Jab1 regulates levels of endothelin type A and B receptors by promoting ubiquitination and degradation

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Key words: yeast two-hybrid screening; G protein-coupled receptor (GPCR); Jun activation domain-binding protein 1 (Jab1); GPCR-interacting protein (GIP); ubiquitination; protein degradation

Abbreviations used: C-tail, carboxyl terminal tail; ERK1/2, extracellular signal-regulated kinase 1/2; IB, immunoblotting; IP, immunoprecipitation.

Abstract

Endothelin type A receptor (ET_AR) plays an important role in some cardiovascular disorders where ET_AR levels are increased. However, regulatory mechanisms for ET_AR levels are unknown. Here, we identified Jun activation domain-binding protein 1 (Jab1) as an ET_AR-interacting protein by yeast two-hybrid screening of human heart cDNA library using carboxyl terminal tail (C-tail) of ET_AR as a bait. The interaction was confirmed by glutathione *S*-transferase pull-down assay, co-immunoprecipitation in HEK293T cells expressing ET_AR-myc and FLAG-Jab1, and confocal microscopy. Jab1 knockdown increased whole cell and cell surface levels of ET_AR and ET-1-induced ERK1/2 phosphorylation in HEK293T cells expressing ET_AR, whereas Jab1 overexpression decreased them. Jab1 overexpression accelerated disappearance rate of ET_AR after protein synthesis inhibition as an index of a degradation rate. ET_AR was constitutively ubiquitinated, and the level of ubiquitination was enhanced by Jab1 overexpression. Long-term ET-1 stimulation markedly accelerated the rate of ET_AR degradation and increased the amount of Jab1 bound to ET_AR with a maximal level of 500% at 3h. In the absence of ET-1 stimulation, the level of ET_BR was lower than that of ET_AR and the degradation rate of ET_BR was markedly faster than that of ET_AR. Notably, the amount of Jab1 bound to ET_BR and ubiquitination level of ET_BR were markedly higher than those for ET_AR. Taken together, these results suggest that the amount of Jab1 bound to ETR regulates the degradation rate of ET_AR and ET_BR by modulating ubiquitination of these receptors, leading to changes in ET_AR and ET_BR levels.

Introduction

G protein-coupled receptors (GPCRs) interact with intracellular proteins such as heterotrimeric G proteins, GPCR kinases, second messenger-dependent protein kinases and β-arrestin [1, 2]. While the functional consequences of these interactions are well characterized, recent studies have raised the possibility that some of the physiological actions of GPCRs are not mediated by the interaction of GPCRs with the proteins listed above [2]. Moreover, recent studies using yeast two-hybrid system and GST pull-down assay have identified a growing number of additional intracellular proteins that directly bind to GPCRs, especially C-tail of GPCRs [3]. While the functional consequences of these interactions have not always been clarified, it seems that the formation of such GPCR and GPCR-interacting protein (GIP) complexes result in the regulation of the activity of GIP, the activity of the associated GPCRs, and intracellular trafficking of GPCRs [4]. Until now, more than 50 GIPs interacting with C-tails of a variety of GPCRs have been identified [3]. In some cases, it has been found that more than 5 GIPs interact with a single GPCR.

Endothelin-1 (ET-1) is a vasoconstricting peptide of 21 amino acids which is synthesized and released in endothelial cells of vessels [5]. ET-1 also possesses the activity to stimulate cell growth [6, 7]. Therefore, ET-1 is considered to play important roles in the physiological control of blood pressure and cardiac function and also in genesis and development of cardiovascular diseases such as atherosclerosis [8], cardiac remodeling accompanying chronic heart failure [7], pulmonary hypertension [9]. There are two types of receptors for ET-1, endothelin type A receptor (ET_AR) and ET_BR, both of which are GPCRs [10, 11]. ET_AR is coupled with G_q and G_s [12, 13], while ET_BR is coupled with G_q and G_i [12, 14]. Notably, levels of ET receptors as well as ET-1 are reported to be elevated in cardiac muscles of chronic heart failure [7] and in infiltrating cells of atherosclerotic lesions such as smooth muscle cells and macrophages [8]. The mechanism for the elevation of levels of ET receptors in these diseases is at present unknown, but the dysregulation of GIPs for ET receptors might contribute to the elevated levels of ET receptors. As for GIPs of ET_AR, only two proteins such as Tip 60 (histon acetyltransferase) and HDAC 7 (histon deacetylase) have been identified using yeast two-hybrid system and implicated in ET_AR intracellular signaling [15].

In this study, to identify the ET_AR C-tail-interacting proteins, we have performed yeast two-hybrid screening of adult human heart cDNA library using C-tail of ET_AR as a bait. Among ET_AR-interacting proteins, we have identified Jun activation domain-binding protein 1 (Jab1). Jab1 was originally discovered as a protein interacting with c-Jun protein, which combined with Fos protein to form a gene regulatory protein complex, AP-1 [16]. In additon, Jab1 was shown to interact with $p27^{Kip1}$ [17], p53 [18] and lutropin/choriogonadotropin receptor (LHR) precursor [19] and to accelerate the degradation of these proteins. Recent studies have shown that Jab1 overexpression induces ubiquitination of some of its interacting partners [20, 21]. Jab1 is reported to be a member of COP9 signalosome (CSN) which interacts with cullin-RING type E3 ubiquitin ligases, and regulates the activity of that enzyme [22, 23].

Here, we report that the amount of Jab1 bound to ETR regulates the degradation rate of ET_AR and ET_BR by modulating ubiquitination of these receptors, leading to changes in ET_AR and ET_BR levels.

Materials and methods

Antibodies

Mouse monoclonal anti-myc-tag antibody, rabbit polyclonal anti-phospho-p44/42 MAPK (p-ERK1/2) antibody and rabbit polyclonal anti-p44/42 MAPK (ERK1/2) antibody were purchased from Cell Signaling. Mouse monoclonal anti-Jab1 antibody, mouse monoclonal anti-GAPDH antibody and mouse monoclonal anti-ubiquitin antibody were purchased from Santa Cruz. Mouse monoclonal anti-FLAG antibody conjugated to horseradish peroxidase (HRP) was purchased from Sigma. Goat anti-mouse IgG or anti-rabbit IgG antibody conjugated to HRP was purchased from Jackson ImmunoResearch.

Plasmid construction

For yeast two-hybrid screening or GST pull-down assay, the C-terminal region of human ET_AR (373-427 amino acids) was amplified by RT-PCR and subcloned into pGBKT7 vector containing the GAL4 DNA-binding domain (Clontech) or pGEX-5X-1 vector containing GST cDNA (GE Healthcare), respectively. For co-immunoprecipitation and Western blotting, PCR products of full-length human ET_AR cDNA or Jab1 cDNA were subcloned into pMXrmv5-$(G_4S)_3$ YFP1-myc retroviral vector (for expression of ET_AR-myc) or pcDNA3-FLAG vector (Invitrogen) (FLAG-Jab1), respectively. For confocal microscopic study, the PCR products were subcloned into pMXrmv5-$(G_4S)_3$YFP retroviral vector (ET_AR-YFP) or pMXrmv5-$(G_4S)_3$CFP retroviral vector (Jab1-CFP), respectively.

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using the MATCHMAKER two-hybrid system 3 (Clontech), according to the manufacturer's protocol.

Cell culture and transfection

All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. For plasmid DNA transfection, the cells (80% confluent) were transiently transfected with pcDNA3-FLAG-Jab1 using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. For siRNA transfection, the cells (80% confluent) were transiently transfected with either scrambled (control) or Jab1 siRNA using DharmaFECT 1 (Dharmacon), according to the manufacturer's protocol. For construction of HEK293T cells stably expressing ET_AR-myc (HEK293T/ET_AR) and for confocal microscopy (Carl Zeiss LSM510 META), retroviral vector constructs were introduced into HEK293T cells.

GST pull-down assay

GST-ET_AR C-tail fusion protein expressed in BL21-Gold bacteria was immobilized to glutathione-Sepharose beads (GE Healthcare), and incubated for 1 h at 4°C with whole cell lysates from HEK293T cells expressing FLAG-Jab1 protein. After washing (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40), the proteins bound to GST-ET_AR C-tail fusion protein were separated by SDS-PAGE, and immunoblotted with anti-FLAG antibody.

Western blotting

Cells were lysed by incubation on ice for 30 min with RIPA (radio-immunoprecipitation assay) buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml Leupeptin/Aprotinin/Pepstatin, 1 mM Na_3VO_4, 20 mM NaF, 1 × protease inhibitor cocktail (Pierce)). After centrifugation, the lysate containing 20 μg protein was dissolved in Laemmli buffer (25 mM Tris-HCl (pH 6.8), 0.8% (w/v) SDS, 2% (v/v) 2-mercaptoethanol (2-ME), 4% (v/v) glycerol, 0.04% (w/v) bromophenol blue (BPB)) and was incubated at 25°C for 20 min. The proteins were separated by SDS-PAGE and immunoblotted with either of primary antibodies, followed by goat anti-mouse IgG or anti-rabbit IgG antibody conjugated to HRP. After washing, the immune complexes were detected by ECL Westerrn blotting substrate (GE healthcare).

Co-immunoprecipitation

The cell lysates in RIPA buffer were incubated with protein G-Sepharose beads (GE Healthcare) for 30 min at 4°C for pre-cleaning. After centrifugation, 1-ml aliquots of the lysates containing 1 mg of protein were incubated with anti-myc-tag antibody (1 μg) or anti-Jab1 antibody (1 μg) overnight at 4°C, followed by incubation with protein G-Sepharose beads (20 μl) for 2 h at 4°C. After washing, the beads were incubated in Laemmli buffer at 25°C for 20 min to elute the bound proteins. After removal of the beads by centrifugation, the eluted proteins were separated by SDS-PAGE and immunoblotted with anti-myc-tag antibody, anti-Jab1 antibody, anti-FLAG antibody or anti-ubiquitin antibody.

Quantitation of cell surface ET_AR

To measue cell surface level of ET_AR-myc, biotinylation of cell surface proteins was performed in HEK293T/ET_AR cells using membrane-impermeable biotin analog, sulfo-NHS-SS-biotin, according to the manufacturer's protocol (Pierce). After washing and lysis in RIPA buffer, biotinylated proteins purified by streptavidin-agarose beads (Sigma) were analyzed by Western blot with anti-myc-tag antibody.

Quantitation of ubiquitinated ET_AR

HEK293T/ET_AR-myc cells were lysed with RIPA buffer containing 20 mM *N*-ethylmaleimide (NEM; Calbiochem) to prevent deubiquitination [24]. The cell lysates were immunoprecipitated with anti-myc-tag antibody, and immunocomplex was subjected to Western blot analysis with anti-ubiquitin antibody (1:1000).

Statistical analysis

The data were expressed as mean $\pm$ S.E.M. Statistical comparisons between two groups were done using Student's *t*-test. $p < 0.05$ (*) or $p < 0.01$ (**) was considered statistically significant.

Results and discussion

Identification of Jab1 as an ET_AR-interacting protein

To identify the proteins that interact with ET_AR C-tail, a yeast two-hybrid screening of a human heart cDNA library was performed using human ET_AR C-tail as a bait. Among these ET_AR C-tail-interacting clones, two independent clones containing the cDNA fragments encoding Jab1 were obtained. FLAG-Jab1 was pulled-down with GST-ET_AR C-tail fusion protein immobilized to the beads (Fig. 1A, lane 3). GST tag protein without ET_AR C-tail did not interact with FLAG-Jab1 (Fig. 1A, lane 2). These results indicate ET_AR C-tail interacts with Jab1 *in vitro*.

We next confirmed the interaction between ET_AR and Jab1 in HEK293T cells, using co-immunoprecipitation experiments. Endogenous Jab1 was co-immunoprecipitated with ET_AR-myc in HEK293T/ET_AR-myc cells, but not in wild-type HEK293T cells (Fig. 1B, upper panel, lanes 1 and 2). Both FLAG-Jab1 and endogenous Jab1 were co-immunoprecipitated with ET_AR-myc in HEK293T/ET_AR-myc cells expressing FLAG-Jab1 (Fig. 1B, upper panel, lane 3). Comparable amount of ET_AR-myc was immunoprecipitated in the absence and presence of FLAG-Jab1 (Fig. 1B, lower panel, lanes 2 and 3). Notably, ET_AR was present as putative monomer, dimer and multimer including molecules with a molecular size >200kDa. Reversing the antibodies used for immunoprecipitation and immunoblotting revealed such interactions in HEK293T/ET_AR-myc cells with or without FLAG-Jab1. Importantly, putative monomer, dimer and multimer of ET_AR interacted with endogenous Jab1 (Fig. 1C, upper panel, lane 1). The immunoprecipitated amount of all forms of ET_AR-myc was more abundant after transfection of FLAG-Jab1 (Fig. 1C, upper panel, lane 2). After reprobing PVDF membrane with anti-Jab1 antibody, endogenous Jab1 and FLAG-Jab1 were found to be immunoprecipitated (Fig. 1C, lower panel, lanes 1 and 2). These results demonstrate that monomer, dimer and multimer of ET_AR interact with Jab1.

Intracellular localization of ET_AR and Jab1 was visualized with confocal microscopy in HEK293T cells expressing ET_AR-YFP and Jab1-CFP. ET_AR-YFP was predominantly localized at the plasma membrane (Fig. 1D, left panel), whereas Jab1-CFP was diffusely distributed in the cytosol and nucleus (Fig. 1D, middle panel). The overlay image shows the merged color (yellow) on the periphery of the cells (Fig.

1D, right panel). Combined with the data by immunoprecipitation study, these results suggest that ET_AR-myc is present in monomer, dimer and multimer mainly on the cell surface, where Jab1 interacts with each form of ET_AR.

Effect of Jab1 overexpression or knockdown on ET_AR levels

Following overexpression of FLAG-Jab1, the level of total ET_AR which is the sum of monomer, dimer and multimer was significantly decreased in comparison with that in the cells transfected with an empty vector (Fig. 2, A and B). The levels of ET_AR dimer and multimer but not that of monomer were significantly decreased (Supplementary Fig. S1, A). In contrast, following Jab1 knockdown with its siRNA, the level of total ET_AR was significantly increased in comparison with that in the cells transfected with scrambled siRNA (control siRNA) (Fig. 2, C and D). The levels of ET_AR dimer and multimer but not that of monomer were significantly increased (Supplementary Fig. S1, B). Following knockdown of endogenous Jab1, the levels of total and each form of ET_AR on cell surface determined by biotinylation assay were significantly increased in comparison with those in HEK293T/ET_AR-myc cells transfected with control siRNA (Supplementary Fig. S1, C and D). Taken together, these results suggest that Jab1 negatively regulates whole cell and cell surface levels of ET_AR.

Effect of Jab1 overexpression or knockdown on ET-1-induced ERK1/2 phosphorylation

In control cells, the level of p-ERK1/2 was negligible before ET-1 stimulation: it reached the maximal level 5 min after ET-1 stimulation and thereafter gradually decreased as previously reported [6, 25]. Jab1 overexpression decreased ET-1-induced ERK1/2 phosphorylation (Supplementary Fig. S2, A and B), whereas Jab1 knockdown increased them (Supplementary Fig. S2, C and D). These results suggest that Jab1 regulates ET_AR-mediated intracellular signaling by regulating ET_AR levels.

Jab1 promotes degradation of ET_AR

To elucidate the mechanism by which Jab1 regulates ET_AR levels, we examined the effect of Jab1 on degradation rate of ET_AR which was determined by the rate of disappearance for ET_AR-myc after treatment with a protein synthesis inhibitor, cycloheximide (CHX, Calbiochem). In the absence of Jab1 overexpression, the level of total ET_AR decreased with time up to 24 h after treatment with CHX, and at 24 h, it was about 46% of control level (Fig. 2E, left panel and Fig. 2F). Following Jab1 overexpression, the rate of disappearance for total ET_AR became significantly faster and at 24 h, it was 24% of control level (Fig. 2E, right panel and Fig. 2F). Similar results were obtained for each form of ET_AR (data not shown). ET_AR was constitutively ubiquitinated, and the level of ubiquitination was enhanced by Jab1 overexpression (Fig. 2, G and H). These results suggest that Jab1 promotes degradation of ET_AR by enhancing its ubiquitination.

Long-term ET-1 stimulation promotes ET_AR degradation and ET_AR-Jab1 interaction

Because long-term agonist exposure was reported to promote degradation for many GPCRs (26), we assessed changes in ET_AR levels, degradation rate and the amount of Jab1 bound to the receptor. Long-term ET-1 stimulation decreased the level of ET_AR in a time-dependent manner (Fig. 3A, lower panel), but it increased the amount of Jab1 bound to the receptor with a maximal level of 500% at 3h (Fig. 3A, upper panel and Fig. 3B). Long-term ET-1 stimulation markedly increased the rate of ET_AR degradation (Fig. 3, C and D). These results suggest that an increase in degradation rate of ET_AR after long-term ET-1 stimulation is mediated by an increase in the amount of Jab1 bound to ET_AR.

Comparison of degradation rate, ubiquitination level and the amount of Jab1 bound to ET_AR and ET_BR

It is reported that in the absence or presence of ET-1 stimulation, ET_AR is primarily targeted to recycling pathway, whereas ET_BR is exclusively targeted to lysosomal pathway for degradation (27, 28). To get insights into a mechanism for a faster degradation rate of ET_BR, we compared the expression level, degradation rate, ubiquitination level and the amount of bound Jab1 of ET_BR with those of ET_AR. In the absence of ET-1 stimulation, the level of ET_BR was lower than that of ET_AR (Fig. 4B) and the rate of ET_BR degradation was faster than that of ET_AR (Fig. 4, A and C), as expected. In immunoprecipitation experiments, Jab1 was confirmed to interact with ET_BR (Fig. 4D). Notably, the amount of Jab1 bound to ET_BR (Fig. 4, D and E) and ubiquitination level of ET_BR (Fig. 4, F and G) were markedly higher than those for ET_AR. These results suggest that the difference of degradation rate between ET_AR and ET_BR is due to the different amount of Jab1 bound to both receptors and subsequent ubiquitination level between ET_AR and ET_BR.

Concluding remarks

We identified Jab1 as a protein interacting with ET_AR and ET_BR and demonstrated that the amount of Jab1 bound to both receptors regulates degradation rate of ET_AR and ET_BR by modulating ubiquitination of these receptors, leading to changes in ET_AR and ET_BR levels. This conclusion is based on the following observations. First, in the absence of ET-1 stimulation, the amount of Jab1 bound to ET_AR and ET_BR was positively correlated with ubiquitination level and degradation rates of ET_AR and ET_BR, causing a change in both receptor levels opposite to that of the amount of Jab1. Second, ET-1 stimulation induced an increase in the amount of Jab1 bound to ET_AR and degradation rate of ET_AR. Third, a faster degradation rate of ET_BR in comparison with that of ET_AR was associated with higher levels of bound Jab1 and ubiquitination for ET_BR.

To elucidate the detailed molecular mechanisms for ubiquitination and degradation of ETR by Jab1, we must clarify the ubiquitination-related enzymes (E2 and E3) specific for ETR, ubiquitination sites of ETR, a role of ubiquitination in ETR trafficking, the relationship between ubiquitination and phosphorylation in the context of ETR trafficking.

Acknowledgements

This work was supported in part by Grant-in-Aids for Scientific Research (B) 18390072 from Japan Society for the Promotion of Science (JSPS) (to S.M.) and in part by grants from the Smoking Research Foundation of Japan (to S.M.).

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Figure legends

Fig. 1. Interaction of Jab1 with ET_AR detected by GST pull-down assay (A), immunoprecipitation (B, C) and confocal microscopic study (D). (A) Cell lysates from HEK293T cells expressing FLAG-Jab1 were incubated with GST protein (GST) or GST-ET_AR C-tail fusion protein (GST-ET_AR-C) immobilized to glutathione-Sepharose beads. The bound proteins were analyzed by Western blot with anti-FLAG antibody. (B, C) Cell lysates from HEK293T/ET_AR-myc cells expressing FLAG-Jab1 or FLAG were immunoprecipitated with anti-myc-tag antibody, followed by Western blot with anti-Jab1 antibody (B, upper panel). For a negative control, wild-type HEK293T cells were used. In (C), the antibodies for

immunoprecipitation and blotting used in (B) were reversed. PVDF membrane was reprobed with anti-myc-tag antibody (B, lower panel), or with anti-Jab1 antibody (C, lower panel). *endo. Jab1*, endogenous Jab1. (D) Confocal microscopic study for cellular localization of ET_AR-YFP and Jab1-CFP proteins in HEK293T cells retrovirally transferred with cDNAs for these proteins.

Fig. 2. Changes in ET_AR levels (A-D), the degradation rate (E, F) and ubiquitination level (G, H) of ET_AR following a change in Jab1 expression level. (A-D) HEK293T/ET_AR-myc cells were transfected with either pcDNA3-FLAG or pcDNA3-FLAG-Jab1 for 48 hours (A) and with either control siRNA or Jab1 siRNA for 96 hours (C). ET_AR-myc (A and C, upper panels), endogenous Jab1 (endo. Jab1) (A and C, middle panels), FLAG-Jab1 (A, middle panel) and GAPDH (A and C, lower panels) were detected by Western blotting. The images were acquired using the Fluor-S MultiImager (Bio-Rad), and the density of each band was quantitated by Quantity One software (Bio-Rad). The ratio for total ET_AR to GAPDH (total ET_AR/GAPDH) was normalized to the values in control cells. Each bar represented the mean ± S.E.M. of three independent experiments (B, D). (E, F) HEK293T/ET_AR-myc cells expressing FLAG (E, left panel) or FLAG-Jab1 (E, right panel) were incubated with 50 μM cycloheximide (CHX) for the indicated time. ET_AR-myc (E, upper panel), endogenous Jab1

(endo. Jab1)/FLAG-Jab1 (E, middle panel) and GAPDH (E, lower panel) were detected by Western blotting. The data were represented (F) as described above. (G, H) Cell lysates from HEK293T/ET_AR-myc cells expressing FLAG or FLAG-Jab1 were immunoprecipitated with anti-myc-tag antibody, followed by Western blotting with anti-ubiquitin antibody (G, left panel). PVDF membrane was reprobed with anti-myc-tag antibody (G, right panel). For a negative control, wild-type HEK293T cells were used. The band around 80 kDa might be the complex between heavy-chain and light-chain of IgG (G, left panel). After densitometric analysis, the level of ubiquitinated ET_AR was normalized by level of total ET_AR (H). *, $p < 0.05$, significantly different (n=3).

Fig. 3. Effects of ET-1 stimulation on ET_AR level (A), the amount of Jab1 bound to ET_AR (A, B) and the rate of ET_AR degradation (C, D) in HEK293T/ET_AR-myc cells expressing FLAG-Jab1. (A, B) The cells were incubated with 100 nM ET-1 for the indicated time, and cell lysates from these cells were immunoprecipitated with anti-myc-tag antibody, followed by Western blotting with anti-Jab1 antibody (A, upper panel). PVDF membrane was reprobed with anti-myc-tag antibody (A, lower panel). The density of each band was quantitatively analyzed as described in Fig. 2, and the amount of bound Jab1 was normalized by total ET_AR (B). (C, D) The cells were incubated with or without 100 nM ET-1 for the indicated time in

the presence of 50 μM CHX. ET_AR-myc (C, upper panel) and GAPDH (C, lower panel) were detected by Western blotting, and the data were represented as described in Fig. 2 (D). *, $p <$ 0.05 or **, $p <$ 0.01; significantly different (n=3).

Fig. 4. Comparison of degradation rate (A, C), expression level (B), the amount of bound Jab1 (D, E) and ubiquitination level (F, G) between ET_AR and ET_BR. (A-C) HEK293T/ET_AR-myc cells and HEK293T/ET_BR-myc cells were incubated with 50 μM cycloheximide (CHX) for the indicated time. ET_AR-myc and ET_BR-myc (A, upper panel) and GAPDH (A, lower panel) were detected by Western blotting, and the ratio (total $ET_{A/B}$R/GAPDH) was represented as described in Fig. 2. In (B), expression levels of ET_AR and ET_BR before treatment with CHX were represented, while changes in the levels of these receptors after the treatment were plotted in (C). (D, E) Cell lysates from HEK293T/ET_AR-myc cells and HEK293T/ET_BR-myc cells were immunoprecipitated with anti-myc-tag antibody, followed by Western blot with anti-Jab1 antibody (D, left panel). Endogenous Jab1 in whole cell lysates (WCL) from these cells was detected by Western blot (D, right panel). After densitometric analysis, the amount of Jab1 bound to ET_AR or ET_BR was normalized by levels of total ETRs (E). The levels of ET_AR and ET_BR were determined by immunoprecipitation with anti-myc-tag antibody, followed by Western blot with anti-myc-tag antibody, as shown in Fig. 4F, right panel. (F, G) The cell

lysates were immunoprecipitated with anti-myc-tag antibody, followed by Western blotting with anti-ubiquitin antibody (F, left panel). *ub*, ubiquitin; *IgG H*, heavy chain of IgG. The expression levels of ET_AR and ET_BR were separately analyzed using part of the same immunoprecipitates by Western blotting with anti-myc-tag antibody (F, right panel). The level of ubiquitinated $ET_{A/B}R$ was normalized by level of total $ET_{A/B}R$ (G). *, $p < 0.05$ or **, $p < 0.01$; significantly different (n=3).

Fig. 1.

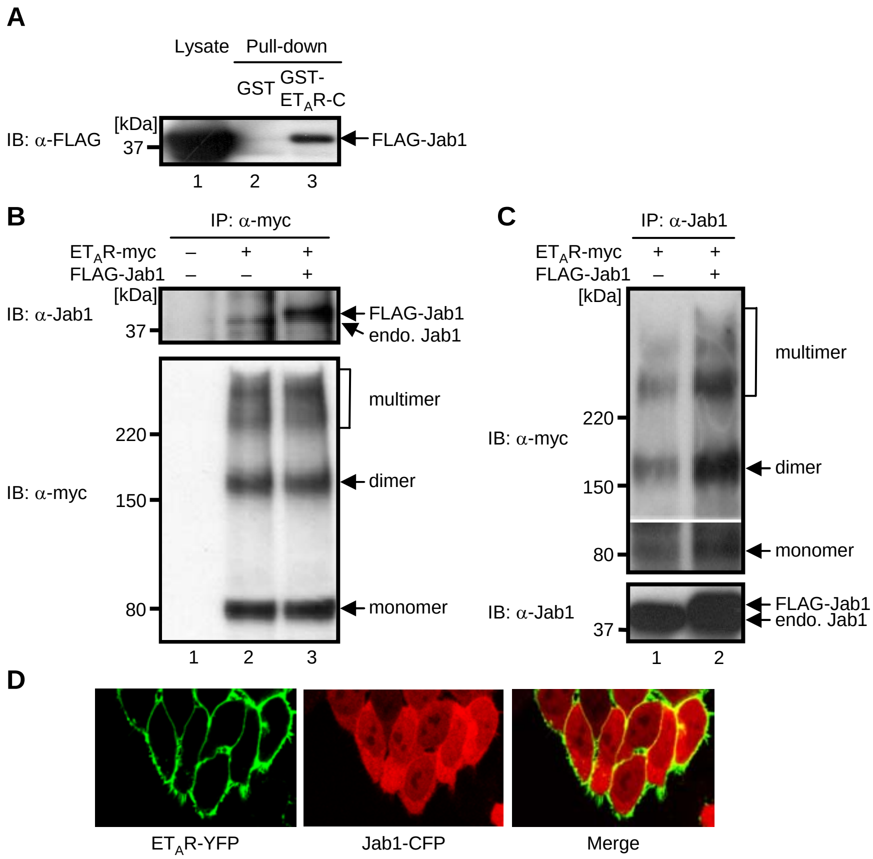

A
Lysate
Pull-down
GST
GST-ET$_A$R-C
IB: α-FLAG
[kDa]
37
FLAG-Jab1
1 2 3
B
IP: α-myc
ET$_A$R-myc – + +
FLAG-Jab1 – – +
IB: α-Jab1
FLAG-Jab1
endo. Jab1
IB: α-myc
220
150
80
multimer
dimer
monomer
1 2 3
C
IP: α-Jab1
ET$_A$R-myc + +
FLAG-Jab1 – +
IB: α-myc
IB: α-Jab1
multimer
dimer
monomer
FLAG-Jab1
endo. Jab1
1 2
D
ET$_A$R-YFP
Jab1-CFP
Merge

Fig. 2.

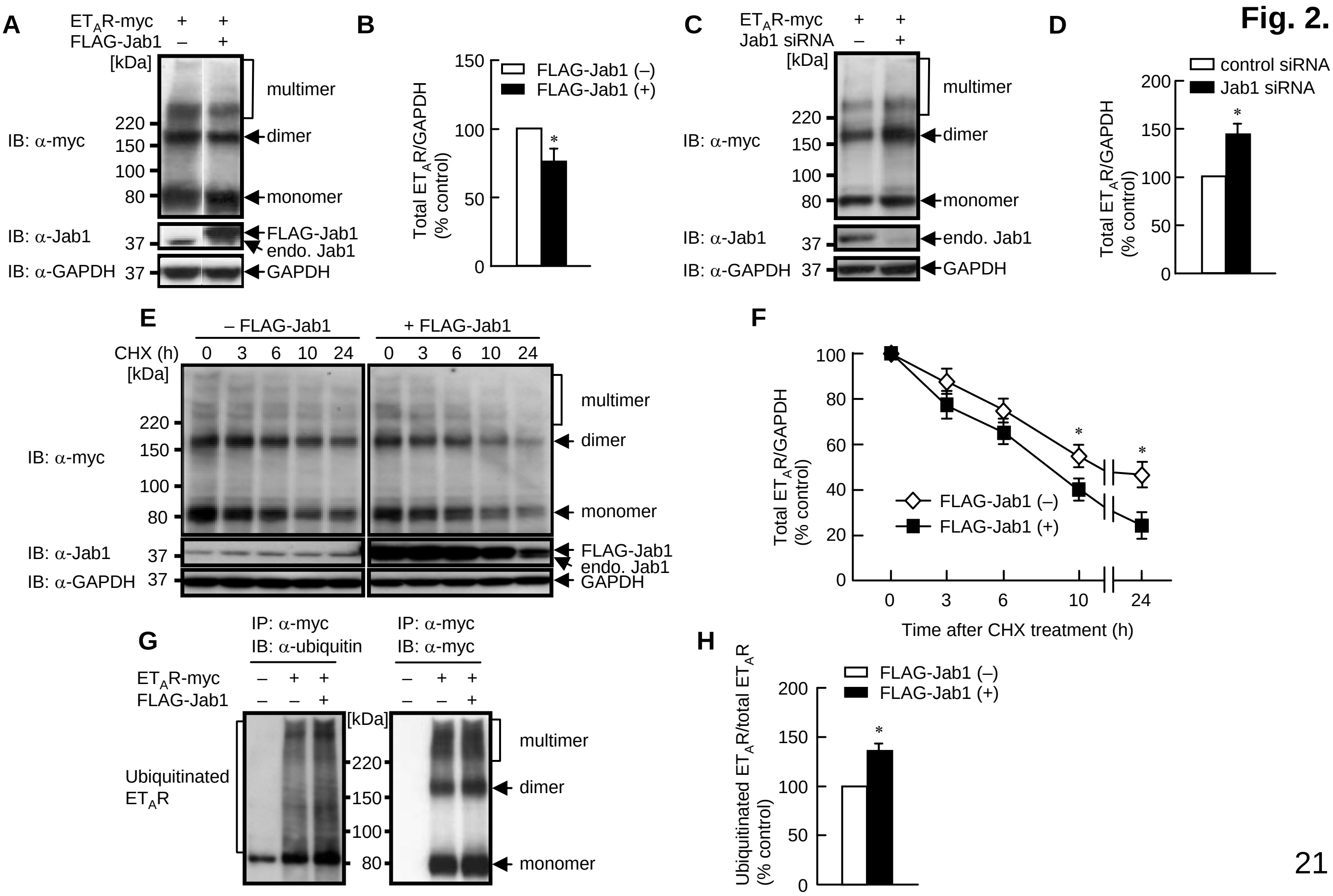

A
ET$_A$R-myc + +
FLAG-Jab1 – +
[kDa]
IB: α-myc
220
150
100
80
multimer
dimer
monomer
IB: α-Jab1
FLAG-Jab1
endo. Jab1
IB: α-GAPDH
GAPDH
B
FLAG-Jab1 (–)
FLAG-Jab1 (+)
Total ET$_A$R/GAPDH (% control)
C
ET$_A$R-myc + +
Jab1 siRNA – +
endo. Jab1
D
control siRNA
Jab1 siRNA
E
– FLAG-Jab1
+ FLAG-Jab1
CHX (h) 0 3 6 10 24 0 3 6 10 24
F
Time after CHX treatment (h)
G
IP: α-myc
IB: α-ubiquitin
IP: α-myc
IB: α-myc
Ubiquitinated ET$_A$R
H
Ubiquitinated ET$_A$R/total ET$_A$R (% control)

Fig.3.

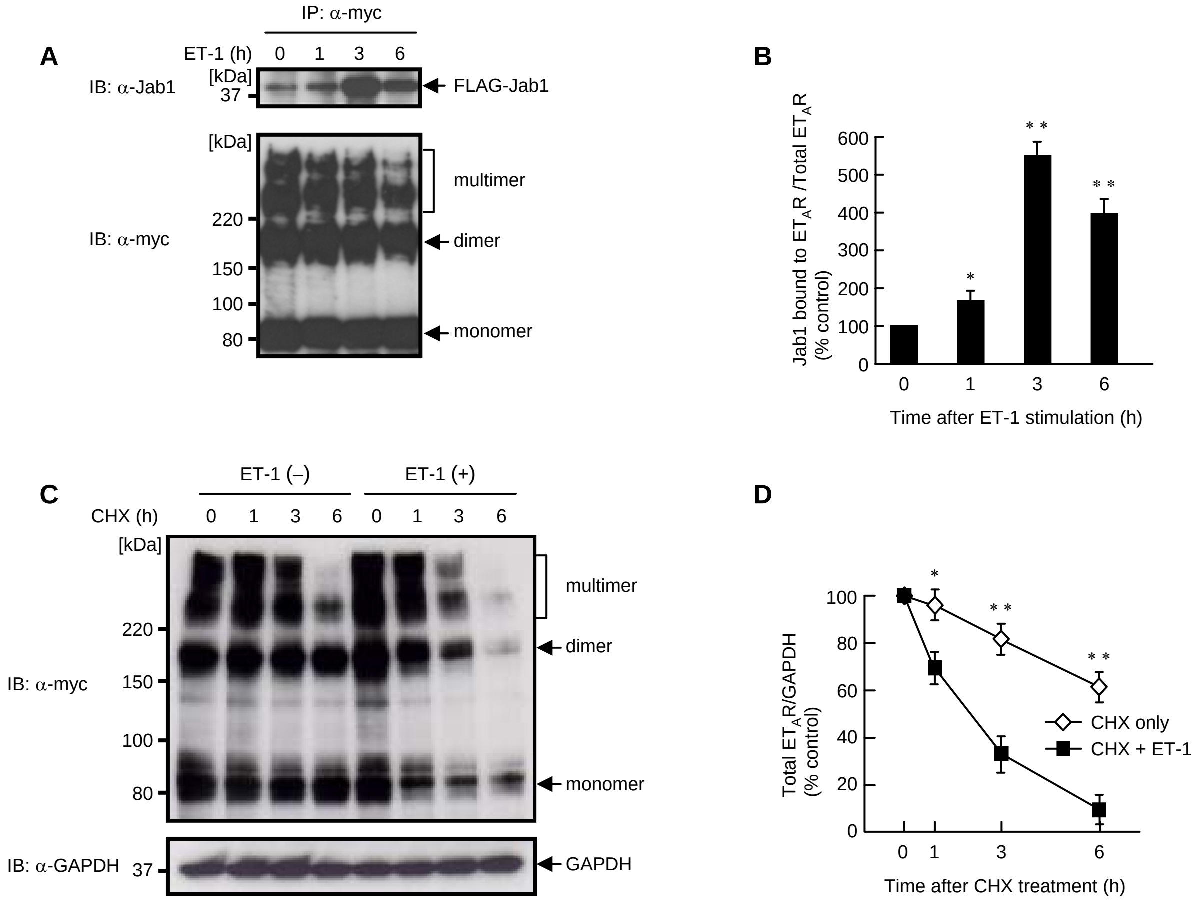
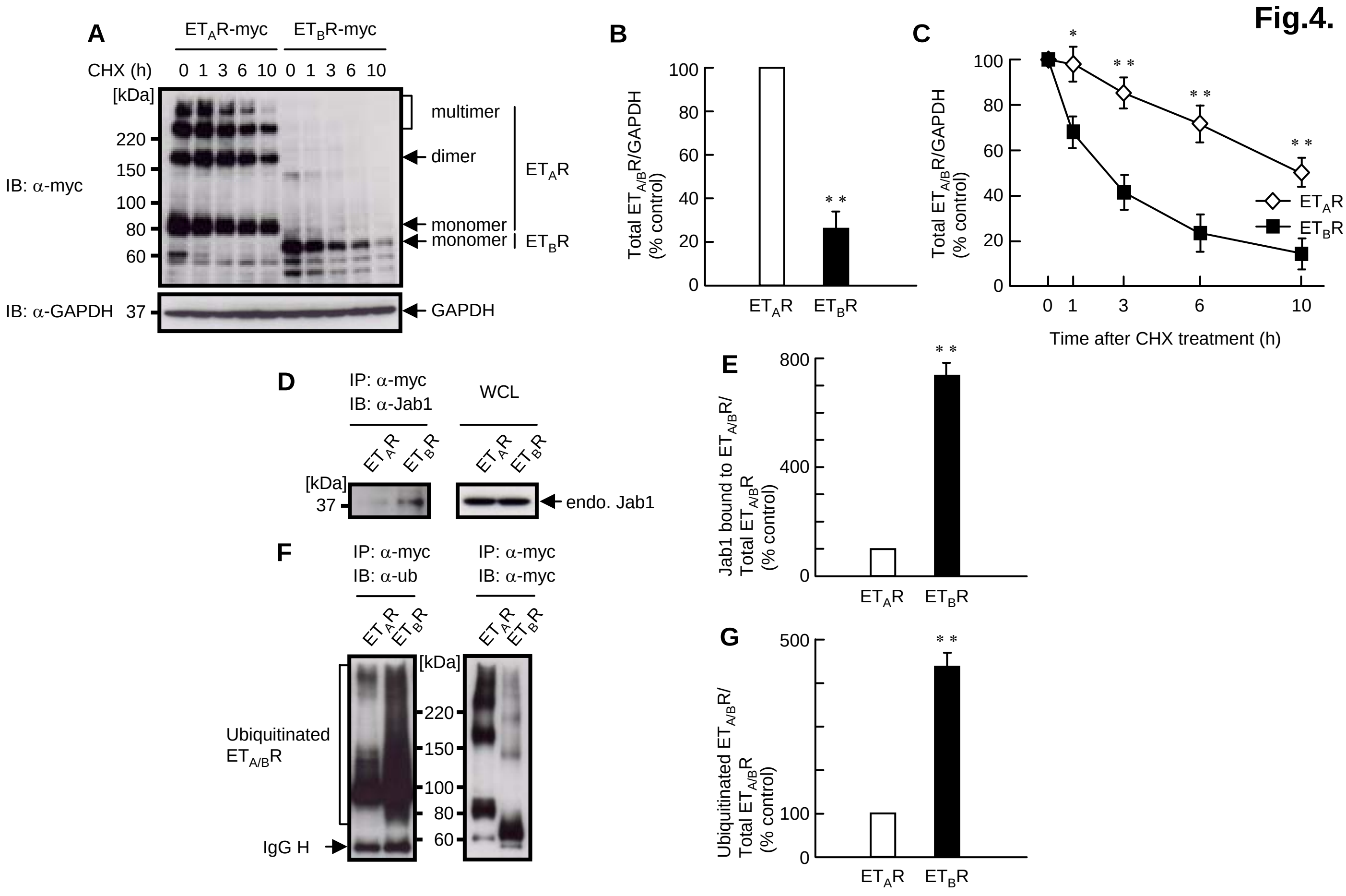
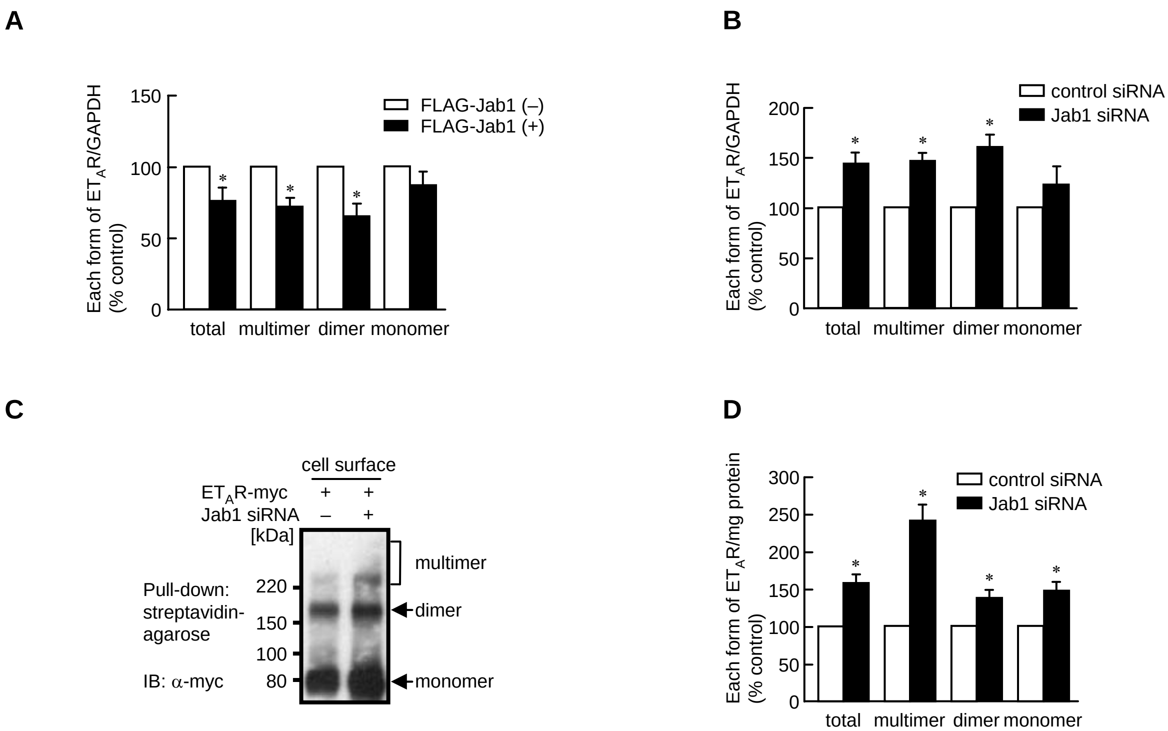

A
IP: α-myc
ET-1 (h) 0 1 3 6
IB: α-Jab1
[kDa]
37
FLAG-Jab1
[kDa]
multimer
220
dimer
IB: α-myc
150
100
monomer
80
B
Jab1 bound to ETAR /Total ETAR
(% control)
600
500
400
300
200
100
0
0 1 3 6
Time after ET-1 stimulation (h)
C
ET-1 (–)
ET-1 (+)
CHX (h) 0 1 3 6 0 1 3 6
[kDa]
multimer
220
dimer
IB: α-myc
150
100
80
monomer
IB: α-GAPDH 37
GAPDH
D
Total ETAR/GAPDH
(% control)
100
80
60
40
20
0
CHX only
CHX + ET-1
0 1 3 6
Time after CHX treatment (h)

Fig.4.

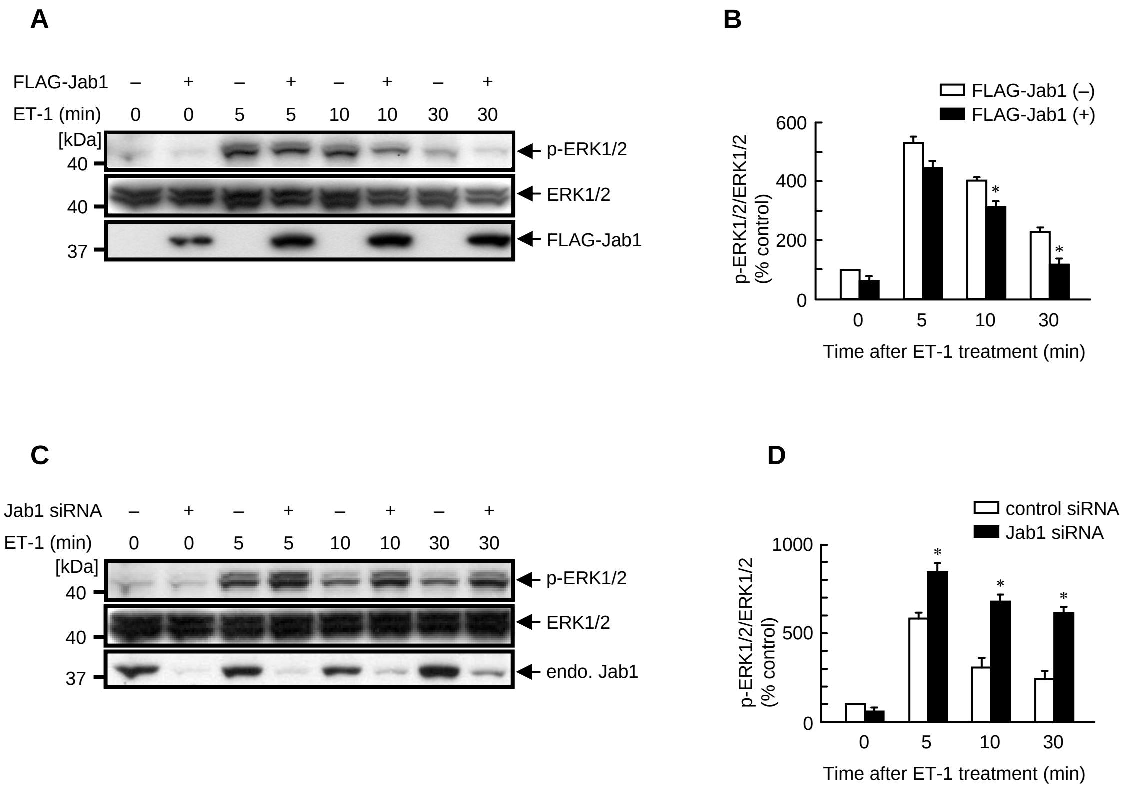

A
ETAR-myc
ETBR-myc
CHX (h) 0 1 3 6 10 0 1 3 6 10
[kDa]
multimer
220
dimer
150
ETAR
IB: α-myc
100
monomer
80
monomer
ETBR
60
IB: α-GAPDH 37
GAPDH
B
Total ETA/BR/GAPDH
(% control)
100
80
60
40
20
0
ETAR ETBR
C
Total ETA/BR/GAPDH
(% control)
100
80
60
40
20
0
ETAR
ETBR
0 1 3 6 10
Time after CHX treatment (h)
D
IP: α-myc
IB: α-Jab1
WCL
ETAR ETBR ETAR ETBR
[kDa]
37
endo. Jab1
E
Jab1 bound to ETA/BR/
Total ETA/BR
(% control)
800
400
0
ETAR ETBR
F
IP: α-myc
IB: α-ub
IP: α-myc
IB: α-myc
ETAR ETBR ETAR ETBR
[kDa]
220
150
Ubiquitinated
ETA/BR
100
80
60
IgG H
G
Ubiquitinated ETA/BR/
Total ETA/BR
(% control)
500
100
0
ETAR ETBR

Supplementary Fig. S1



Supplementary Fig. S1. Effect of overexpression or knockdown of Jab1 on the level of total and each form ET_AR in whole cell (A, B) and cell surface (C, D). Experiments for overexpression (A) or knockdown (B) of Jab1 and the processing of the data were performed as described in legends for Fig. 2A-B or Fig. 2C-D, respectively. (C, D) HEK293T/ET_AR-myc cells were transfected with either control siRNA or Jab1 siRNA for 96 hours. Cell surface ET_AR-myc was isolated by biotinylation assay and detected by Western blot with anti-myc-tag antibody as described in “Materials and methods” (C). After densitometric analysis, the level of total and each form ET_AR was normalized by protein amount used in biotinylation assay (D). *, $p < 0.05$, significantly different from control values (n=3).

Supplementary Fig. S2



Supplementary Fig. S2. Effect of overexpression (A, B) or knockdown (C, D) of Jab1 on ET-1-induced ERK1/2 phosphorylation. HEK293T/ET_AR-myc cells were transfected with either pcDNA3-FLAG or pcDNA3-FLAG-Jab1 for 48 hours (A) and with either control siRNA or Jab1 siRNA for 96 hours (C). After starvation for 16 h, the cells were stimulated with 100 nM ET-1 for the indicated time. Phosphorylated ERK1/2 (p-ERK1/2), ERK1/2 (A and C, upper and middle panels), FLAG-Jab1 (A, lower panel) and endogenous Jab1 (endo. Jab1) (C, lower panel) were detected by Western blotting. After densitometric analysis, the levels of p-ERK1/2 was normalized by levels of ERK1/2 (B, D). *, $p < 0.05$, significantly different (n=3).