Stat3 transcriptional activity stimulated by EGF

We next investigated whether Grb2-mediated inhibition of Stat3 tyrosine phosphorylation could lead to repression of Stat3 transcriptional activity. COS-1 cells were co-transfected with a reporter plasmid containing a CAT gene driven by three copies of a high-affinity binding site of Stat3, hSIE, together with the Stat3 expression plasmid in the absence or presence of Grb2. The CAT assay results showed that transcriptional activity of Stat3 was stimulated by EGF to 2.5-fold above the unstimulated level (Figure 1C, cf. lanes 2 and 6), but the induction was abolished in the presence of Grb2 (lane 8). Together, these results suggest that in addition to Stat3 tyrosine phosphorylation, Grb2 also has a negative effect on EGF-induced Stat3 transcriptional activity.

Depletion of endogenous Grb2 expression enhances Stat3 tyrosine phosphorylation stimulated by EGF

The inhibitory effect of Grb2 shown above was based on its overexpression. We speculated whether this effect could occur under physiological conditions. To address this issue, we designed an siRNA for Grb2 and a non-specific siRNA as control, and introduced them into the epidermoid carcinoma cell line A431 [24]. The effect of the siRNA on the EGF-mediated tyrosine phosphorylation of Stat3 was examined. Tyrosine phosphorylation of Stat3 by EGF (Figure 2, top panel, lane 1) was further enhanced by co-transfection of the Grb2-specific siRNA (lane 3). Correspondingly, the expression of the Grb2 protein was effectively blocked by Grb2 siRNA (third panel, lane 3). In contrast, the control siRNA had no effect on the Grb2 expression and tyrosine phosphorylation of Stat3 (lane 2). Interestingly, the EGF-induced ERK phosphorylation was diminished by Grb2 siRNA (fourth panel, lane 3). Although the siRNA was based on the human Grb2, it also inhibited endogenous Grb2 and enhanced Stat3 tyrosine phosphorylation in the monkey cell line COS-1 (results not shown). These results provide direct evidence of a negative role for the endogenous Grb2 in regulating EGF-mediated Stat3 activation.

Repression of Stat3 activity by Grb2 does not involve a direct interaction

In the following experiments, we attempted to unravel the mechanisms of the inhibitory effect of Grb2. First, we examined whether this inhibition was caused by a direct association between Stat3 and Grb2. To this end, cells were transfected with Myc-tagged Stat3 and/or HA-tagged Grb2 or HA-tagged GRIM-19 as a positive control. The lysate was immunoprecipitated with anti-Myc antibody, and the co-immunoprecipitated proteins were subjected to Western-blot analysis using anti-HA as a probe. The results indicated that Grb2 did not co-immunoprecipitate with Stat3 in cells either left untreated or treated with EGF (Figure 3, top panel, lanes 5 and 6). In contrast, GRIM-19, a newly identified Stat3-interacting protein [19], co-precipitated with Stat3 (lane 8). These results suggest that the negative regulatory effect of Grb2 on Stat3 is unlikely to be mediated by a physical interaction between the two proteins.

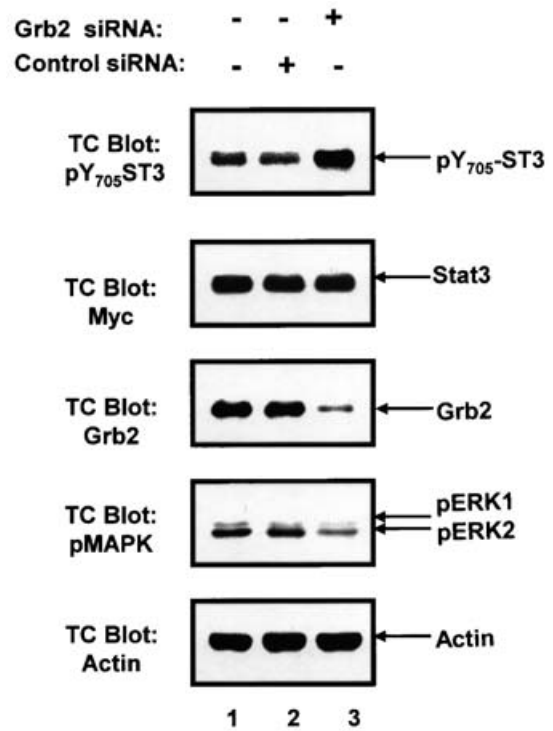

Figure 2 Depletion of Grb2 enhances tyrosine phosphorylation of Stat3 stimulated by EGF

A431 cells were transfected with Myc-tagged Stat3 alone (lane 1) or with Myc-tagged Stat3 in the presence of control (lane 2) or Grb2 siRNA (lane 3) and then stimulated with EGF for 30 min as described in the Materials and methods section. Total cell lysates were subjected to Western-blot analysis using various antibodies as labelled on the left of each panel. Actin was used as a control to demonstrate that the amount of protein in the lysates is equal.

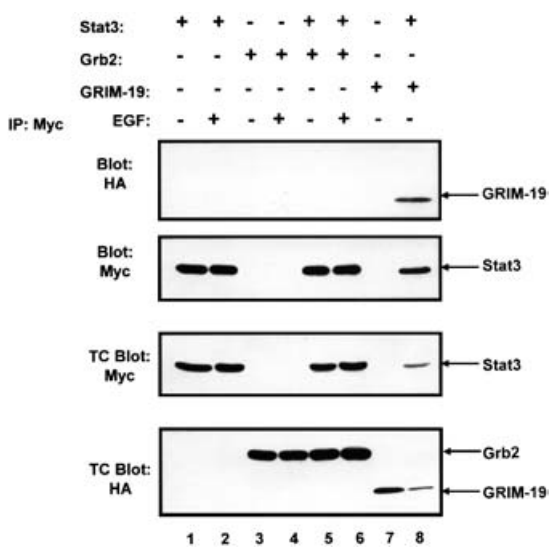

Figure 3 Lack of interaction between Stat3 and Grb2

COS-1 cells were transfected with Myc-tagged Stat3 and/or HA-tagged Grb2 or HA-tagged GRIM-19 plasmid. The cell lysates were immunoprecipitated (IP) with anti-Myc antibody. The precipitates were separated by SDS/PAGE, and blotted with anti-HA antibody (uppermost panel). The blot was stripped and reblotted with anti-Myc antibody (second panel). Total cell lysates were subjected to Western-blot analysis to show the expression level of Stat3 (third panel) and Grb2/GRIM-19 (fourth panel).

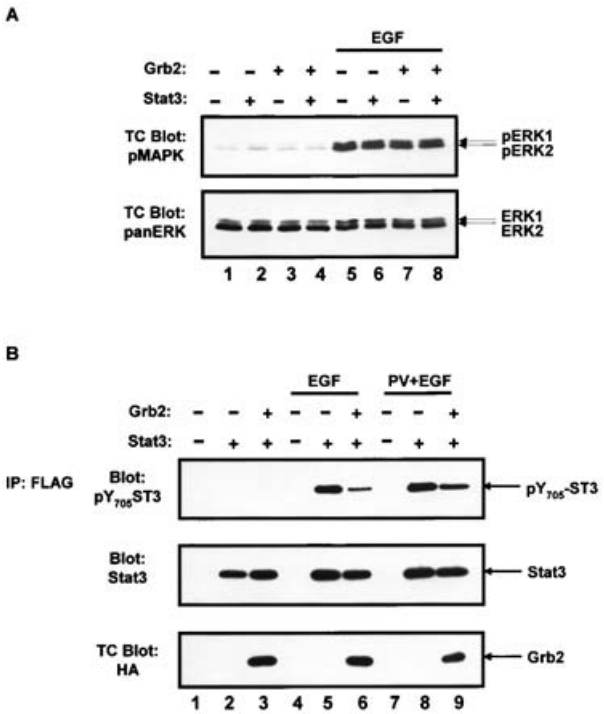

Figure 4 Effect of MAPK and protein tyrosine phosphatase inhibitor on the Grb2-mediated repression

(**A**) COS-1 cells were transfected with Stat3 expression plasmid and/or Grb2 plasmid. The cells were left untreated (lanes 1–4) or treated with EGF (lanes 5–8) for 15 min. The cell lysates were subjected to Western-blot analysis using antibody raised against phospho-MAPK (pMAPK; upper panel) or pan-ERK (lower panel). (**B**) The cells were left untreated (lanes 1–3) or treated with EGF for 15 min (lanes 4–6) or treated with pervanadate (0.1 mM Na_3VO_4/0.2 mM H_2O_2) for 15 min before EGF treatment (lanes 7–9). Pervanadate (PV) was freshly prepared by incubating Na_3VO_4 and H_2O_2 for 20 min at room temperature (23 °C). The cell lysates were immunoprecipitated (IP) with anti-FLAG and probed with anti-pY_{705}ST3 antibody (upper panel). The blot was stripped and reprobed with an anti-Stat3 antibody (middle panel) to show the amount of Stat3 immunoprecipitated. The expression of HA–Grb2 was detected by Western-blot analysis as shown in the lower panel.

Effect of ERKs and protein tyrosine phosphatases on the repression of Stat3 by Grb2

It has been reported that activation of MAPK (ERK2) results in repression of Stat3 activation [23,25]. Since the MAPK pathway is activated by EGF via Grb2, we next examined whether Grb2 inhibits Stat3 tyrosine phosphorylation via activation of ERKs by measuring their phosphorylation. The phosphorylation levels of ERK1 and ERK2 were undetectable in the unstimulated COS-1 cells, but were strongly stimulated by EGF. However, the phosphorylation levels of ERKs were not further increased by the overexpression of Grb2 (Figure 4A, upper panel, cf. lanes 6 and 8), suggesting that repression due to activation of ERKs is unlikely. No further activation of ERKs by Grb2 could be due to the limited amount of factors such as EGFR and/or Son-of-sevenless in the cells.

Another possibility is that when Grb2 is bound to the EGFR after EGF stimulation, it might recruit phosphatases to the receptor via its SH3 domains and thereby dephosphorylate Stat3. To address this issue, cells were treated with the protein tyrosine phosphatase inhibitor, pervanadate, before EGF treatment. As shown in Figure 4(B), Stat3 tyrosine phosphorylation was still repressed, albeit less effectively, in the presence of the phosphatase inhibitor. These results indicate that protein tyrosine phosphatase does not

play a major role but probably a partial role in Grb2-mediated inhibition of Stat3 phosphorylation.

Grb2 down-regulates Stat3 tyrosine phosphorylation through its SH2 domain

We further studied the domains of Grb2 that might be responsible for its inhibitory effect on Stat3. To this end, mutants that contain point mutations in the SH2 or SH3 domains of Grb2 were utilized. The mutations were designed such that binding of the proteins to the mutant domain was abolished specifically without affecting the binding activity of the non-mutated domains [26]. In addition to the wild-type Grb2, mutants with mutations in the SH3 domains as shown in Figure 5(A) were fused to GST, and co-transfected into COS-1 cells with Stat3. Their effects on Stat3 tyrosine phosphorylation were examined. Expression of mutants with either a single mutation [W36K (Trp-36 → Lys) and W193K] or a double mutation (W36, 193K) in the SH3 domains attenuated Stat3 tyrosine phosphorylation stimulated by EGF to a similar level as the wild-type Grb2 (Figure 5B, top panel). In contrast, the repressive effect of Grb2 on Stat3 tyrosine phosphorylation was ablated by a single mutation (R86L) within the SH2 domain of Grb2 (Figure 5C, top panel, lane 5). Therefore the inhibition of Stat3 phosphorylation is mediated by the SH2 domain, but not the SH3 domains of Grb2.

Grb2 inhibits the association of Stat3 and EGFR by competitive interaction with EGFR

It has been reported that Grb2 binds to the phosphorylated Tyr-1068 and Tyr-1086 sites of EGFR via its SH2 domain [4]. On the other hand, these residues have also been identified to be the binding sites for Stat3 [18]. Since the inhibitory effect of Grb2 was shown to be mediated by its SH2 domain, it is possible that the inhibition could be due to a competitive binding between Grb2 and Stat3 to the same sites on the EGFR. To address this hypothesis, we tested the association of Stat3 with endogenous EGFR in the absence or presence of increased doses of Grb2 in COS-1 cells. The EGFR was pulled down by the antibody conjugated with agarose beads and the associated Stat3 was detected by immunoblotting with anti-Myc antibody. As shown in Figure 6, Stat3 co-precipitated with EGFR under the normal growth condition (uppermost panel, lane 2). However, this association was diminished in a dose-dependent manner by co-transfection of increasing amounts of Grb2 (lanes 4–6). Concomitantly, the interaction between Grb2 and endogenous EGFR was enhanced gradually (second panel, lanes 4–6). The amount of EGFR pulled down in each sample was comparable (third panel). Consistent with this result, the interaction between Stat3 and EGFR was further increased in the EGF-stimulated cells, which was also inhibited by Grb2 (uppermost panel, lanes 8 and 10). These results indicate that Grb2 inhibits Stat3 tyrosine phosphorylation by competitive binding to the EGFR.

Grb2 does not affect Stat3 tyrosine phosphorylation stimulated by IL-6

Besides growth factors, Stat3 is strongly activated by IL-6 cytokines. The cytoplasmic domain of the IL-6 receptor subunit, gp130, contains six specific tyrosine residues that are phosphorylated after stimulation of IL-6. Stat3 can be recruited to gp130 by binding to any of the four tyrosine residues at the C-terminal region of gp130 after IL-6 stimulation [27,28]. However, Grb2 does not directly interact with gp130 [29]. We next examined

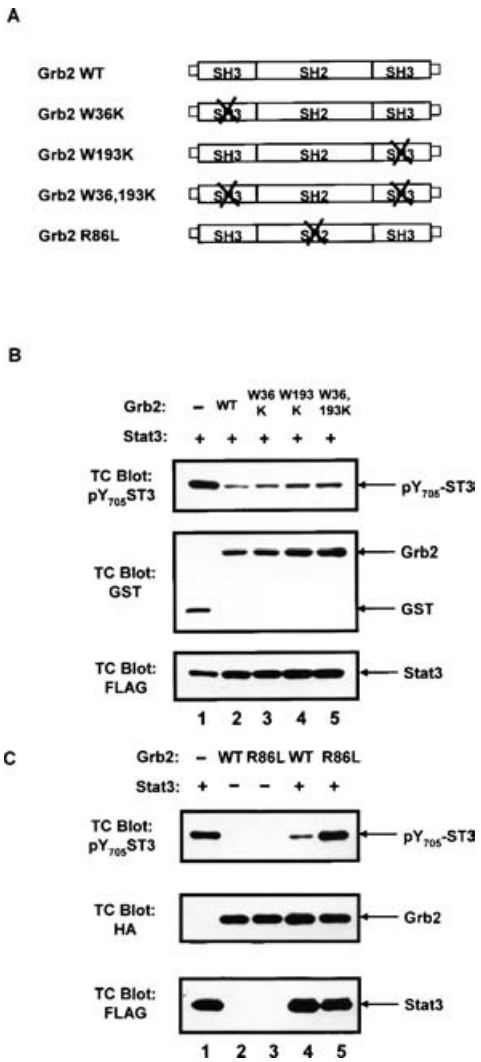

Figure 5 Effect of SH2/SH3 domain mutants of Grb2 on tyrosine phosphorylation of Stat3

(**A**) Schematic representation of wild-type Grb2 (WT) and its dominant-negative mutants of SH2/SH3 domains. Mutated domains in which their binding activity has been ablated are indicated by crosses. Tryptophan (W) residues on amino acid 36 and/or 193 or arginine (R) residues on amino acid 86 were substituted by lysine (K) and leucine (L) residues respectively as indicated on the left. The FLAG-tagged Stat3 was co-transfected with the fusion constructs containing GST-tagged SH3 domain mutants of Grb2 in (**B**) or its SH2 domain mutant tagged by HA in (**C**) into COS-1 cells, as labelled on the top of the Figures. Plasmids containing the wild-type Grb2 (WT) and GST were also transfected and used as controls. The cells were treated with EGF for 15 min. Tyrosine phosphorylation of Stat3 and protein expression of Stat3 and Grb2 were monitored by Western-blot analysis.

whether Grb2 can also affect IL-6-induced Stat3 activation. The human liver hepatoma cell line, HepG2, was transfected with Stat3 alone or Stat3 and Grb2, and tyrosine phosphorylation of Stat3 was examined. The results showed that IL-6-induced tyrosine phosphorylation of Stat3 was not inhibited by co-transfection of Grb2 (Figure 7, top panel, lanes 6 and 8). Our experiments in HepG2 cells indicate that Grb2 did not inhibit Stat3 tyrosine phosphorylation in a system where Grb2 and Stat3 do not share the same binding site with the receptor. These results suggest that Grb2 selectively represses Stat3 phosphorylation in EGF signalling, and support the hypothesis that the repression involves a competitive binding to the receptor.

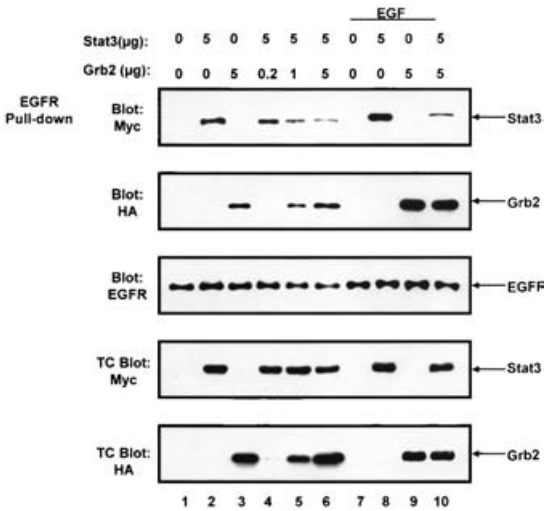

Figure 6 Grb2-inhibited interaction of Stat3 and EGFR by competitive interaction

(**A**) The Myc-tagged Stat3 (5 μg) was co-transfected with various amounts of HA-tagged Grb2 (0.2, 1 or 5 μg) into COS-1 cells. The cells were either left untreated (lanes 1–6) or treated with EGF for 30 min (lanes 7–10). The cells were harvested and the lysates were incubated with anti-EGFR antibody conjugated with agarose beads. The precipitates were subjected to Western-blot analysis and the co-precipitated Stat3 or Grb2 was detected by anti-Myc (uppermost panel) or anti-HA (second panel) antibody. The blot was also probed by anti-EGFR to indicate the amount of EGFR in each pull-down experiment. Total cell lysates were subjected to Western-blot analysis using anti-Myc or anti-HA antibody to show the expression levels of Stat3 and Grb2 (fourth and fifth panels).

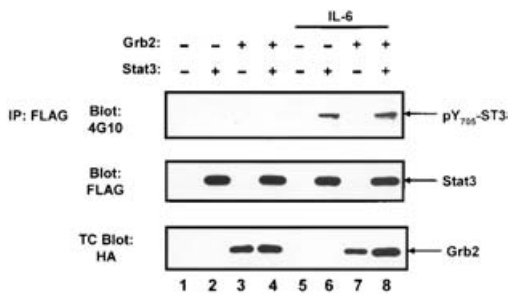

Figure 7 Stat3 tyrosine phosphorylation is not repressed by Grb2

HepG2 cells were transfected with FLAG-tagged Stat3 and/or HA-tagged Grb2 plasmids. The cells were left untreated (lanes 1–4) or treated with 100 ng/ml IL-6 for 15 min (lanes 5–8). Tyrosine phosphorylation of Stat3 was examined by immunoprecipitation (IP) of Stat3 with anti-FLAG antibody and by immunoblotting with the anti-phosphotyrosine antibody 4G10. The bottom panel shows a Western-blot analysis of the total cell lysate using anti-HA antibody for Grb2 expression.

Phosphorylation of Stat1, but not Stat5a, is repressed by Grb2

Other than Stat3, activations of Stat1 and Stat5 by EGF were detected in mouse liver and in some cancer cell lines [12,13]. We next examined how Grb2 affects the phosphorylation of other STAT members in EGF signalling. FLAG-tagged Stat1 and Stat5a were transfected in the absence or presence of HA-tagged Grb2 expression plasmid. Phosphorylation of Stat1 and Stat5a in response to EGF stimulation was examined. As shown in Figure 8, both Stat1 and Stat5a were tyrosine-phosphorylated after EGF stimulation (upper panels, lanes 2 and 6). However, once Grb2 was co-transfected, tyrosine phosphorylation of Stat1 (lane 4) was significantly decreased, whereas phosphorylation of Stat5a (lane 8) remained unchanged. These results indicate that the inhibitory effect of Grb2 is not applicable to all the STAT members, but is selective towards Stat1 and Stat3 in EGF signalling.

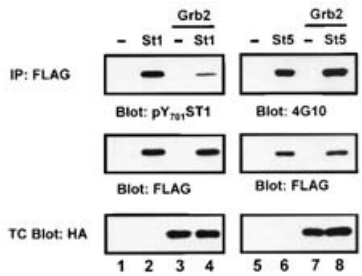

Figure 8 Differential effects of Grb2 on tyrosine phosphorylation of Stat1 and Stat5

COS-1 cells were transfected with FLAG–Stat1 (St1) or FLAG–Stat5a (St5) and/or HA–Grb2 expression plasmids, as labelled on the top of the Figure. The cells were treated with EGF for 15 min, and the lysates were immunoprecipitated (IP) with anti-FLAG antibody and probed with either anti-phospho-Tyr-701-Stat1 ($pY_{701}ST1$) or anti-phosphotyrosine (4G10) antibody to detect the tyrosine-phosphorylated Stat1 and Stat5a (upper panels). The blot was stripped and reprobed with anti-FLAG antibody (middle panels) to show the amount of immunoprecipitated Stat1 and Stat5a. The expression of HA–Grb2 is shown in the lower panels.

DISCUSSION

EGFR has been extensively investigated over many years, and it serves as an excellent model of a receptor that mediates different signalling pathways, leading to diverse cellular processes such as proliferation and differentiation [2]. However, it is less clear how cells co-ordinate these signalling pathways. Ras-MAPK and STAT are two important pathways to be activated in response to EGF stimulation. Cross-talk between these two pathways has been reported. For instance, Stat3 phosphorylation by MAPK on Ser-727 enhances the tyrosine phosphorylation-dependent transcriptional activity of Stat3 [30]. In addition, other members in the Ras-MAPK cascade, such as MEK (MAPK kinase) kinase 1 and c-Jun N-terminal kinase, also phosphorylate Stat3 on Ser-727 [31,32]. On the other hand, we and others also found that activation of MAPK leads to a negative regulation of Stat3 tyrosine phosphorylation and its activities, stimulated by EGF and IL-6 [23,25,33], and the mechanism mediating this inhibitory effect may involve activation of the specific inhibitors of Janus kinase–STAT pathways [25]. Our present study shows that Grb2 represses Stat3 tyrosine phosphorylation and the subsequent transcriptional activity (Figure 1). Also, Grb2 has a similar effect on Stat1, but not on Stat5a (Figure 8). More importantly, this inhibition could occur under physiological conditions (Figure 2). Our results also indicate that Stat activation could be regulated at the receptor level. Together, these results suggest that the Ras-MAPK pathway interacts intimately with Stat pathways and regulates Stat activity at multiple layers in RTK signalling.

The mechanism of this inhibition was further studied. No direct interaction between Stat3 and Grb2 was detected (Figure 3), and no Grb2-interacting proteins and tyrosine phosphatases seemed to be involved. Instead, a functional SH2 domain of Grb2 is necessary for this repression (Figures 4 and 5), suggesting the involvement of a receptor-binding event. Indeed, the association of Stat3 and endogenous EGFR was observed and this interaction was reduced by Grb2, whereas the interaction of Grb2 with

EGFR was enhanced in a dose-dependent manner (Figure 6). These results suggest that Grb2 inhibits Stat3 activation via a competitive binding to the EGFR. Although the mechanisms for this are not completely understood, a few points can be considered. First, the competition could occur at Tyr-1068 and Tyr-1086 of EGFR. Among the multiple sites at EGFR, Grb2 binding was shown to be mediated via phospho-Tyr-1068 and -1086 [4]. On the other hand, activation of Stat1 and Stat3 by EGFR was analysed in detail and a rather complicated regulation was reported [17]. Nevertheless, although other sites at the EGFR cannot be completely excluded, Tyr-1068 and Tyr-1086 seem to be the major sties for Stat3 activation [17,18]. Furthermore, Shao et al. [18] recently identified Tyr-1068 and Tyr-1086 as the recruitment sites of Stat3 in the EGFR by showing a direct interaction between Stat3 and phosphopeptides derived from these two sites. In support of this, our results demonstrating that Grb2 very efficiently inhibits Stat3 binding and activation suggest that Grb2 blocks Stat3 binding at these two sites. Secondly, whereas our results demonstrate that Grb2 inhibits Stat3, Stat3 seems to have little effect on Grb2, since overexpression of Stat3 does not seem to affect phosphorylation of ERKs induced by EGF (Figure 4). This could be due to a lower affinity for Stat3–EGFR interaction. Indeed, Shao et al. [18] reported that the binding affinities of Stat3 for phospho-Tyr-1068 and -1086 are 135 and 243 nM respectively, whereas the binding affinities of Grb2 for these sites are 30 and 60 nM respectively [18], indicating that Grb2 binds to these sites four times more efficiently than Stat3. This provides a basis for the predominant role of Grb2 in the regulation of Stat proteins, but not vice versa. Thirdly, we observed that Stat3 and Grb2 can interact with endogenous EGFR in unstimulated cells (Figure 5). This is in agreement with a previous report showing a constitutive interaction between endogenous EGFR and Stat1, Stat3 and Stat5 in A431 cells [34]. This suggests that Stat proteins may interact with the unphosphorylated EGFR in certain cases, and Grb2 can also inhibit such interaction by competitive binding.

Stat proteins can be activated by EGF in certain cell types, such as COS-1 and A431, but not in others, although they express EGFR. For instance, Stat3 cannot be activated in the rat phaeochromocytoma PC12 cells by EGF, in which the Ras-MAPK pathway is strongly activated. On the other hand, Stat3 is constitutively activated in various tumours and tumour cell lines in which EGFR is overexpressed. In the present study, we provide evidence that in growth factor signalling, the adaptor protein Grb2 can ablate the tyrosine phosphorylation of Stat1 and Stat3, which reveals a new mechanism for negative regulation of Stat proteins. It will be interesting to investigate further whether Grb2 plays a role in the inhibition of Stat3 activation in certain cell types and also in the constitutive activation of Stat3 in tumour cells mediated by EGF signalling.

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