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Regulation of stress granule dynamics by Grb7 and FAK signalling pathway

Nien-Pei Tsai, Ping-Chih Ho and Li-Na Wei*

Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN, USA

Cells form stress granules (SGs) in response to environmental stresses, which constitute cytoplasmic domains where mRNAs are stored and translation is halted. Although several components are found in SGs, it is poorly understood as to how SGs are formed and dissolved. We identified growth factor receptor-bound protein 7 (Grb7), an RNA-binding, translational regulator, as an integral component of SGs, which directly interacts with Hu antigen R (HuR) and is required for cells to form SGs. When stress is terminated, Grb7 is hyperphosphorylated by focal adhesion kinase (FAK), loses its ability to directly interact with HuR and is dissociated from SG components, thereby disrupting SGs in recovering cells. Consistently, dominant-negative hypophospho mutants of FAK and Grb7 significantly attenuate SG disassembly during recovery. FAK activation followed by its phosphorylating Grb7 constitutes a cell-autonomous signalling pathway that regulates the disassembly of SGs and translational stimulation during recovery. This is the first reported pathway actively regulating the dynamics of SGs.

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Introduction

Stress granules (SGs), formed in response to various environmental stresses, constitute cytoplasmic domains where mRNAs are stored and their translation is halted under unfavourable conditions (Kedersha and Anderson, 2002; Moore, 2005; Anderson and Kedersha, 2006; Kiebler and Bassell, 2006). In mammalian cells, SG assembly is initiated by TIA-1 that binds to the 48S complex and promotes polysome disassembly and routing of mRNA into the aggregated SGs (Kedersha *et al*, 2000; Gilks *et al*, 2004). In addition, studies have shown that cells form SGs in response to other signals, such as phosphorylation of initiation factor eIF2α (Kedersha *et al*, 2002; Anderson and Kedersha, 2006), a shift in the cellular redox potential during stress (Cande *et al*, 2004), and so on. It is also known that housekeeping gene transcripts are stored in SGs whereas stress-responsive gene transcripts are excluded from these structures in stressed cells (Kedersha and Anderson, 2002).

An increasing number of components have recently been found in SGs and can regulate cell physiology. For example, components of the transcription machinery can also be found in SGs and have a role in translational control (Anderson and Kedersha, 2007; Kwon *et al*, 2007; Yu *et al*, 2007), suggesting that SG components might regulate other processes involving mRNAs. Further, SGs can be involved in cellular responses upon viral infection (Schutz and Sarnow, 2007; White *et al*, 2007). Despite the identification of an increasing number of SG components (Wilczynska *et al*, 2005; Guil *et al*, 2006; Stohr *et al*, 2006; Vessey *et al*, 2006; Gallois-Montbrun *et al*, 2007; Lin *et al*, 2007), the regulation of SG dynamics remains poorly understood. What has been described is that during recovery, SGs disappear and cells then re-engage in normal translation (Brengues *et al*, 2005; Bhattacharyya *et al*, 2006). It remains elusive whether any mechanism exists for cells to actively dissolve SGs when stress is terminated, and if the recovering phase, in terms of the changing dynamics of these important subcellular structures, can be regulated at all.

Recently, we have identified a new RNA binding protein, which is an adaptor protein named growth factor receptor-bound protein 7 (Grb7) (Tsai *et al*, 2007), and surprisingly found its colocalization with several typical SG markers. Further, its specific upstream signal stimulator, focal adhesion kinase (FAK), was also found to be colocalized with these SG markers. We now report the two key players in SG formation, Grb7 and FAK, whose signal transduction pathway constitutes the first identified cell-autonomous process when cells encounter stress and when the stress is terminated. This process is initiated by the recruitment of hypophosphorylated Grb7 by Hu antigen R (HuR) to SGs, which stabilizes TIA-1 aggregates when cells are under stress; upon termination of stress, FAK is activated, which subsequently phosphorylates Grb7. The phosphorylated Grb7 then leaves HuR, as well as other SG components, thereby disrupting SGs. This represents the first cellular signalling pathway that can directly and actively regulate the dynamics of SGs, and is able to integrate environmental factors to coordinate changes in these subcellular structures in cells under stress and during recovery.

Results

Grb7 is a critical component of SGs

The adaptor protein Grb7, in its hypophosphorylated state, is a specific RNA-binding translational repressor. It can be phosphorylated by FAK and loses its RNA-binding activity, thereby releasing the otherwise silenced transcripts to be translated (Tsai *et al*, 2007). In a heat-shock-triggered stress model of a mouse embryonal carcinoma cell line P19, we first found the colocalization of Grb7 with two known SG markers, TIA-1 and HuR. As shown in Figure 1A, the endogenous Grb7 formed distinct foci mostly colocalized with TIA-1

*Corresponding author. Department of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455, USA. Tel.: +1 612 6259402; Fax: +1 612 6258408; E-mail: weixx009@umn.edu

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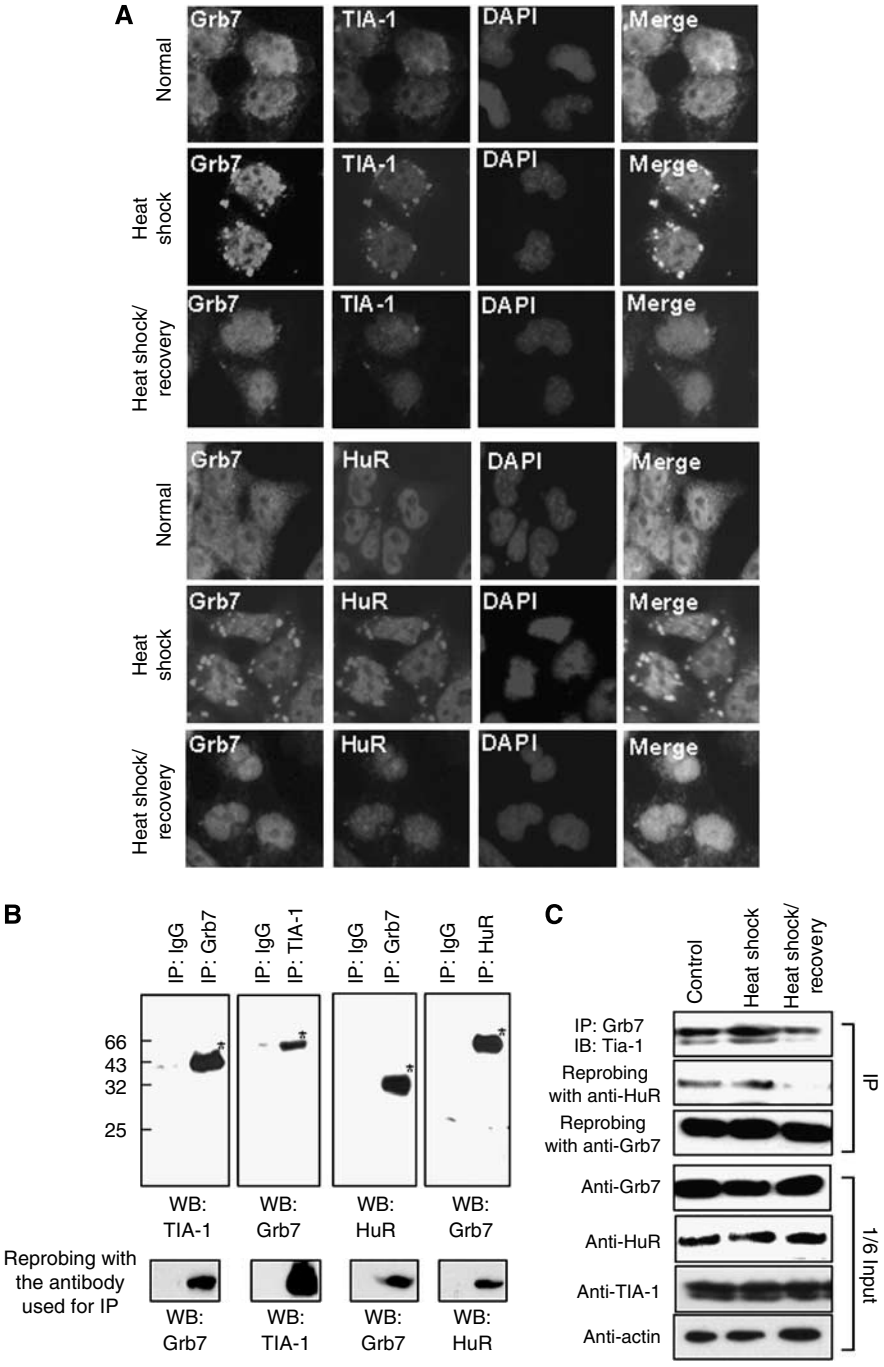

Figure 1 Grb7 is localized in SG. (**A**) Immunohistochemistry of endogenous Grb7 and known SG components, TIA-1 (three upper lines) and HuR (three lower lines), in cells under the condition of normal, 30 min heat shock or additional 60 min recovery. (**B**) Western blots of reciprocal co-immunoprecipitates of endogenous Grb7 with anti-TIA-1 antibody, anti-HuR antibody (second and fourth columns), TIA-1 with anti-Grb7 (first column) or HuR with anti-Grb7 antibody (third column) in cells under heat-shock treatment. Specifically associated proteins are marked by *. Precipitation efficiency was monitored by re-probing the membrane with the antibodies used for precipitation as shown in the bottom panels. (**C**) Western blots of co-immunoprecipitates of endogenous TIA-1 or HuR with Grb7 in cells before, during and after heat-shock treatment. A one-sixth of protein input is shown in the lower four panels.

(three upper lines) and HuR (three lower lines) in the cytoplasm of stressed cells but not in normal cells or cells recovering from stress for 60 min. A few partially overlapping or non-overlapping punctae were noticed, suggesting that SG components are probably not evenly distributed and some granules are heterogeneous. Complex formation of endogenous Grb7 with TIA-1/HuR was validated in a co-immunoprecipitation experiment shown in Figure 1B. The amount of co-precipitated Grb7/TIA-1 complex increased in stressed cultures (to 135% of the normal condition, $P<0.05$) but decreased in cultures during recovery (to 51% of the stressed condition, $P>0.05$) (Figure 1C, top). Interestingly, the amount of co-precipitated Grb7/HuR complex also decreased in recovering cultures (to 11% of the normal condition, $P<0.05$), whereas the association of Grb7 and HuR was maintained relatively constant in normal and stressed

(at 110% of the normal condition, $P>0.05$) cultures (Figure 1C, bottom). This suggests that Grb7 and HuR are constantly associated with each other in normal and stressed conditions. During the transition from stress to recovery, Grb7 and HuR are dissociated from each other, which has been subsequently validated (see later, Figure 7). These results reveal that Grb7 is an SG component and forms a complex with other SG components in stressed cells. It is then dissociated from these SG components during recovery.

To determine if Grb7 has any functional role in SG formation, we conducted Grb7 silencing in P19 cultures, subjected these cultures to stress and monitored the formation of SGs in these stressed cells. As shown in Figure 2A, SGs mostly disappeared in cells whose endogenous Grb7 was transiently knocked down (the cell marked by an arrowhead) and the level of an SG marker, TIA-1, was maintained (Supplementary Figure S1), as compared to cells whose endogenous Grb7 was retained (the cell marked by an arrow) in the same culture under the same stress condition. To validate this finding, we employed stable Grb7-silencing P19 cells (silencing efficiency validated with anti-Grb7 staining; Supplementary Figure S2) and cultures rescued with siRNA-insensitive Flag–Grb7 to determine if SGs could be restored by re-expressing Grb7. Data in Figure 2B show that the Flag-positive cell (marked by an arrow) indeed restored SGs as compared to untransfected cells (marked by an arrowhead). Quantification was conducted by scoring cells that formed SGs that were bigger than 1 μm in diameter (Ivanov *et al*, 2003), which confirmed the significantly (more than a 50% reduction) reduced SG-positive cell counts in Grb7-silencing cultures, and expression of siRNA-insensitive Flag–Grb7 restored this defect (Figure 2C). We then performed immunoprecipitation to assess the formation of SGs, HuR/TIA-1 complex, in the Grb7-silencing culture and that receiving siRNA-insensitive Flag–Grb7 (Figure 2D). The relative amount of co-precipitated HuR/TIA-1 complex was apparently reduced in the Grb7-silenced P19 culture, which could be effectively restored by expressing the siRNA-insensitive Flag–Grb7.

To validate this result with respect to the well-established role of SGs, translational repression, metabolic labelling was performed to monitor *de novo*-synthesized protein during stress (Figure 2E). The known SG-exclusive transcript, mRNA of heat-shock protein 70 (HSP70) (Kedersha and Anderson, 2002), was used as control and two SG-localized transcripts, kappa opioid receptor (KOR) (SG association shown later, Figure 6A) and Actin mRNAs (Stohr *et al*, 2006), were examined. The data (upper set of panel E showing the gels and lower set of panel E showing the quantitative results) showed that in Grb7-silencing cells (green), *de novo*-synthesized KOR and Actin, as compared to HSP70, were enhanced (i.e., de-repressed translation), which was effectively reversed by the expression of siRNA-resistant Flag–Grb7 (blue). The efficiency of stable silencing and transient restoration was monitored and is shown in Supplementary Figure S3A.

To shed light on how Grb7 affects cells to form SGs under stress, we conducted gain-of-function experiments to assess its effect on the integrity (protease sensitivity) of SGs (Gilks *et al*, 2004) or ribonuclear protein (RNP) complexes before and after stress, which was typically reflected by the aggregation of TIA-1 (Gilks *et al*, 2004). As shown in Figure 2F (the left panel showing gels and the right panel showing quantitative data by normalizing TIA-1 to Actin), in control cells, protease sensitivity of TIA-1 and HuR aggregates under stress was enhanced, as also observed in a previous study (Gilks *et al*, 2004). In the normal culture condition, these aggregates were resistant to protease in cells overexpressing Grb7 within the concentration range similar to that of control cells. But under stress, these aggregates were more resistant to protease in Grb7-overexpressing cells than in control cells. It was interesting that the anti-Grb7 detected (for both endogenous and overexpressed Grb7) pattern exhibited resistance to protease at the concentration even as high as 1.6 μg/ml, whereas the anti-Flag detected pattern (detecting exogenous Flag–Grb7) indicated that Flag had much weaker resistance to protease than Grb7. This might be caused by the greater sensitivity of the Flag tag linked to the N terminus that might protrude outside the compact Grb7 molecule. Nevertheless, the overall results (protease sensitivity of three SG components including TIA-1, HuR and Grb7) are in agreement and suggest a role for Grb7 in enhancing the integrity of RNP complexes or stabilizing TIA-1 aggregates. This is further supported by the increased distribution of TIA-1 in the insoluble fraction (indicative of SGs) in cells overexpressing Grb7 (Supplementary Figure S4). An elevated level of HSP27 was also detected in this insoluble fraction, consistent with the predicted behaviour of this chaperon protein in response to stress. It is concluded that Grb7 has an active role in stimulating SG formation, mediated, at least partially, by stabilizing TIA-1 aggregates and enhancing SG or RNP integrity.

FAK, the activator of Grb7, is also localized in SGs and is important for dissolving SGs during recovery

Previously, we showed that the specific upstream activator of Grb7, FAK, could phosphorylate Grb7 and the phosphorylated Grb7 lost its RNA-binding activity (Tsai *et al*, 2007). To examine if this regulator of Grb7 was involved in stress response, we monitored FAK in stressed cells. As shown in Figure 3A, FAK was also recruited into SGs in cells under stress, and the punctuated FAK signals disappeared as cells recovered and SGs were dissolved. It was surprising that using immunohistochemistry, FAK was detected, mainly, in the P19 nucleus but not in focal contacts as seen in other cell types. We then conducted cell fractionation experiments to examine its distribution (Supplementary Figure S5), which confirmed its primarily nuclear distribution in P19 as in neurons or neuronal-like cells (Contestabile *et al*, 2003; Xie *et al*, 2003). This is consistent with our previous studies where P19 cells were found to behave very similar to neuron precursors (Bi *et al*, 2001, 2003; Tsai *et al*, 2006). To confirm the association of FAK with SGs, biochemical assays were conducted, which supported that FAK was associated with SGs during heat shock and dissociated from SG components during recovery (Figure 3B).

To determine if this was a merely correlative phenomenon, or in fact FAK could possibly regulate the dynamics of SGs, we performed transient siRNA knockdown of endogenous FAK in cells before stress induction, which were then stressed and allowed to recover. Interestingly, silencing FAK did not affect SG formation in cells under stress, because cells still formed SGs effectively based upon scoring the SG-positive cell number (control, 83%; FAK silencing, 78%; restoring

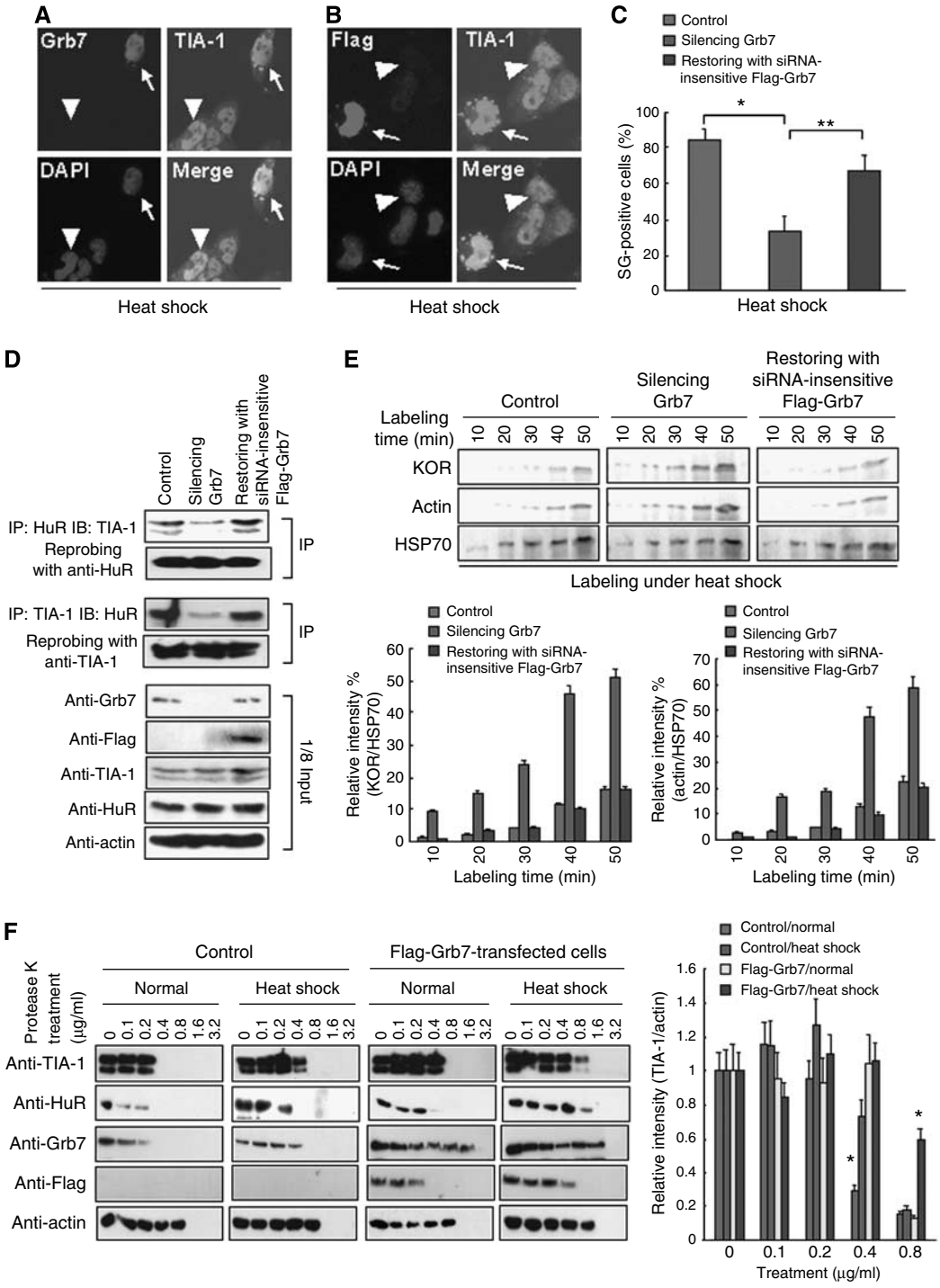

Figure 2 Grb7 is critical for SG formation. (**A**) Immunohistochemistry of Grb7 and TIA-1 of Grb7-silenced P19 cultures after heat-shock treatment. Grb7-knockdown cells and those inefficiently silenced are marked by arrowheads and arrows, respectively. (**B**) Immunohistochemistry of Flag and TIA-1 of stable Grb7-silenced P19 cultures transfected with siRNA-insensitive Flag–Grb7 after heat-shock treatment. Grb7-knockdown cells and those receiving siRNA-insensitive Flag–Grb7 are marked by arrowheads and arrows, respectively. (**C**) Quantification of SG-positive cells in normal, Grb7-knockdown or cultures rescued with siRNA-insensitive Flag–Grb7. Data are presented as mean ± s.e.m. (*$P<0.05$). (**D**) Western blots of reciprocal precipitates of TIA-1 with HuR from control cells, Grb7-silenced cells or cells transfected with siRNA-insensitive Flag–Grb7 that restored Grb7 function (top). Precipitation efficiency was monitored by re-probing the same membrane with the antibodies used for precipitation. A one-eighth input was monitored (bottom). (**E**) Metabolic labelling to monitor KOR, Actin and HSP70 translation in control, Grb7 silencing or cultures receiving siRNA-insensitive Flag–Grb7 in 10, 20, 30, 40 or 50 min ^{35}S-Met/Cys incubation under heat shock. The relative HSP70-normalized values are shown in the bottom panels ($P<0.05$ for Grb7 silencing compared to normal or cultures receiving siRNA-insensitive Flag–Grb7). (**F**) Western blots of endogenous TIA-1, HuR, Grb7, Actin and Flag–Grb7 from the lysates of control or Flag–Grb7-transfected P19 cells treated with Protease K at the concentration of 0, 0.1, 0.2, 0.4, 0.8, 1.6 or 3.2 μg/ml for 5 min. TIA-1 intensity normalized with Actin is shown on the right. Data are presented as mean ± s.e.m. (*$P<0.05$).

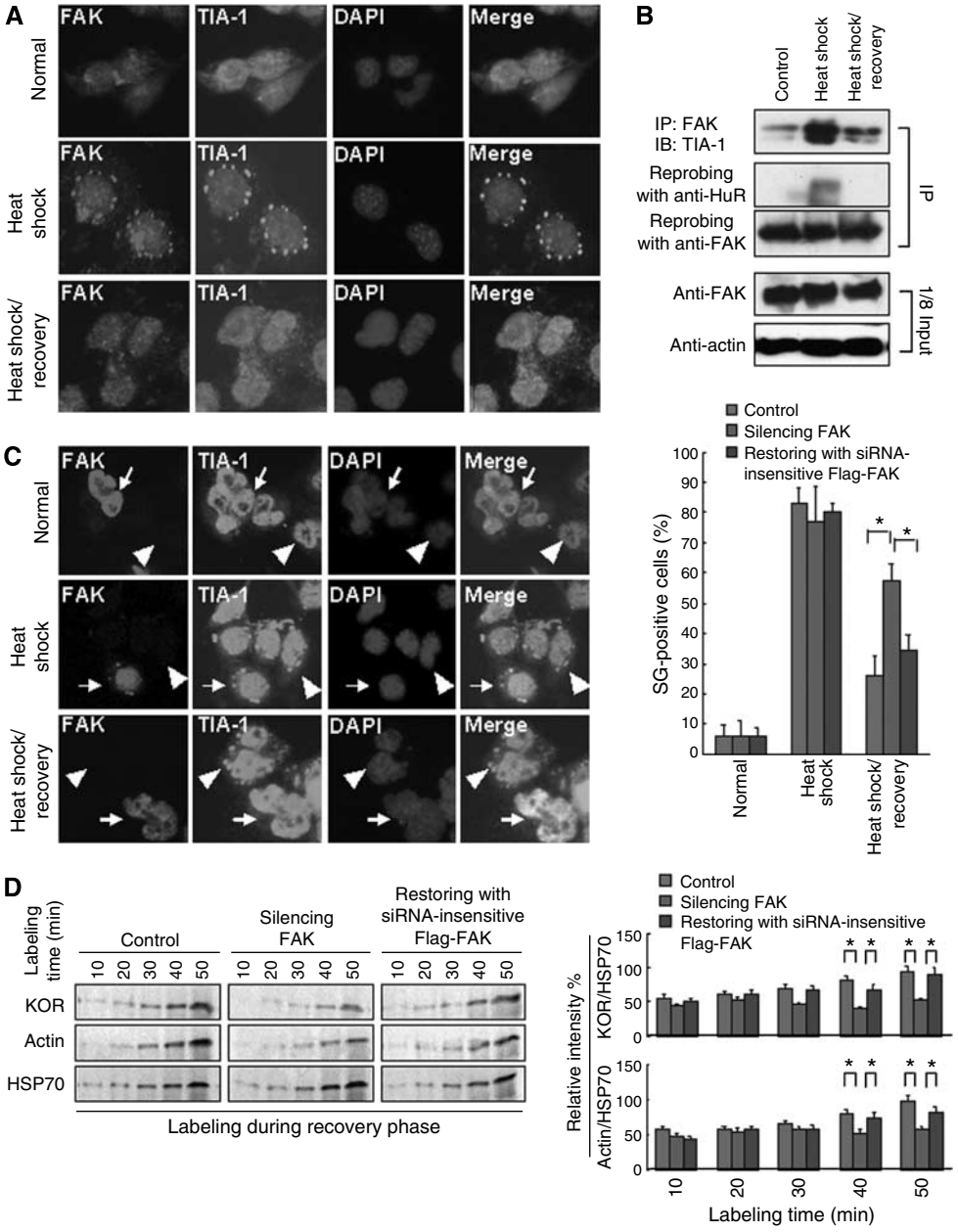

Figure 3 FAK is recruited to SGs during stress. (**A**) Immunohistochemistry of endogenous FAK and SG marker, TIA-1, in cells in normal, 30 min heat-shock or additional 60 min recovery conditions. (**B**) Western blots of co-immunoprecipitates of endogenous TIA-1 with FAK in cells before, during and after heat-shock treatment. Precipitation efficiency was monitored by re-probing the same membrane with the antibodies used for precipitation. A one-eighth input was monitored (bottom). (**C**) Immunohistochemistry of endogenous FAK and SG marker, TIA-1, on FAK-silencing cells in normal, 30 min heat-shock or additional 60 min recovery conditions. FAK-knockdown cells and those inefficiently silenced are marked by arrowheads and arrows, respectively. Quantification of SG-positive cells in normal, FAK-knockdown and cultures rescued with siRNA-insensitive Flag–FAK under normal, 30 min heat-shock or additional 60 min recovery conditions. Data are presented as mean $\pm$ s.e.m. ($^*P<0.05$). (**D**) Metabolic labelling to monitor KOR, Actin and HSP70 translation in control, FAK silencing or cultures rescued with siRNA-insensitive Flag–FAK in 10, 20, 30, 40 or 50 min ^{35}S-Met/Cys incubation during recovery. Relative HSP70-normalized values are shown in the right panels. Data are presented as mean $\pm$ s.e.m. ($^*P<0.05$).

with siRNA-insensitive Flag–FAK, 80%) and the number of SGs per cell (control, 7.3 $\pm$ 0.6; FAK silencing, 6.8 $\pm$ 0.9; restoring with siRNA-insensitive Flag–FAK, 6.7 $\pm$ 0.7) (Figure 3C, top two panels). However, FAK significantly delayed SG disassembly during the recovery phase (after 60 min recovery) (Figure 3C, bottom, the arrowhead pointing to a FAK-silenced cell with apparent SGs and the arrow pointing to a cell where FAK was not silenced and SGs were dissolved effectively during recovery). Importantly, the delay in SG disassembly in FAK-silencing cells was effectively rescued by expressing siRNA-insensitive Flag–FAK in the stable FAK-silencing cells (validated by anti-FAK staining; Supplementary Figure S6), because the SG-positive cell count was decreased to 35% (close to that of control, 29%) after a 60 min recovery as compared to that of FAK-silencing cells (57%) (Figure 3C, right, and Supplementary Figure S7). The number of SGs per cell in the FAK-silencing culture was also significantly different from that of the control or cultures restored by expressing siRNA-insensitive Flag–FAK (control, 2.2 $\pm$ 0.6; FAK silencing, 4.4 $\pm$ 0.5; restoring with

siRNA-insensitive Flag–FAK, 1.8±0.7). We further conducted a time-course experiment to examine SG-positive cells in the normal and FAK-silencing cultures during recovery for 100 min. The data showed that SG-positive cell counts decreased in parallel, suggesting that the recovery process was delayed but not completely abolished (Supplementary Figure S8).

To study the effects of silencing FAK with respect to translation, we conducted metabolic labelling experiments to monitor *de novo*-synthesized protein during recovery. In Figure 3D, by normalizing to HSP70, translation of KOR and Actin mRNAs was found to decrease in FAK-silencing cells as compared to control cells, which could be effectively restored by expressing siRNA-insensitive Flag–FAK. The efficiency of silencing and restoration is shown in Supplementary Figure S3B. Together, these results confirm that FAK has a critical role, particularly during stress recovery.

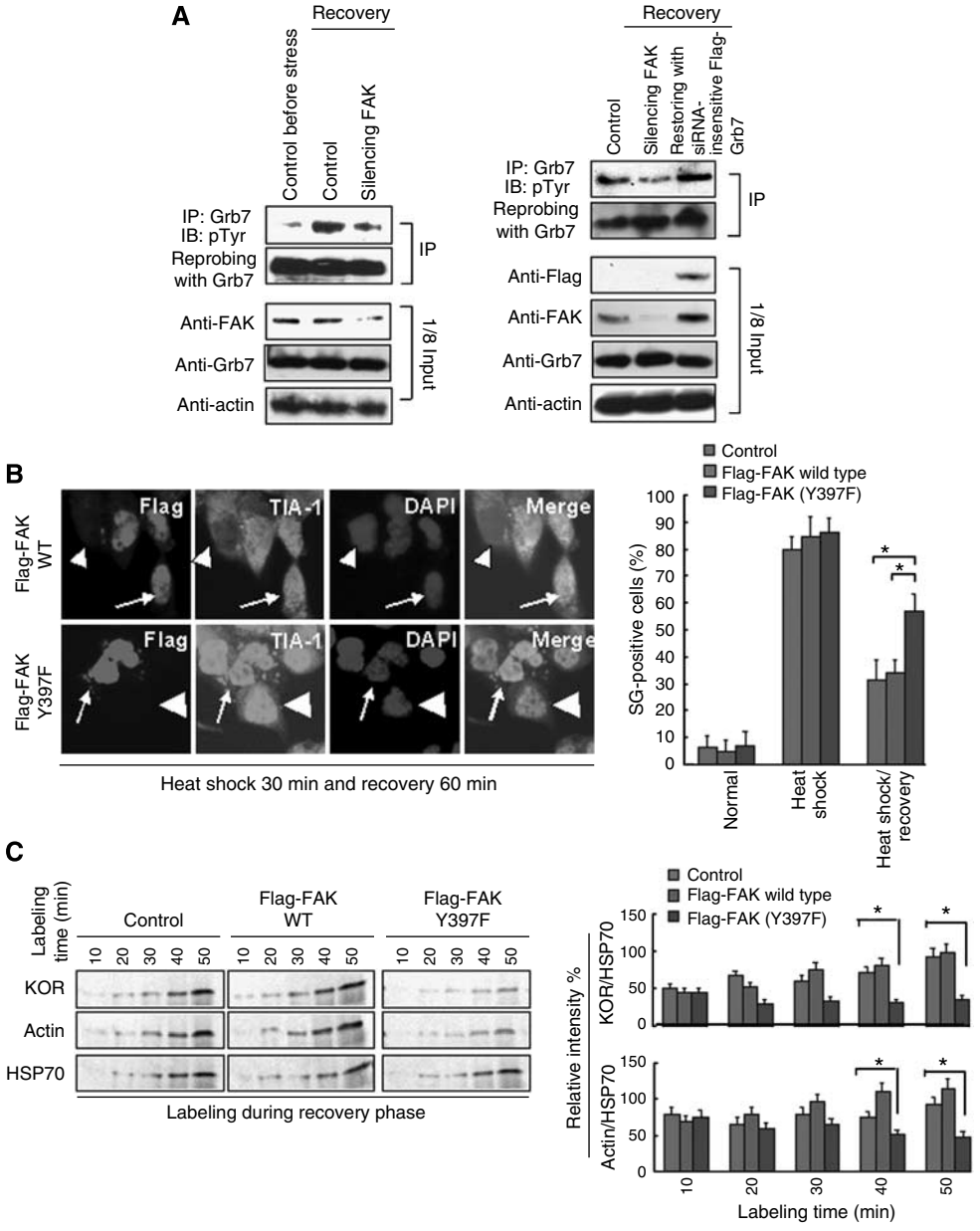

Figure 4 FAK is critical for dissolving SGs during recovery. (**A**) Left: western blots, detected with anti-Grb7 and anti-phosphor-tyrosine, of immunoprecipitated Grb7 in control before stress (first lane) and control (second lane) or FAK-silenced (third lane) P19 cultures during recovery; right: western blots, detected with anti-Grb7 and anti-phosphor-tyrosine, of immunoprecipitated Grb7 in control, FAK-silenced cultures or cultures receiving siRNA of FAK together with siRNA-insensitive Flag–FAK that restored FAK function during recovery. (**B**) Immunohistochemistry of SGs (marked with TIA-1) and anti-Flag in P19 transiently transfected with the WT Flag–FAK (upper panel) or the Flag–FAK mutant (Y397F, lower panel) after 30 min heat shock and 60 min recovery. Control cells and cells receiving transfected proteins are marked by arrowheads and arrows, respectively. Quantification of SG-positive cells in control, Flag–FAK WT- or Flag–FAK (Y397F)-transfected cultures under normal, 30 min heat-shock or additional 60 min recovery conditions. Data are presented as mean±s.e.m. ($^*P<0.05$). (**C**) Metabolic labelling to monitor KOR, Actin and HSP70 in control, Flag–FAK WT- or Flag–FAK (Y397F)-transfected P19 cells in 10, 20, 30, 40 and 50 min ^{35}S-Met/Cys incubation during recovery. Relative HSP70-normalized values are shown in the right panels. Data are presented as mean±s.e.m. ($^*P<0.05$).

To examine if FAK activity (phosphorylating Grb7) was indeed knocked down by the siRNA, Grb7 phosphorylation was assessed in cultures silenced by either one of two independent FAK siRNAs (one representative set is shown in Figure 4A, left). Apparently, phosphorylation of Grb7 in control, recovering cultures was increased significantly as predicted, but was lowered to approximately 42% in cultures treated with siRNA of FAK ($P<0.05$ from three experiments) (FAK expression was knocked down to approximately 26%). Importantly, this reduction was reversed by introducing the siRNA-insensitive Flag–FAK (Figure 4A, right), supporting the specific effect of FAK on the phosphorylation of Grb7 in recovering cells.

As this was the first time a kinase, FAK, was found in, and could regulate, SGs, it was important to determine if it was the specific activity or the protein *per se* that contributed to the detected effect on the dynamics of SGs. We thus introduced into P19 cells a wild-type (WT) Flag-tagged FAK or an enzymatically inactive (dominant negative) FAK point mutant (Flag–FAK Y397F) and monitored SGs in recovering cells after stress. It appeared that cultures transfected with either the WT or the mutant FAK exhibited no significant difference in SG dynamics under normal or after heat-shock treatment (Supplementary Figure S9), supporting that FAK activity is not required for SG formation during heat shock. However, in recovering cultures (Figure 4B), cells harbouring the dominant-negative mutant FAK (Y397F, Flag-positive, lower panel, arrows) retained significantly more SGs as compared to the control cells (Flag-negative, lower panel, arrowheads). The time required for dissolving SGs was prolonged in the dominant-negative FAK-expressing culture (Supplementary Figure S10). Cells harbouring additional WT FAK did not significantly increase the efficiency of dissolving SGs during recovery (Supplementary Figure S11), suggesting that endogenous FAK was perhaps at the saturated level. Finally, to validate the effect of FAK activity on *de novo* protein synthesis, metabolic labelling was conducted (Figure 4C). Also by normalizing to HSP70, labelled KOR and Actin were less in the Flag–FAK (Y397F)-expressing culture than in the control or cultures harbouring the WT FAK. The efficiency of transfection is shown in Supplementary Figure S3C. Together, these results confirm that FAK activity triggers Grb7 phosphorylation and subsequent SG disassembly in recovering cells. Blocking or knocking down the enzymatic activity of FAK delays SG disassembly in recovering cells. This is the first reported cell-autonomous signalling pathway that employs a kinase to regulate SG dynamics in post-stress recovery.

Phosphorylation of Grb7 facilitates SG disassembly during recovery

Phosphorylation of Grb7 is a known downstream effect of FAK activation. We then determined if phosphorylation of Grb7 indeed mediated the effect of FAK on the disassembly of SGs. We introduced into cultures the WT Grb7 (Flag–Grb7 WT) or the tyrosine phosphorylation double mutant Grb7 (Flag–Grb7 Y483F/Y495F), subjected the cultures to heat-shock stress and allowed them to recover for 60 min. As shown in Supplementary Figure S12, cells harbouring the WT or mutant Grb7 (Flag–Grb7 Y483F/Y495F) were equally efficient in forming SGs during stress. However, during recovery (Figure 5A), cells harbouring the mutant Grb7 retained more SGs (lower panel, marked by an arrow, approximately 60% based on SG-positive cell counts shown on the right), whereas fewer cells harbouring the WT Grb7 (upper panel, marked by an arrow, 28% by SG-positive cell counts) or without additional Grb7 (marked by arrowheads, 35% by SG-positive cell counts) retained SGs. The effect of Grb7 phosphorylation on SG aggregation was further supported in co-immunoprecipitation experiments shown in Figure 5B. In WT Grb7-transfected cultures, phosphorylation of Grb7 occurred normally, whereas in the double mutant (Y483F/Y495F) Grb7-transfected cultures, no phosphorylation of the introduced Flag–Grb7 was detected (second panel). This confirmed the predicted phosphorylation status of Flag–Grb7 used in these experimental systems. The association of the introduced Flag–Grb7 with SG components such as TIA-1 and HuR in these cells was then monitored

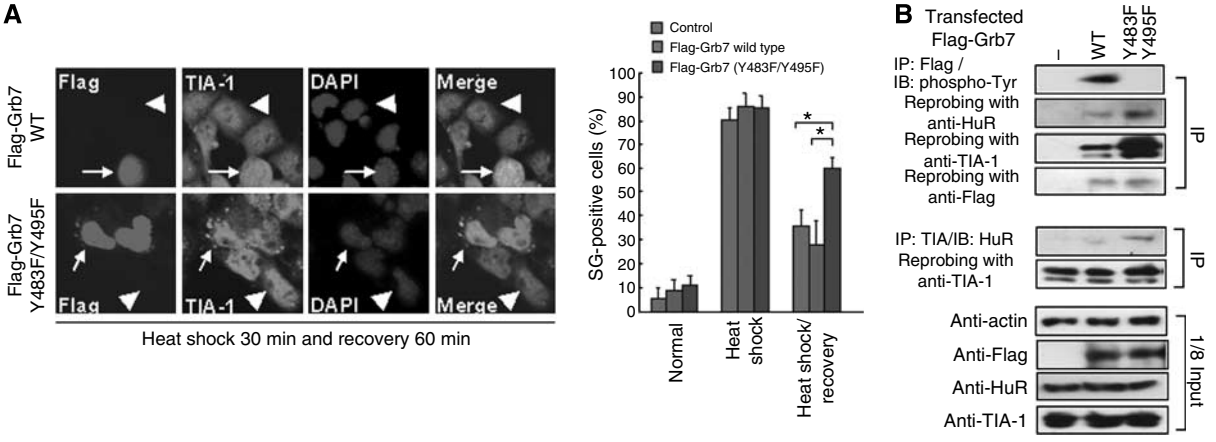

Figure 5 Phosphorylation of Grb7 triggers dissociation of SG. (**A**) Immunohistochemistry of SGs (marked with TIA-1) and anti-Flag in P19 transiently transfected with WT Flag–Grb7 or Flag–Grb7 (Y483F/Y495F) after 30 min heat shock and 60 min recovery. Control cells and cells receiving transfected proteins are marked by arrowheads and arrows, respectively. Quantification of SG-positive cells in control, Flag–Grb7 WT or Flag–Grb7 (Y485F/Y493F)-transfected under normal, 30 min heat-shock or additional 60 min recovery conditions. Data are presented as mean ± s.e.m. (*$P<0.05$). (**B**) Western blots of anti-Flag-precipitated phosphor-tyrosine, HuR and TIA-1 (first, second and third panels) and co-immunoprecipitation of TIA-1 with HuR from untransfected or transfected P19 (fifth panel). Precipitation efficiency was monitored by re-probing the same membrane as shown in the fourth and sixth panels. Input controls are shown in the bottom four panels.

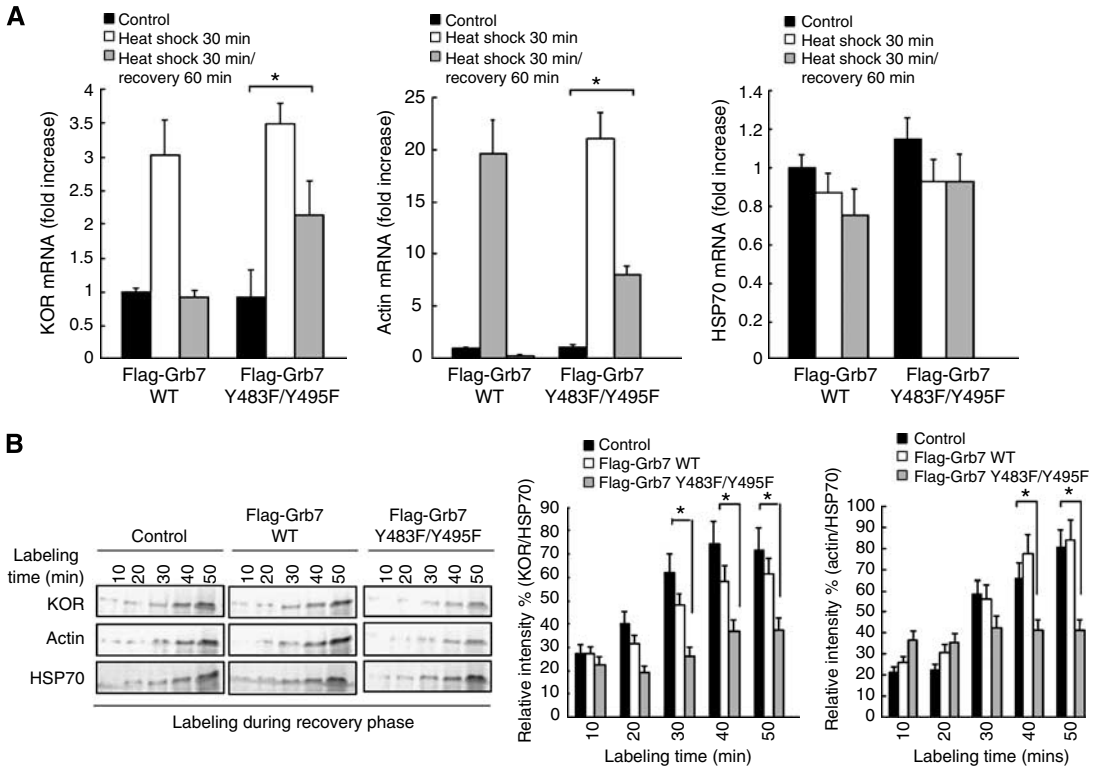

Figure 6 Phosphorylation of Grb7 triggers mRNA localization and *de novo* protein synthesis. (**A**) Real-time RT–PCR of RNA precipitated with anti-TIA-1 antibody from Flag–Grb7 WT- or Flag–Grb7 (Y485F/Y493F)-transfected P19 cells under normal, 30 min heat-shock or additional 60 min recovery conditions. Data are presented by fold increases using the level of mRNA precipitated from cultures transfected with Flag–Grb7 WT under normal condition as 1 (*$P<0.05$). (**B**) Metabolic labelling to monitor KOR, Actin and HSP70 in control, Flag–Grb7 WT- or Flag–Grb7 (Y483F/Y495F)-transfected P19 cultures in 10, 20, 30, 40 and 50 min ^{35}S-Met/Cys incubation during recovery. Relative HSP70-normalized values are shown in the right panels. Data are presented as mean ± s.e.m. (*$P<0.05$).

by a series of immunoprecipitation experiments (the third and fifth panels from the top of Figure 5B). Indeed, hypophosphorylation of Grb7 significantly elevated its association with these SG components including HuR and TIA-1, and also enhanced the association of HuR with TIA-1.

We next determined the effects of Grb7 phosphorylation on the subcellular distribution of specific Grb7-targeted mRNA and certain known SG-recruited mRNAs. As shown in Figure 6A, the specific mRNA target of Grb7, KOR mRNA, was found to be enriched in TIA-1 precipitates (indicative of SGs) from both WT and hypophosphorylated (Y483F/Y495F) Flag–Grb7-overexpressing cultures during stress, but it was then efficiently dissociated from TIA-1 during recovery in cultures transfected with the WT Flag–Grb7 (Figure 6A, left) but not in the hypophosphorylated Grb7 (Y483F/Y495F)-expressing cultures. The controls for input, transfection and precipitation efficiency are shown in Supplementary Figure S13. A similar effect was also detected for Actin mRNA (Figure 6A, middle) but not for the SG-excluded mRNA, HSP70 mRNA (Figure 6A, right), suggesting that the phosphorylation state of Grb7 regulates SG dynamics not only for its target mRNA but also for other mRNAs recruited to SGs. Finally, the effect on translation was monitored (Figure 6B). Translation of KOR and Actin was decreased in hypophosphorylated Grb7 (Y483F/Y495F)-transfected cells as compared to that in control or Flag–Grb7 WT-transfected cultures. The efficiency of transfection is shown in Supplementary Figure S3D. Altogether, these results confirm the role of phosphorylation of Grb7 in SG recovery.

Grb7 directly interacts with HuR, which is inhibited by phosphorylation of Grb7

Previous results (Figure 1C) have suggested that Grb7 constitutively forms a complex with HuR. We examined an intriguing possibility of a direct interaction of Grb7 with HuR, which might have a role in regulating SG dynamics. We prepared GST–Grb7 and GST–HuR for reciprocal GST pull-down experiments (Figure 7A). The results clearly confirmed the constitutive and direct interaction between Grb7 and HuR. We further determined the interacting domains of Grb7 and HuR by testing the dissected Grb7 and HuR fragments in reciprocal pull-down experiments. As shown in Figure 6B, the C-terminal 120 residues of HuR (construct D) were sufficient to be pulled down by GST–Grb7. As shown in Figure 6C, the N-terminal 150 residues of Grb7 (construct C) were responsible for interacting with HuR. The effect of Grb7 phosphorylation on its interaction with HuR was further evaluated in GST pull-down tests (Figure 6D). The WT and mutant GST–Grb7s were both subjected to *in vitro* phosphorylation with FAK (enriched by immunoprecipitation) and tested in pull-down assays. The efficiency of *in vitro* phosphorylation was estimated to be approximately 80% based upon western blot analysis of the remaining fraction following the removal of phosphorylated protein with anti-phospho-Tyr antibody as shown in Figure 7D, right. In the *in vitro* protein interaction assay, Grb7 and its hypophosphorylated version (Y485F/Y493F-Grb7 or Y485F/Y493F-(p)Grb7), but not the phosphorylated version ((p)Grb7), effectively and directly interacted with HuR (first GST pull-down). The effect

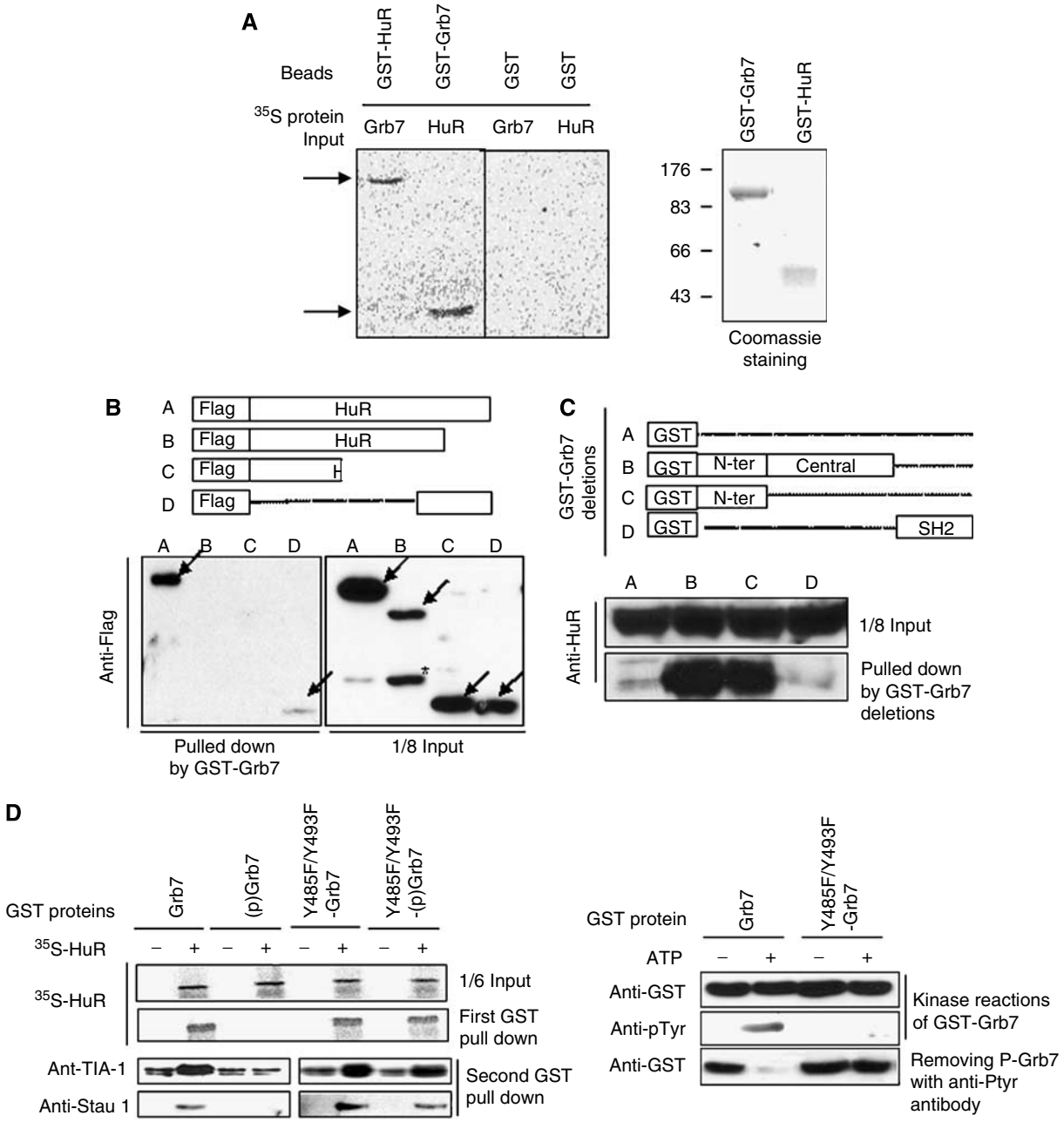

Figure 7 Hypophosphorylated Grb7 directly interacts with HuR, forming a complex with SG components, whereas phosphorylation of Grb7 disrupt its interaction with HuR and complex formation with SG components. (**A**) ^{35}S-labelled Grb7 or HuR detected in reciprocal GST pull-down assays with GST, GST–Grb7 or GST–HuR. The specific Grb7 and HuR bands are marked with arrows. Input of GST-fused proteins is shown on the right. (**B, C**) Western blot analyses of various domains of HuR pulled down by GST–Grb7 (B) or HuR pulled down by various domains of GST–Grb7 (C). (**D**) right: western blots (two upper panels) showing *in vitro* kinase reaction of GST–Grb7 (Grb7, two columns on the left) or the phosphor-mutant GST–Grb7 (Y485F/Y493F-Grb7, two columns to the right). The efficiency of phosphorylation was monitored by removing the phosphorylated protein with anti-phospho-Tyr and detecting the remaining fraction with anti-GST shown in the bottom panel. The anti-GST-positive signals indicated those unphosphorylated portions. Top left: GST pull-down of *in vitro*-synthesized and labelled HuR by GST–Grb7 or phosphor-mutant GST–Grb7 after *in vitro* kinase reaction as shown in the right. Bottom left: western blots of TIA-1 and Staufen 1 precipitated by the complex of GST–Grb7–HuR following incubation with lysates from stressed P19.

of Grb7 phosphorylation on the interaction of Grb7/HuR complex with other SG components was then monitored in a sequential (second) pull-down experiment where the complex pulled down by GST–Grb7 (GST–Grb7–HuR) was subsequently incubated with extracts from heat-shock-stressed P19 cells that contained endogenous SG components. The pull-down complex was then detected with antibodies specific to several SG components such as TIA-1 (anti-TIA-1) and Staufen 1 (anti-Stau 1). Indeed, all the three hypophosphorylated Grb7/HuR complexes (Grb7, Y485F/Y493F-Grb7 or Y485F/Y493F-(p)Grb7), but not the hyperphosphorylated Grb7 ((p)Grb7), effectively pulled down TIA-1 and Staufen 1 (an independent, reproducible data set is shown in Supplementary Figure S14).

Together, these data confirm that hypophosphorylated Grb7 directly interacts with HuR and facilitates the formation of SGs in stress cells and that knocking down of endogenous Grb7 reduces SG formation. In stressed cells, FAK is recruited

to SGs and trigger phosphorylation of Grb7 during recovery, which is a critical step for dissolving SGs by dissociating Grb7 from HuR and other SG components subsequently. Knockdown of endogenous FAK or expressing the phosphorylation mutant of Grb7 attenuates the disassembly of SGs even when cells are allowed to recover. Consistently, the specific mRNA targets are increasingly associated with SGs during stress, but are dissociated from SGs during recovery. However, in cells expressing the mutant Grb7, which cannot be phosphorylated by FAK, the specific KOR transcripts remained highly associated with SGs even during recovery, supporting the retention of specific transcripts by hypophosphorylated Grb7 in the SGs. These results establish a cell-autonomous signalling pathway mediated by the FAK–Grb7 signalling pathway that regulates the assembly and disassembly of SGs, as well as sorting of mRNA targets into SGs in stressed cells, as well as the release of these mRNA targets from SGs during recovery.

Discussion

Altogether, this study demonstrates (a) Grb7 as a new SG component, (b) a new role for hypophosphorylated Grb7 in facilitating SG formation by directly interacting with HuR and stabilizing TIA-1 aggregates, (c) FAK recruitment to SGs during stress and (d) an activity of FAK-phosphorylated Grb7, as well as the enzymatic activity of FAK, in actively disassembling SGs and sorting of mRNA targets during the phase of recovery. This represents the first reported cell-autonomous signalling pathway, FAK activation followed by Grb7 phosphorylation, which can actively trigger the dissociation of SG components in post-stress recovery. However, altering FAK and Grb7 signalling pathway delayed, but not abolished, SG recovery (Supplementary Figure S10). Other signalling pathways can also be involved in this process. Further, Grb7 and FAK were both reported to be involved in other signalling pathways including cell migration. It is tempting to speculate that SG might have an even more general role in regulating various physiological processes, in addition to translational control. Given the fact that FAK and Grb7 are significantly recruited to SGs under stress, their upstream or downstream signals may also have a role. It will be an important issue to address whether these pathways also provide protective or regulatory functions in addition to regulating the stress response.

Grb7 was initially identified as a cytosolic adaptor molecule and a translational regulator that binds to specific mRNA targets (Tsai *et al*, 2007). The current study reports Grb7 as a new and unique category of RNA-binding, SG component that regulates the integrity, or formation, of TIA-1 aggregates and SGs. The hypophosphorylated Grb7 exerts a default activity to directly interact with HuR and to promote SG formation during stress, whereas the hyperphosphorylated (by FAK) Grb7 loses its ability to interact with HuR and other SG components (such as TIA-1 and Staufen 1; Figure 7D) and actively disrupts the integrity of SGs or RNP complexes during recovery. In the current study, we also attempted to generate phospho-mimetic Flag–Grb7 (Y483E/Y495E) and tested its function. However, this mutant retained its translational repressive activity for KOR mRNA as well as its interaction with HuR (data not shown). This could be due to the fact that the phosphorylated domain was distant from the N terminus that was responsible for these activities. Alternatively, glutamate might not be able to fully substitute phosphorylated tyrosine in regulating these activities of Grb7.

Intriguingly, overexpression of Grb7 alone failed to induce SG formation without stress stimulation (Supplementary Figure S12). Presumably, the Grb7–HuR complex functions to facilitate the integrity of RNP complexes before the formation of more visible large aggregates of SGs, which is stimulated by stress. Notably, Grb7 also triggers relocalization of a chaperon protein, HSP27, to the insoluble fraction (Supplementary Figure S4), which is also seen in cells with the prion-like aggregated SGs (Gilks *et al*, 2004) and thought to represent a self-defence mechanism because HSP27 is an ATP-independent chaperon protein that prevents the formation of apoptosome and caspase activities (Garrido *et al*, 2003). Incidentally, studies have shown co-amplification of Grb7 with other oncogenic proteins, which can contribute to cancer formation (Haran *et al*, 2004; Vinatzer *et al*, 2005; Tanaka *et al*, 2006). Whether Grb7 triggers anti-apoptotic pathways remains to be determined.

Although it has long been recognized that SGs can be detected in all the stressed cells where global translation is halted, it is poorly understood how SG formation and disassembly are regulated and how they relate to the sorting of specific mRNA targets. We have identified the first kinase system, FAK, that directly regulates SG dynamics. The endogenous FAK is relatively hypophosphorylated in cells under stress, consistent with the hypophosphorylation of Grb7 under this condition (Figure 4A). Interestingly, it has been reported that phosphatase is indispensable for stress responses (Hahn and Thiele, 2002) and increased phosphatase activity is detected during heat shock (Winkler *et al*, 2002; Mortaz *et al*, 2005). In this regard, it would be interesting to examine if there is any specific phosphatase that dephosphorylates pFAK or pGrb7 during stress.

As several growth factors, including axon guidance factor, netrin-1 (Ren *et al*, 2004; Tsai *et al*, 2006), epidermal growth factor (Chen *et al*, 2003) and insulin (Kasus-Jacobi *et al*, 2000), have been found to regulate FAK/Grb7 signalling pathway, it will be of an enormous interest in the future to investigate the status of these growth factors in cultures confronting environmental stresses. It will be also important to examine whether and how these extracellular factors may affect this cell-autonomous signalling pathway in terms of the regulation of SG dynamics.

Materials and methods

Antibodies and reagents

Antibodies were from Santa Cruz Biotechnology (anti-Grb7, anti-TIA-1, anti-HuR, anti-HSP27, anti-HSP70 and anti-FAK), Sigma-Aldrich (anti-Actin and anti-Flag) and Upstate Biotechnology (anti-FAK and anti-phosphor-Tyr). DAPI was from Roche Applied Science.

Cell culture

P19 embryonal carcinoma cells were maintained as described previously (Tsai *et al*, 2006).

Plasmid constructs

The IL2R-FAK and IL2R-FAK-Y397F were kind gifts from Dr K Yamada (National Institute of Dental and Craniofacial Research). Grb7-silencing construct, FAK-silencing construct, Flag–Grb7 and Grb7 phosphor-mutants were as described (Tsai *et al*, 2006, 2007). siRNA-insensitive Flag–FAK was constructed by using IL2R-FAK as template and primer pair (5′-CTATCAACAGGTGAAGTCCGACTA

CATGCAAGAAATAGC-3′ and 5′-GCTATTTCTTGCATGTAGTCGGA CTTCACCTGTTGATAG-3′). After obtaining the construct, both WT and siRNA-insensitive Flag–FAK were amplified with primer pair (5′-TAGGATCCGCAGCTGCTTATCTTGACCC-3′ and 5′-TAGCTAGCT CAGTGTGGCCGTGTCTGC-3′) and subcloned into Flag tag-inserted pCMX vector. HuR construct for *in vitro* preparation was made by inserting the full-length HuR cDNA amplified with a primer pair (5′-CCAGATCATATGGTTATGAAGACCAC-3′ and 5′-CTGCTAGCGAGTT ATTTGTGGGACTTG-3′) into *Bgl*II/*Xba*I sites of pCMX. Truncated HuRs were constructed using different primer sets (5′-GCTAGC CACTCATGTGATCTACACC-3′, 5′-GCTAGCCCACTGATGTATAAGTT GGC-3′ and 5′-CTAGATCTTTTGGAGGCCCTGTACACCAC-3′) and PCR-amplified with full-length HuR cDNA as template into *Bgl*II/*Xba*I sites of pCMX. GST–HuR was made by inserting a PCR fragment (5′-CCAGATCATATGGTTATGAAGACCAC-3′ and 5′-TAAGCGGCCGCGAGTTATTTGTGGGACTTG-3′) into *Bgl*II/*Not*I of pGEX-2T (Amersham).

Immunoprecipitation and western blotting

Immunoprecipitation was performed as described (Tsai *et al*, 2007) with slight modification. In brief, 200 μg cell lysate was extensively sonicated for 20 s in a buffer (0.5% SDS, 1% Triton X-100, 1 mM EDTA, 1 M NaCl, 20 mM Tris–HCl, pH 8.0) and incubated with the specific antibody overnight at 4°C with continuous shaking. Protein G beads (Millipore) were then added for another 1 h. Beads were collected, washed three times with cold PBS and subjected to western blotting. Western blotting was conducted as described (Tsai *et al*, 2006).

GST pull-down, in vitro kinase reaction, metabolic labelling and real-time RT–PCR

The above assays were performed as described (Tsai *et al*, 2006, 2007; Hwang *et al*, 2007).

Stress induction, recovery and immunohistochemistry

Stress was induced by heating to 43°C for 30 min and recovery was performed by additional 60 min normal condition culture. Cells were then fixed with methanol (stored at −20°C) for 15 min. After blocking with 5% calf serum for 30 min, primary antibodies were diluted 1:200 with 1% bovine serum albumin and applied onto fixed cells for at least 4 h. After three PBS washes, 1:500 diluted Cy3- or FITC-conjugated secondary antibodies were applied onto cells for another 2 h in dark. DAPI in PBS (1:500) was added at the final 5 min of secondary antibody incubation. After washing three times, the cells were observed under an inverted fluorescence microscope (Nikon).

Transfection and siRNA experiments

Transfection was conducted with Lipofectamine 2000 (Invitrogen) as described (Mostaqul Huq *et al*, 2006). With this method, transfection in P19 was in the range of 50–60% for Flag–Grb7 or Flag–HuR, and 40% for IL2R-FAK or IL2R-FAK-Y397F. HuR siRNAs were from Qiagen with transfection using Hyperfect Reagent (Qiagen). All siRNA experiments were performed with at least two independent target sequences and were repeated three times.

Protease digestion and cell fractionation

Protease digestion and soluble–insoluble fractionation were as described (Gilks *et al*, 2004). In brief, 100 μg of total cytoplasmic protein was taken and subjected to Protease K digestion for 5 min at 37°C. Samples were quickly subjected to SDS–PAGE and western blotting analyses.

Cell counting and statistical analysis

In each experiment, 100 cells were scored to determine the percentage of cells with SGs (defined as distinct overlapping granules with approximately 1 μm in diameter). For Grb7- or FAK-silencing cells, only cells that retain 30% or lower are considered as efficiently silenced cells. For transfected cells, only cells that show detectable Flag are considered as transfected cells. Three experiments were conducted to obtain statistical results, presented as means ± s.d. and analysed with Student's *t*-test where $P < 0.05$ was considered as significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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