Involvement of growth factor receptor bound protein-2 in rat hepatocyte growth

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Abstract

Growth factor receptor bound protein-2 (GRB-2) is a protein linking receptor tyrosine kinase and Sos (*Son of Sevenless* gene; Ras GDP/GTP exchange protein), leading to activation of Ras-mitogen-activated protein kinase (MAPK) cascade. So far, it remains unclarified how GRB-2 plays a role in signal transduction pathways evoked by hepatotrophic factors. This study was attempted to evaluate the involvement of GRB-2 in signaling in rat hepatocyte growth. Using rat cultured hepatocytes stimulated by hepatotrophic factors and regenerating livers after partial hepatectomy (PH), we examined GRB-2-mediated linkage of hepatotrophic factor receptors to signal transducing molecules such as Sos or dynamin-Ⅱ by immuno-precipitation and Western blot analysis. In primary cultured hepatocytes stimulated with HGF or EGF, GRB-2 linked HGF receptor or EGF receptor, respectively, to Sos which will activate mitogen-activated protein kinase (MAPK) cascade. In contrast, in primary cultured hepatocytes stimulated with insulin GRB-2 linked insulin receptor substrate-1 (IRS-1) to dynamin-Ⅱ besides Sos. In the early phase after PH, GRB-2 activated Ras-MAPK cascade by linking HGF receptor, IRS-1, or EGF receptor to Sos. In the late phase after PH, a complex of IRS-1-GRB-2 associated with dynamin-Ⅱ, indicating that GRB-2 may transduce signals from IRS-1 to dynamin-Ⅱ. We conclude that GRB-2 may play a role in transmitting signals from hepatotrophic factors to not only MAPK but also to other signaling pathways in hepatocyte growth.

Key words: dynamin-Ⅱ, EGF receptor, GRB-2, HGF receptor, IRS-1, Liver regeneration, Partial hepatectomy, Primary cultured hepatocyte, Rat hepatocyte growth.

Introduction

While hepatocytes are quiescent in normal livers, they rapidly proliferate following stimulation with mitogens or the partial removal of liver tissue.[1, 2] In this regard, a number of growth factors have been identified, and considerable experimental evidence supports the requirement for HGF, EGF, and insulin in liver regeneration *in vivo* and *in vitro* .[3-8] However, the exact mechanisms of intracellular signal transduction generated by these growth factors have so far remained unidentified.

Growth factor receptor bound protein-2 (GRB-2) has been identified as a 25-kDa adaptor molecule containing one Src homology 2 (SH2) domain and two flanking SH3 domains.[9, 10] Using various kinds of cell lines, it has been revealed that through the SH2 domain, GRB-2 binds to activated growth factor receptors such as the hepatocyte growth factor receptor (MET) [11] and the epidermal growth factor receptor (EGF-R) [12-14] or their substrates, such as insulin receptor substrate-1 (IRS-1).[15-17] Subsequently, GRB-2 activates Ras by forming a complex with mammalian *Son of Sevenless* (Sos), a *ras*-specific guanine nucleotide exchange factor,[18-21] leading to activation of the Ras-mitogen-activated protein kinase (MAPK) cascade. Thus, GRB-2 binding to growth factor receptors or their substrates may be an important step in activating MAPK cascade. So far the role of GRB-2 in this cascade has not been clarified in hepatocyte. Therefore, our study was aimed to explore the involvement of GRB-2 in hepatocyte growth using rat primary cultured hepatocytes and a rat liver regeneration model induced by two-thirds partial hepatectomy (PH).

Methods

Antibodies

Anti-MET rabbit polyclonal antibody and anti-GRB-2 rabbit polyclonal antibody were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-EGF-R sheep polyclonal antibody, anti-IRS-1 rabbit polyclonal antibody, and anti-Sos rabbit polyclonal antibody were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phosphotyrosine mouse monoclonal antibody was purchased from Leinco Technologies, Inc. (Ballwin, MO). Anti-dynamin-Ⅱ mouse monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY).

Hepatocyte Isolation and Culture

Hepatocytes were isolated from the livers of 180-200 gr. male Sprague-Dawley rats (Kiwa Laboratory Animals Co., Wakayama, Japan); the portal vein was perfused with Liver Perfusion Medium (GIBCO/BRL, Life Technologies, Inc., Gaithersburg, MD) for 10 min. at 23 ml/min., followed by perfusion with Liver Digest Medium (GIBCO/BRL, Life Technologies, Inc., MD) for 10 min. Subsequently, the liver was excised, and the hepatocytes were detached. Isolated cells were centrifuged at 50 g, for 2 min. and resuspended in Hepatocyte Wash Medium (GIBCO/BRL, Life Technologies, Inc., MD). The cells were filtered, recentrifuged, and plated onto 75 cm^2 collagen-coated dishes (Iwaki Glass Co., Chiba, Japan) at a cell density of 1×10^7 cells. Cell viability exceeded 90%, as determined by the trypan blue exclusion test. The cells were maintained in Williams' E medium (Dainippon Pharmaceutical Co., Osaka, Japan) containing 26.2 mM $NaHCO_3$, 10% fetal calf serum, 100 nM insulin and 10 nM dexamethasone. After being placed in 5% CO_2/air at 37℃ for a few hours, the cultures were shifted to serum-free medium and incubated overnight.

Treatment of Cultures

Recombinant rat HGF (Toyobo Biochemicals, Inc., Osaka, Japan), recombinant EGF (Sigma Chemical Company, St. Louis, MO) and recombinant insulin (Sigma Chemical Company, St. Louis, MO) were dissolved in culture medium at a final concentration of 10 ng/ml, 10 ng/ml, and 100 nM, respectively. Incubation of hepatocytes with each growth factor started after overnight serum starvation. At the indicated times, the cells were harvested and analyzed.

Partial Hepatectomy

A total of 55 male Sprague-Dawley rats (Kiwa Laboratory Animals Co., Wakayama, Japan), weighing approximately 200 to 220 grams, were housed under standard laboratory conditions and provided with food and water *ad libitum* before the experiment. The rats were divided into two groups; 33 experimental rats were subjected to two-thirds partial hepatectomy (PH) according to the Higgins and Anderson method,[22] and 22 rats were sham-operated at the indicated times. At the indicated times after PH, the remaining rat liver was rapidly excised to prepare tissue homogenates for immunoprecipitation and Western blot analysis. These experiments were performed according to the guidelines of the Committee on Experimental Animals of Osaka University.

Immunoprecipitation and Western Blot Analysis

Liver tissue homogenates and cell lysates were prepared in Triton lysis buffer [50 mM Tris-HCl (pH 7.5), 1%Triton X-100, 2 mM ethylene glycol-bis (β -aminoethyl ether)-N,N,N,N'-tetraacetic acid (EGTA), 10 mM EDTA, 100 mM NaF, 1 mM $Na_4P_2O_7$, 2 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml aprotinin, 3.5 mg/ml pepstatin A, 25 mg/ml leupeptin]. These samples were then subjected to immunoprecipitation followed by Western blot analysis as previously described.[23] Protein concentration was determined using the BCA protein assay system (Pierce, Rockford, IL). Four-hundreds mg of the

homogenates were allowed to react with anti-MET antibody, anti-EGF-R antibody, anti-IRS-1 antibody, or anti-GRB-2 antibody at 4℃ overnight and incubated with protein A Sepharose beads (Pharmacia LKB, Uppsala, Sweden) to precipitate the antigen-antibody complexes. The beads were washed 3 times in Triton lysis buffer. The immunoprecipitates were boiled in 6X concentrated Quench buffer containing 60 mM EDTA, 44 mM dithiothreitol, 30% sucrose, 6.6% SDS, and 60 mg/ml pyronine Y, separated by 7.5% or 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF filters (Millipore Corp., Bedford, MA). The filters were blocked in a Tris-buffered saline (TBS) buffer containing 4% skim milk at 4℃ overnight, and incubated with anti-phosphotyrosine antibody, anti-GRB-2 antibody, or anti-Sos antibody diluted in TBS buffer at 4℃ overnight. Bound antibodies were detected with ^{125}I-labeled protein A (ICN Pharmaceuticals, Inc., Irvine, CA). Autoradiography was carried out at -80℃ for 12 h. The time-dependent change in protein expression or TP relative to the initial level (time zero) was determined with a densitometer (MCID imaging analyzer; Fuji Photo Film Co., Tokyo, Japan). The data are expressed as the mean ± the standard deviation for three separate experiments.

Results

I. Experiments on primary cultured hepatocytes

Tyrosyl phosphorylation of MET protein, its association with GRB-2, and the association of GRB-2 with Sos after HGF stimulation in rat primary cultured hepatocytes

First, we examined MET activation and its association with GRB-2. After stimulation with HGF, tyrosyl phosphorylation of MET protein was enhanced 2.5-fold over the initial level peaking at 5 min. (Fig. 1A and B), and the association of MET protein with GRB-2 increased 1.6-fold over the initial level, peaking at 10 min. (Fig. 1A and C). Next, we determined the association of GRB-2 with Sos. The association of GRB-2 with Sos increased 1.5-fold over the initial level peaking at 5 min. after stimulation (Fig. 1A and D). In this way, the association of MET protein with GRB-2, and that of GRB-2 with Sos, occurred almost in parallel with the degree of TP of the MET protein (Fig. 1A).

Tyrosyl phosphorylation of EGF-R protein, its association with GRB-2, and the association of GRB-2 with Sos after EGF stimulation in rat primary cultured hepatocytes

TP of EGF-R protein was enhanced 8.3-fold over the initial level, peaking at 10 min. after stimulation with EGF. The association of EGF-R protein with GRB-2 was enhanced 2.0-fold over the initial level peaking at 10 min. after stimulation, and the association of GRB-2 with Sos increased 2.4-fold over the initial level, also peaking at 10 min. after stimulation. The association of EGF-R protein with GRB-2, and that of GRB-2 with Sos, peaked in parallel with the state of TP of EGF-R (Fig. 2).

Tyrosyl phosphorylation of IRS-1 protein, its association with GRB-2, and the association of GRB-2 with Sos after insulin stimulation in rat primary cultured hepatocytes

TP of IRS-1 protein was up-regulated 1.8-fold over the initial level, peaking at 10 min. after stimulation, and returning to the initial level thereafter. The association of IRS-1 protein with GRB-2 was enhanced from 5 min. to 90 min., peaking once at 20 min. and again at 60 min. after stimulation. Furthermore, this association was sustained after the remission of TP. The association of GRB-2 with Sos was not enhanced, but a gradual dissociation of GRB-2 from Sos was detected after insulin stimulation (Fig. 3).

Association of GRB-2 with dynamin-Ⅱ, and GRB-2 with IRS-1 protein after insulin stimulation in rat primary cultured hepatocytes

GRB-2 has previously been reported to associate with signaling molecules other than Sos through its SH3 domains. GRB-2 has been shown to bind *in vitro* and *in vivo* to dynamin-Ⅱ via its SH3 domains[26-28]. To determine how GRB-2 may transduce signals from IRS-1 protein to dynamin-Ⅱ after insulin stimulation in rat primary cultured hepatocytes, immuno-precipitates with the anti-GRB-2 antibody were separated by SDS-PAGE and detected by anti-dynamin-Ⅱ antibody and anti-IRS-1 antibody on the same blotting. The association of GRB-2 with dynamin-Ⅱ gradually increased and was enhanced from 30 min. to 90 min. after stimulation, while the association of GRB-2 with IRS-1 protein was enhanced from 10 min. to 90 min. after stimulation (Fig. 4).

II. Experiments on regenerating rat liver after partial hepatectomy

Association of MET, EGF-R, and IRS-1 proteins with GRB-2 after partial hepatectomy

For consistency the association of MET protein with GRB-2 was enhanced up to 2.8-fold over the initial level peaking at 4 h. after PH.

The association of EGF-R protein with GRB-2 was enhanced 2.3-fold over the initial level, peaking at 18 h. after PH. The association of IRS-1 protein with GRB-2 increased between 1.5-3-fold over the initial level from 2 h. to 48 h. after PH (Fig. 5). In contrast, no change in the association of GRB-2 with the MET, EGF-R, or IRS-1 proteins was detected in the sham-operated control groups (data not shown).

Association of GRB-2 with Sos or dynamin-II after partial hepatectomy

GRB-2 displayed dissociation from Sos at 0.5 h. after PH, followed by increased association with Sos that peaked at 6 h. after PH. The association of GRB-2 with dynamin-II also displayed an increase, peaking at 1 h. and 18 h. after PH (Fig. 6). However, no change in the association of GRB-2 with Sos or dynamin-II was detected in the sham-operated control groups (data not shown).

Discussion

This is the first report demonstrating dynamics of GRB-2 function in hepatocyte proliferation, especially *in vivo* regeneration; GRB-2 converges signals generated by many hepatotrophic factors and transmits them to the Ras-MAPK cascade as well as to other intracellular signal pathways. In the first set of experiments, we used rat primary cultured hepatocytes to investigate the role of GRB-2 under stimulation with a single growth factor. In order to examine how GRB-2 is involved in intracellular signal transduction after stimulation with mitogens such as HGF and EGF, or co-mitogens such as insulin, we measured tyrosine phosphorylation levels of MET, EGF-R, and IRS-1 proteins, their association with GRB-2, and the association of GRB-2 with Sos after stimulation with HGF, EGF, and insulin, respectively. After stimulation by HGF or EGF, the corresponding receptor displayed autophosphorylation accompanied by enhancement of the link with GRB-2. The association of GRB-2 with Sos was also enhanced under stimulation with HGF or EGF. Afterwards, the association of GRB-2 with MET protein or EGF-R protein declined in parallel with the level of the association between GRB-2 and Sos (Fig. 1 and 2). These findings indicate that the association of GRB-2 with tyrosine-phosphorylated MET protein or EGF-R protein may contribute to activation of the Ras-MAPK cascade. In contrast, there was no enhanced association of GRB-2 with Sos after insulin stimulation, but rather a gradual dissociation between GRB-2 and Sos was observed (Fig. 3). This is compatible with the findings of previous studies that GRB-2 binds constitutively to Sos even in the absence of extracellular stimuli, and that insulin stimulation results in a decreased affinity of Sos for GRB-2.[24, 25] Interestingly, the association of GRB-2 with IRS-1 protein was enhanced and sustained after insulin stimulation while GRB-2 dissociated from Sos (Fig. 3). These data suggest that the association of GRB-2 with tyrosine-phosphorylated IRS-1 may contribute little to MAPK cascade in rat primary cultured hepatocytes. Besides binding to Sos, GRB-2 has been

reported to interact with the proline-rich region of dynamin-Ⅱ, which regulates membrane trafficking through SH3 domains *in vivo* and *in vitro*.[26-28] In order to examine whether GRB-2 may transduce signals from IRS-1 proteins downstream to dynamin-Ⅱ rather than to Sos after insulin stimulation, immuno-precipitates with the anti-GRB-2 antibody were separated by SDS-PAGE and detected by anti-dynamin-Ⅱ antibody and anti-IRS-1 antibody on the same blotting. When the association of GRB-2 with IRS-1 protein was enhanced, the association of GRB-2 with dynamin-Ⅱ was enhanced as well (Fig. 4), indicating that GRB-2 transduces signals from IRS-1 proteins to dynamin-Ⅱ by forming a complex of IRS-1-GRB-2-dynamin-Ⅱ. This finding suggests that insulin may exert its role in regulating membrane trafficking in terms of forming this trinary complex. Moreover, recent works have shown that microinjection of cells with an anti-GRB-2 antibody abolishes the cyto-architecture reorganization including pericellular accumulation of actin fibers and membrane ruffling dependent on Rho/Rac proteins.[29, 30] All these findings, taken together, lead us to speculate that GRB-2 may have a role in not only the Ras-MAPK cascade but also in other signaling pathways in rat hepatocyte growth.

To confirm whether GRB-2 may have an important role *in vivo* as well, we examined the role of GRB-2 in intracellular signal transduction in liver regeneration after PH. In contrast to cell growth in primary cultured hepatocytes, the mechanisms whereby intracellular signal transduction is activated in liver regeneration appear to be much more complicated; not only HGF, EGF, and insulin, but also other hepatotrophic factors, including TGF-α or IGF-Ⅰ, may contribute to liver regeneration in a time dependent fashion.[4] It has previously been reported that TGF-α shares a receptor with EGF,[31] and

IGF-Ⅰ can phosphorylate IRS-1 proteins.[32,33] These findings indicate that determining the association of MET, EGF-R, and IRS-1 proteins with GRB-2 may decipher the network of intracellular signal transduction pathways activated by hepatotrophic factors in liver regeneration. This study has demonstrated how in the early phase after PH, prior to the major wave of DNA synthesis, the association of GRB-2 with MET, IRS-1, or EGF-R proteins was enhanced in a sequential manner (Fig. 5). It is important to stress that the association of GRB-2 and Sos was increased mainly in the early phase after PH (Fig. 6A). These observations suggest that GRB-2 may contribute to activation of the Ras-MAPK cascade by binding to MET, IRS-1, or EGF-R proteins in the early phase of liver regeneration. In addition, the association of IRS-1 protein with GRB-2 was sustained until the late phase after PH, indicating that GRB-2 may mediate signals generated by IRS-1 proteins to other signal transduction cascades; between 18 h. and 72 h. after PH, GRB-2 showed enhanced association with IRS-1 and dynamin-Ⅱ (Fig. 5 and 6B). During this period, GRB-2 may transduce signals from IRS-1 to dynamin-Ⅱ. On the other hand, association of GRB-2 with dynamin-Ⅱ was enhanced with two peaks during liver regeneration (Fig. 6B). The first peak may reflect the contribution of GRB-2 to the receptor-mediated endocytosis of signal-transducing receptors.[34] Interestingly, enhanced association of GRB-2 with dynamin-Ⅱ was followed by that of GRB-2 with Sos in the early phase of liver regeneration (Fig. 6A and B). Recently it has been reported that dynamin-Ⅱ binds to the C-terminal and N-terminal SH3 domain of GRB-2,[35] and Sos binds to GRB-2 mainly through the N-terminal SH3 domain of GRB-2.[36] Taken together, these findings suggest that Sos may compete with dynamin-Ⅱ for binding to GRB-2, which results in signals being delivered downstream to Sos or dynamin-Ⅱ in a time

specific manner in the early phase of liver regeneration, while the role of the enhanced association of GRB-2 with dynamin-Ⅱ in the late phase of liver regeneration remains unknown.

Our findings indicate that during rat liver regeneration, GRB-2 converges signals generated by many hepatotrophic factors and transmits them to the Ras-MAPK cascade as well as to other intracellular signal pathways including dynamin-Ⅱ. In this way, GRB-2 may play an important role in expressing specific biological effects of hepatotrophic growth factors. Our studies may shed new light on the possibility of regulating cell growth by controlling the function of the GRB-2 protein.

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Figure legends

Figure 1. Changes in tyrosyl phosphorylation of MET protein, association of MET protein with GRB-2, and association of GRB-2 with Sos after stimulation with HGF. A. Changes in tyrosyl phosphorylation (TP) of MET protein (□), its association with GRB-2 (○), and the association of GRB-2 with Sos (△) after stimulation with HGF (10ng/ml). Differences in the abundance of proteins in the corresponding autoradiographs were quantified with a densitometer (MCID imaging analyzer; Fuji Photo Film Co., Tokyo, Japan). Scanning values are expressed as n-fold induction compared to the initial level (time zero). Data shown in A are expressed as the mean ± the standard deviation for three separate experiments. There was a 2.5-fold increase in TP of MET protein peaking at 5 min. after stimulation with HGF. The association of MET protein with GRB-2 increased 1.6-fold over the initial level peaking at 10 min. after stimulation. The association of GRB-2 with Sos increased 1.5-fold over the initial level peaking at 5 min. after stimulation. The association of MET protein with GRB-2, and of GRB-2 with Sos, occurred almost in parallel with the state of TP of MET. **B.** Tyrosyl phosphorylation (TP) of the MET protein after stimulation with HGF. **C.** Association of MET protein with GRB-2 after stimulation with HGF. **D.** Association of GRB-2 with Sos after stimulation with HGF. The autoradiographs shown in B, C, and D are representative of three separate experiments. Numbers below the panel indicate the times after stimulation with HGF.

Figure 2. Changes in tyrosyl phosphorylation of EGF-R protein, association of EGF-R protein with GRB-2, and association of GRB-2 with Sos after stimulation with EGF. Changes in TP of EGF-R protein (□), its association with GRB-2 (○), and the association of GRB-2 with Sos (△) after

stimulation with EGF(10ng/ml). There was a 8.3-fold increase in TP of the EGF-R protein peaking at 10 min. after stimulation with EGF. The association of EGF-R protein with GRB-2 increased 2.0-fold over the initial level, peaking at 10 min. after stimulation. The association of GRB-2 with Sos increased 2.4-fold over the initial level peaking at 10 min. The association of EGF-R protein with GRB-2 and the association of GRB-2 with Sos occurred almost in parallel with the state of TP of EGF-R protein. The data are expressed as the mean ± the standard deviation of three separate experiments.

Figure 3. Changes in tyrosyl phosphorylation of IRS-1 protein, association of IRS-1 protein with GRB-2, and association of GRB-2 with Sos after stimulation with insulin. Changes in TP of IRS-1 protein (□), its association with GRB-2 (○), and the association of GRB-2 with Sos (△) after stimulation with insulin (100nM). There was a 1.8-fold increase in TP of the IRS-1 protein peaking at 10 min. after stimulation with insulin. The association of IRS-1 protein with GRB-2 was increased from 5 min. to 90 min., peaking at 60 min. after stimulation. GRB-2 displayed a gradual disassociation from Sos after the stimulation with insulin. The association of IRS-1 protein with GRB-2 began almost in parallel with the level of TP of the IRS-1 protein and this association continued displaying a higher level without decreasing. There was a gradual decrease in the linking between GRB-2 and Sos. The data are expressed as the mean ± the standard deviation of three separate experiments.

Figure 4. Changes in the association of GRB-2 with dynamin-Ⅱ, and GRB-2 with IRS-1 protein, after stimulation with insulin. Changes in the association of GRB-2 with dynamin-Ⅱ (◇), and GRB-2 with IRS-1 (○), after stimulation with insulin. The association of GRB-2 with dynamin-Ⅱ gradually increased

and was enhanced from 30 min. to 90 min. after stimulation. The association of GRB-2 with IRS-1 occurred almost in parallel with the state of the association of GRB-2 with dynamin-Ⅱ. The data shown are expressed as the mean ± the standard deviation of three separate experiments.

Figure 5. Changes in the association of MET, EGF-R, and IRS-1 proteins with GRB-2 during liver regeneration. Changes in the association of the MET protein (○), EGF-R protein (△), and IRS-1 protein (□) with GRB-2 after PH. The association of the MET protein with GRB-2 increased 2.8-fold over the initial level, peaking at 4 h. after PH. The association of the EGF-R protein with GRB-2 increased 2.3-fold over the initial level peaking at 18 h. after PH. The association of the IRS-1 protein with GRB-2 increased about 1.5-3-fold over the initial level from 2 h. to 48 h. The data are expressed as the mean ± the standard deviation of three separate experiments.

Figure 6. Changes in the association of GRB-2 with Sos or dynamin-Ⅱ during liver regeneration. A. Changes in the association of GRB-2 with Sos after PH. In the early phase after PH, while there was an enhancement of the association of the MET, EGF-R, and IRS-1 proteins with GRB-2, GRB-2 also displayed an increased association with Sos. In the late phase while the association of IRS-1 protein with GRB-2 continued, GRB-2 dissociated from Sos. **B.** Changes in the association of GRB-2 with dynamin-Ⅱ after PH. The association of GRB-2 with dynamin-Ⅱ increased, peaking at 1 h., and returned to the initial level at 12 h. Afterwards, this association increased again and peaked at 18 h. after PH. The data are expressed as the mean ± the standard deviation of three separate experiments.

Fig. 1

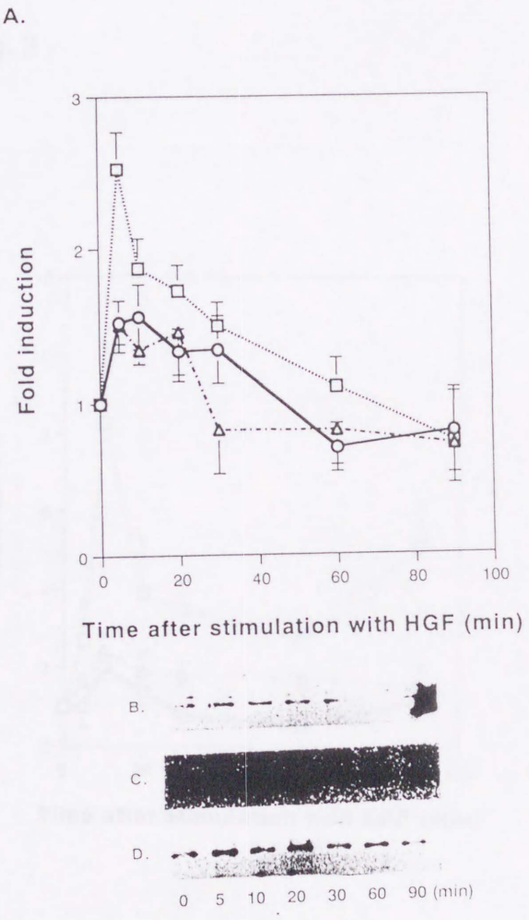

A.
Fold induction
3
2
1
0
0
20
40
60
80
100
Time after stimulation with HGF (min)
B.
C.
D.
0 5 10 20 30 60 90 (min)

Fig. 2

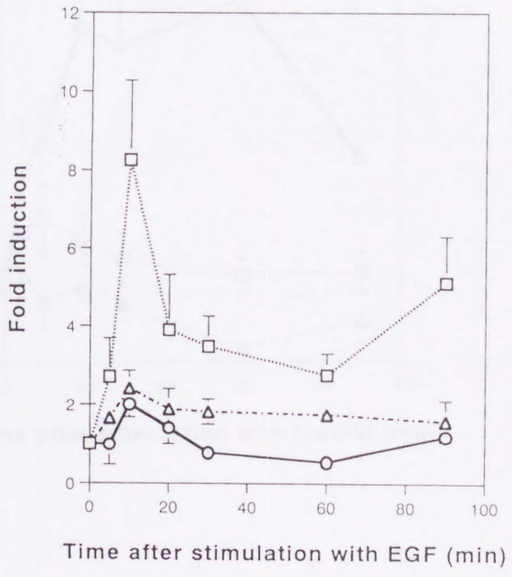

Fold induction
12
10
8
6
4
2
0
0
20
40
60
80
100
Time after stimulation with EGF (min)

Fig. 3

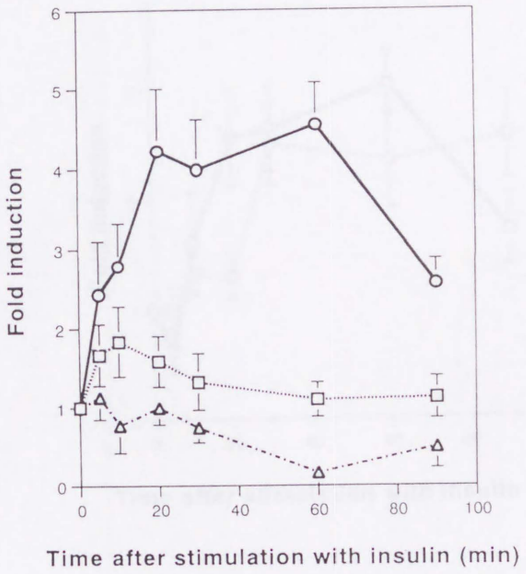

Fold induction
0
1
2
3
4
5
6
0
20
40
60
80
100
Time after stimulation with insulin (min)

Fig. 4

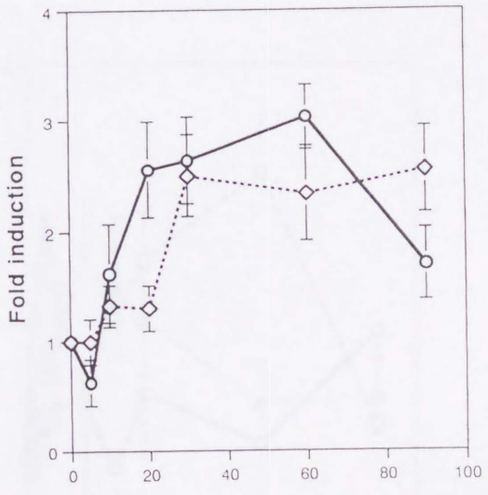

Fold induction
0
1
2
3
4
0
20
40
60
80
100
Time after stimulation with insulin (min)

Fig. 5

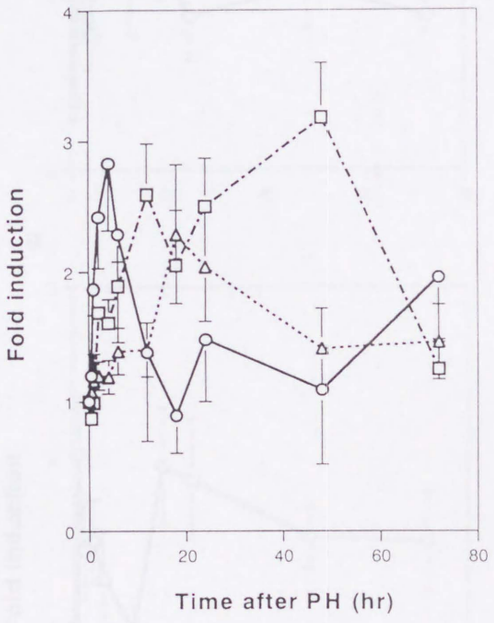
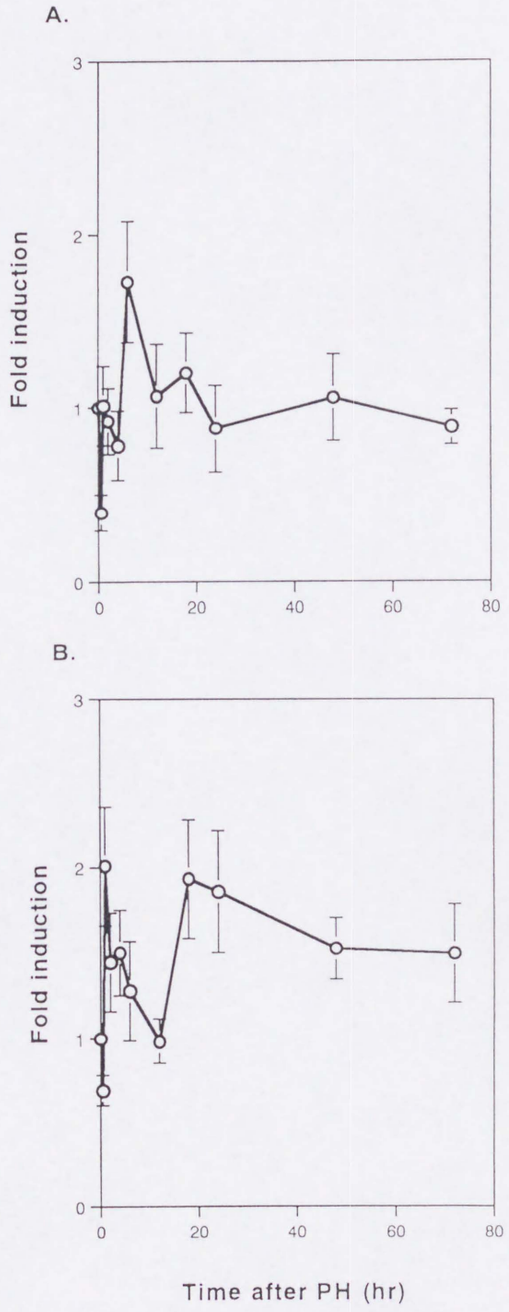


Fig. 6


A.
Fold induction
3
2
1
0
0
20
40
60
80
B.
Fold induction
3
2
1
0
0
20
40
60
80
Time after PH (hr)