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A complex of GRB2–dynamin binds to tyrosine-phosphorylated insulin receptor substrate-1 after insulin treatment

Akifumi Ando, Kazuyoshi Yonezawa[1], Ivan Gout[2], Takao Nakata[3], Hiroo Ueda, Kenta Hara, Yukari Kitamura, Yasuko Noda[3], Tadaomi Takenawa[4], Nobutaka Hirokawa[3], Michael D.Waterfield[2,5] and Masato Kasuga[6]

The Second Department of Internal Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650, [2]Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, [5]Department of Biochemistry and Molecular Biology, University College, Gower Street, London WC1E 6BT, UK, [3]Department of Anatomy and Cell Biology, Faculty of Medicine, University of Tokyo, Hongo, Tokyo 113 and [4]Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan

[1]Present address: Cardiovascular Research Center, Massachusetts General Hospital - East, 149 13th Street, 4th Floor, Charlestown, MA 02129, USA

[6]Corresponding author

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Insulin drives the formation of a complex between tyrosine-phosphorylated IRS-1 and SH2-containing proteins. The SH2-containing protein Grb2 also possesses adjacent SH3 domains, which bind the Ras guanine nucleotide exchange factor Sos. In this report, we examined the involvement of another SH3 binding protein, dynamin, in insulin signal transduction. SH3 domains of Grb2 as GST fusion proteins bound dynamin from lysates of CHO cells expressing wild-type insulin receptor (IR) (CHO-IR cells) in a cell-free system (*in vitro*). Immunoprecipitation studies using specific antibodies against Grb2 revealed that Grb2 was co-immunoprecipitated with dynamin from unstimulated CHO-IR cells. After insulin treatment of CHO-IR cells, anti-dynamin antibodies co-immunoprecipitated the IR β-subunit and IRS-1, as tyrosine-phosphorylated proteins and PI 3-kinase activity. However, purified rat brain dynamin did not bind directly to either the IR, IRS-1 or the p85 subunit of PI 3-kinase *in vitro*. Together, these results suggest that in CHO-IR cells, insulin stimulates the binding of dynamin to tyrosine-phosphorylated IRS-1 via Grb2 and that IRS-1 also associates with PI 3-kinase in response to insulin. This complex formation was reconstituted *in vitro* using recombinant baculovirus-expressed IRS-1, GST–Grb2 fusion proteins and dynamin peptides containing proline-rich sequences. Furthermore, dynamin GTPase activity was found to be stimulated when an IRS-1-derived phosphopeptide, containing the Grb2 binding site, was added to the dynamin–Grb2 complex *in vitro*. These findings provide evidence that dynamin is complexed with Grb2 in CHO-IR cells and, after insulin stimulation, the IRS-1 molecule is able to bind this Grb2–dynamin complex and may regulate dynamin GTPase activity in the complex in intact cells (*in vivo*).

Key words: dynamin/Grb2/GTPase/insulin/IRS-1

Introduction

The insulin receptor (IR) belongs to the family of structurally related transmembrane growth factor receptors with ligand-activated protein tyrosine kinase activity (Kasuga *et al.*, 1983; Roth and Cassell, 1983). Several lines of evidence suggest that this kinase activity is essential in eliciting the complex cellular response to insulin (Becker and Roth, 1990; Kasuga *et al.*, 1990). Insulin treatment of cells has been found to increase phosphoinositide (PI) 3-kinase activity in immunoprecipitates carried out using an anti-phosphotyrosine antibody (Endemann *et al.*, 1990; Ruderman *et al.*, 1990). Insulin treatment of various intact cells causes the rapid tyrosine phosphorylation of a high molecular weight protein (M_r = 160 000–185 000) (White *et al.*, 1985; Kadowaki *et al.*, 1987), the IR substrate-1 (IRS-1). The amino acid sequence, deduced by cDNA cloning, revealed that IRS-1 contains at least 20 potential tyrosine phosphorylation sites, including a YMXM motif, and these potential phosphorylation sites may associate with cellular proteins that contain SH2 domains (Sun *et al.*, 1991). Recent studies have demonstrated that insulin drives the formation of a complex between tyrosine-phosphorylated IRS-1 and SH2-containing proteins, such as the 85 kDa subunit (p85) of PI 3-kinase (Backer *et al.*, 1992; Lavan *et al.*, 1992; Yonezawa *et al.*, 1992b), Grb2 (also known as ASH) (Skolnik *et al.*, 1993a; Tobe *et al.*, 1993), Syp (Kuhne *et al.*, 1993) and Nck (Lee *et al.*, 1993) via specific binding sites on IRS-1 (Sun *et al.*, 1993).

p85, Grb2 and Nck also contain adjacent SH3 domains; the latter two molecules consist almost entirely of SH2 and SH3 domains (Lehmann *et al.*, 1990; Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992). Recent reports have demonstrated that SH3 domains are modular domains which appear to be responsible for the assembly of protein–protein complexes (Birge and Hanafusa, 1993). For instance, Grb2 was shown to complex the Ras guanine nucleotide exchange factor Sos and to act as an adaptor protein linking Sos to tyrosine-phosphorylated IRS-1 (Skolnik *et al.*, 1993b).

We examined whether SH3-containing adaptor proteins interact with other signalling proteins thereby providing additional links between the IR and other signalling pathways. One such candidate is dynamin (Obar *et al.*, 1990; Nakata *et al.*, 1991), which possesses intrinsic GTPase activity (Maeda *et al.*, 1992) and is able to bind

a subset of SH3 domains through a proline-rich sequence in its C-terminal domain (Gout *et al.*, 1993; Herskovits *et al.*, 1993b). In addition, its GTPase activity was found to be stimulated by the binding of SH3 domains (Gout *et al.*, 1993; Herskovits *et al.*, 1993b).

In this report, we show that dynamin makes a complex with Grb2 in CHO cells overexpressing the human IR (CHO-IR cells) and that insulin stimulation results in the formation of a complex between IRS-1 and the Grb2–dynamin complex. Furthermore, dynamin GTPase activity was found to be stimulated when an IRS-1-derived phosphopeptide, containing the Grb2 binding site, was added to the dynamin–Grb2 complex in a cell-free system (*in vitro*).

Results and discussion

To examine whether dynamin was present in CHO-IR cells, a detergent lysate of CHO-IR cells was immunoprecipitated with rabbit antiserum against rat brain dynamin and subsequently immunoblotted with the same antibody. As shown in Figure 1A, compared with a control in which normal rabbit serum was used, a 110 kDa molecule was specifically recognized by the anti-dynamin antibody. The size of dynamin in CHO-IR cells appears to be slightly different from that of rat brain, which is a 100 kDa protein. Then, to examine whether dynamin in CHO-IR cells is capable of binding to SH3 domains, immobilized GST fusion proteins of full-length Grb2 (Full Grb2 in Figure 1B), the individual SH3 domains (SH3N and SH3C Grb2 in Figure 1B) of Grb2 or the SH3 domain of p85α (SH3 p85 in Figure 1B), were incubated with detergent lysates of CHO-IR cells and then immunoblotted with the anti-dynamin antibody. As shown in Figure 1B, all of these SH3-containing proteins were found to bind dynamin in CHO-IR cells. In contrast, neither the GST fusion protein expressing the first SH2 domain of p85 (SH2N p85) nor GST alone bound dynamin. These results indicated that CHO-IR cells contain dynamin which is able to bind to the SH3 domains of Grb2 and p85α in a cell-free system (*in vitro*).

To determine whether dynamin associates with SH3-containing proteins in intact cells (*in vivo*), CHO-IR cells were labelled with ^{35}S, lysed and immunoprecipitation was performed with polyclonal antibodies against Grb2 (Figure 2A and B). As shown in Figure 2A and B, the analysis of the immunoprecipitates from ^{35}S-labelled cells revealed that compared with controls in which normal rabbit serum was used, the major proteins specifically immunoprecipitated with polyclonal antibodies to Grb2 are a 110 kDa protein, a doublet of 95 kDa, a 40 kDa protein and a 25 kDa protein which is Grb2. Western blot analysis of the anti-Grb2 immunoprecipitates from untreated or insulin-treated CHO-IR cells with rabbit antiserum against rat brain dynamin showed that the 110 kDa protein is dynamin (Figure 2C). The association of dynamin with p85 of PI 3-kinase was also examined by Western blot analysis of immunoprecipitates obtained from

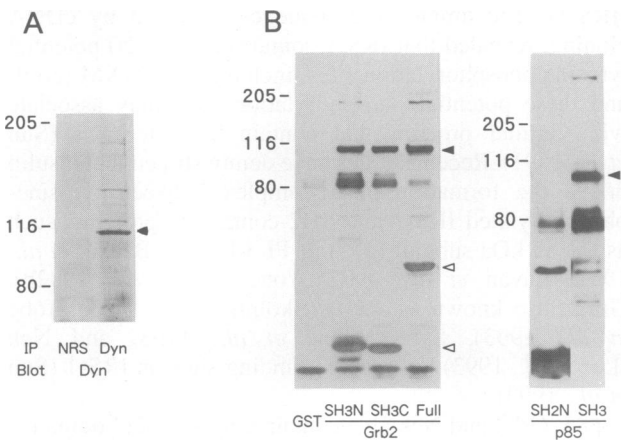

Fig. 1. Detection of dynamin in CHO-IR cells. (**A**) Immunoprecipitation of dynamin from CHO-IR cells. CHO-IR cell lysates were immunoprecipitated (IP) with normal rabbit serum (NRS) or rabbit antiserum against rat brain dynamin (Dyn) bound to protein A–Sepharose. The immunoprecipitates were electrophoresed on 8.5% SDS–polyacrylamide gels and immunoblotted with the anti-dynamin antibody. The dynamin is indicated by a closed arrow. The positions of pre-stained molecular mass markers (in kDa) are also indicated. (**B**) Binding of dynamin from CHO-IR cells to SH3 domains *in vitro*. CHO-IR cell lysates were incubated for 4 h at 4°C with GST fusion proteins immobilized on glutathione–Sepharose beads. This was followed by immunoblotting with rabbit antiserum against rat brain dynamin. From left to right, CHO-IR cell lysates mixed with GST alone, Grb2 N-terminal SH3 domain (SH3N), C-terminal SH3 domain (SH3C), full-length (Full), p85α N-terminal SH2 domain (SH2N) and SH3 domain (SH3). The dynamin and GST fusion proteins are indicated by a closed arrow or open arrows, respectively. The positions of pre-stained molecular mass markers (in kDa) are also indicated.

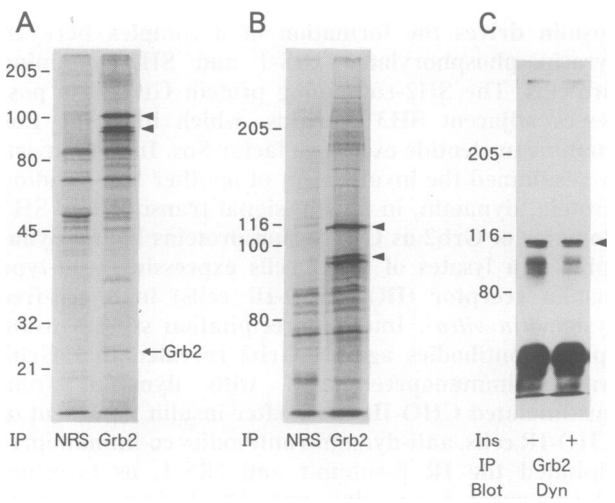

Fig. 2. Association of dynamin with SH3-containing molecules in intact CHO-IR cells. (**A** and **B**) CHO-IR cells were labelled with Tran^{35}S label as shown in Materials and methods. Cells were frozen with liquid nitrogen and then lysed. The lysates were immunoprecipitated (IP) with control normal rabbit serum (NRS) or with polyclonal anti-Grb2 antibodies (Grb2) bound to protein A–Sepharose. The immunoprecipitates were washed, electrophoresed on 14% (A) or 8.5% (B) SDS–polyacrylamide gels and autoradiographed. The 110 kDa protein, the doublet at 95 kDa and the 25 kDa Grb2 are indicated by arrows. The positions of pre-stained molecular mass markers (in kDa) are also indicated. (**C**) CHO-IR cells without treatment or treated with 10^{-7} M insulin for 1 min at 37°C were frozen with liquid nitrogen and then lysed. The lysates were immunoprecipitated with polyclonal anti-Grb2 antibodies (Grb2) bound to protein A–Sepharose. The immunoprecipitates were electrophoresed on 8.5% SDS–polyacrylamide gels and immunoblotted with rabbit antiserum against dynamin (Dyn). The dynamin is indicated by the arrow. The positions of pre-stained molecular mass markers (in kDa) are also indicated.

CHO-IR cells by monoclonal antibodies to p85 (Yonezawa *et al.*, 1992b) with the anti-dynamin antibody. However, we failed to detect any association of dynamin with p85 in CHO-IR cells or even in CHO-IR cells overexpressing wild-type bovine p85α before or after insulin treatment. Moreover, the anti-dynamin antibody did not co-immunoprecipitate PI 3-kinase activity from CHO-IR cells before insulin treatment as shown in Figure 5, even though the PI 3-kinase assay is much more sensitive than Western blotting analysis. We were also unable to show the *in vitro* binding of full-length p85α, expressed in insect cells using baculovirus vectors, with dynamin from CHO-IR cells (data not shown), indicating that the affinity for dynamin of the p85 SH3 domain in full-length p85 is much weaker than that of the isolated p85 SH3.

Since mSos has been reported to bind Grb2 (Skolnik *et al.*, 1993b), the amounts of Grb2–Sos and Grb2–dynamin in CHO-IR cells were compared. A band of 150 kDa was not seen in the immunoprecipitates from ^{35}S-labelled CHO-IR cells obtained with anti-Grb2 antibodies (Figure 2A and B). In addition, mSos was hardly detectable in anti-Grb2 immunoprecipitates from CHO-IR cell lysates blotted with polyclonal antibodies to mSos, although these polyclonal antibodies were able to immunoblot a 150 kDa mSos molecule in detergent lysates of CHO-IR cells (data not shown). These results suggest that dynamin forms a complex with Grb2 but not with p85 and that the amount of the Grb2–dynamin complex is much greater than that of the Grb2–mSos complex in CHO-IR cells.

To examine the effect of insulin on the association of the Grb2–dynamin complex with tyrosine-phosphorylated proteins *in vivo*, lysates from CHO-IR cells untreated or treated with insulin were immunoprecipitated with the rabbit antiserum against dynamin. These immunoprecipitates were then immunoblotted with a monoclonal anti-phosphotyrosine antibody. As illustrated in Figure 3, compared with a control in which normal rabbit serum was used, the anti-dynamin antibodies precipitated 95 and 180 kDa phosphoproteins from insulin-treated cells but not from untreated cells. The 95 and 180 kDa proteins appear to be the β-subunit of the IR and the IRS-1, respectively, since they migrate to the same positions as the β-subunit of the IR and the IRS-1 immunoprecipitated from insulin-treated CHO-IR cells with specific monoclonal antibodies (2F3 or 1D6, respectively; Yonezawa *et al.*, 1994). In a cell-free system, purified rat brain dynamin did not directly bind to either purified IR or recombinant IRS-1 tyrosine-phosphorylated by purified IR (data not shown). In addition, the GST–full-length Grb2 fusion protein was capable of binding tyrosine-phosphorylated IRS-1 but not IR *in vitro* (data not shown). These data suggest that tyrosine-phosphorylated IRS-1 binds to the Grb2–dynamin complex via Grb2. It is unlikely that the Grb2–dynamin complex and the IR associate directly. It is most likely that the 95 kDa protein which co-precipitates with dynamin represents an indirect association of the Grb2–dynamin complex, via IRS-1, with the IR β-subunit. This is consistent with the previous observation that Grb2 indirectly associates with the IR via tyrosine-phosphorylated IRS-1 (Skolnik *et al.*, 1993a).

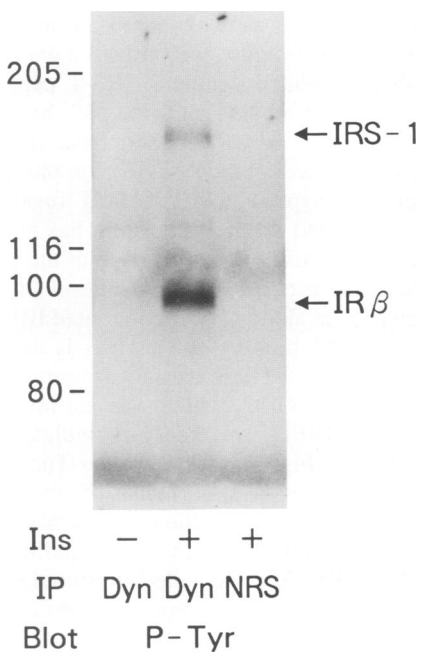

Fig. 3. Effect of insulin on the association of the Grb2–dynamin complex with tyrosine-phosphorylated IRS-1 *in vivo*. CHO-IR cells without treatment or treated with 10^{-7} M insulin for 2 min at 37°C were frozen with liquid nitrogen and then lysed. The lysates were immunoprecipitated (IP) with normal rabbit serum or rabbit antiserum against dynamin (Dyn) bound to protein A–Sepharose. The immunoprecipitates were immunoblotted with a monoclonal anti-phosphotyrosine antibody (P-Tyr). The IRS-1 and the β-subunit of the IR (IRβ) are indicated by arrows. The positions of pre-stained molecular mass markers (in kDa) are also indicated.

There was no detectable change in tyrosine phosphorylation of dynamin after insulin treatment, as shown in Figure 3. No change in the phosphorylation of dynamin was detected in immunoprecipitates with anti-dynamin antibodies of ^{32}P-labelled CHO-IR cells before and after insulin treatment (data not shown).

To reconstitute the association of the Grb2–dynamin complex with tyrosine-phosphorylated IRS-1 *in vitro*, a synthesized P2 peptide, which contains the proline-rich SH3 domain binding motif from dynamin, coupled to an Actigel matrix (Gout *et al.*, 1993), was incubated with phosphorylated or unphosphorylated recombinant IRS-1 in the absence or presence of Grb2 and then immunoblotted with anti-phosphotyrosine antibody. As shown in Figure 4, tyrosine-phosphorylated IRS-1 bound to the dynamin peptide only in the presence of Grb2, suggesting that formation of this complex occurred via the interaction between tyrosine phosphorylation sites of IRS-1 and the SH2 domain of Grb2 and the interaction between the SH3 domains of Grb2 and proline-rich sites of dynamin. The broad band around 140–160 kDa may be a degradation product of IRS-1, since the same band has been observed when IRS-1, which bound to p85 subunit of PI 3-kinase from lysates of insulin-treated CHO-IR cells, was immunoblotted with anti-IRS-1 antibodies (Yonezawa *et al.*, 1992b).

PI 3-kinase is known to associate with IRS-1 in insulin-stimulated cells (Backer *et al.*, 1992; Lavan *et al.*, 1992; Yonezawa *et al.*, 1992b). To determine whether the Grb2–dynamin complex and PI 3-kinase could interact with IRS-1, we examined whether PI 3-kinase activity could be co-immunoprecipitated using antibodies to dynamin. As shown in Figure 5, compared with a control in which

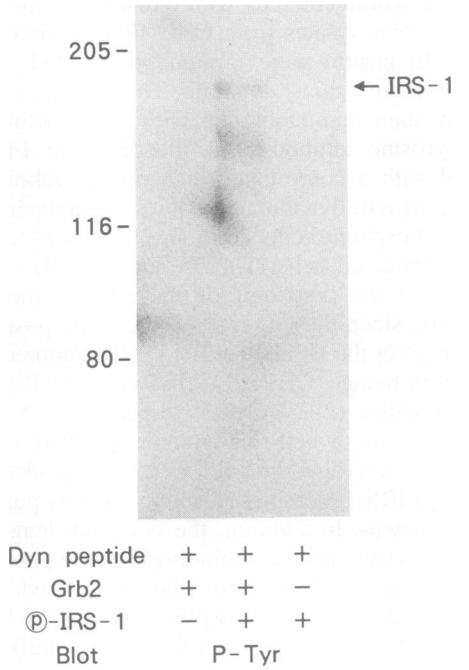

Fig. 4. Association of the Grb2–dynamin complex with tyrosine-phosphorylated IRS-1 *in vitro*. Synthesized P2 peptides (SPTPQRRAPAVPPARPGS), derived from dynamin (Dyn peptide) coupled to Actigel resin were incubated in the absence or presence of 2 μg of GST–full-length Grb2 fusion proteins. After extensive washing, recombinant baculovirus-expressed rat IRS-1 (p-IRS-1), which had been tyrosine-phosphorylated by human IRs immunoprecipitated with a monoclonal anti-IR antibody, was added to the beads. Immunoblotting was then performed with a monoclonal antibody against phosphotyrosine residues (P-Tyr). IRS-1 is indicated by an arrow. The positions of pre-stained molecular mass markers (in kDa) are also indicated.

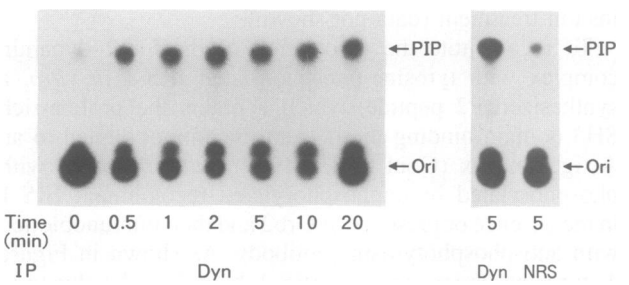

Fig. 5. Effect of insulin on the association of dynamin with PI 3-kinase activity *in vivo*. CHO-IR cells without treatment or treated with 10^{-7} M insulin for indicated times at 37°C were frozen with liquid nitrogen and then lysed. The lysates were immunoprecipitated (IP) with normal rabbit serum (NRS) or rabbit antiserum against dynamin (Dyn) bound to protein A–Sepharose. The immunoprecipitates were subjected to PI 3-kinase assay. Ori and PIP markers indicate the positions of the TLC origin and migration of a PI-4-P standard, respectively.

normal rabbit serum was used, insulin stimulation resulted in an increase in the amount of PI 3-kinase activity that was co-immunoprecipitated with anti-dynamin antibodies. As described above, it is unlikely that p85 associates directly with dynamin. Therefore, this result indicates that after insulin stimulation, the same IRS-1 molecule is able to simultaneously bind the Grb2–dynamin complex and PI 3-kinase *in vivo*. This is consistent with the previous reports that the binding site of Grb2 on IRS-1 is different

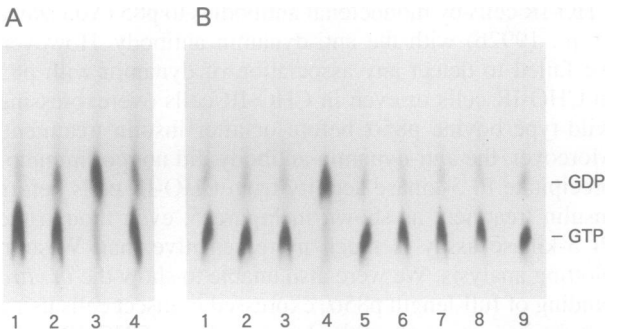

Fig. 6. Effects of synthetic IRS-1 phosphopeptides on dynamin GTPase activity in the Grb2–dynamin complex. GST–Grb2 (1 μg) was pre-incubated with 500 μM of the phosphorylated or non-phosphorylated synthetic IRS-1 peptides for 2 h at 4°C and then added to the reaction mixture which contained 0.5 μg of dynamin in the presence of [α-^{32}P]GTP for 1 h at 37°C (final concentration of peptides: 100 μM). The extent of GTP hydrolysis was assessed by thin layer chromatography and subsequent analysis with a BAS2000 Image Analyzer. (**A**) Effect of a phosphopeptide (IRP-7) corresponding to Grb2 binding site of IRS-1 on dynamin GTPase activity. Lane 1, GST alone; lane 2, GST–Grb2 alone; lane 3, GST–Grb2 plus phosphorylated IRP-7; lane 4, phosphorylated IRP-7 alone. (**B**) Effects of phosphorylated and non-phosphorylated IRS-1 peptides on dynamin GTPase activity in the Grb2–dynamin complex. Lane 1, GST–Grb2 alone; lanes 2–9, GST–Grb2 plus peptides (lane 2, phosphorylated IRP-6; lane 3, non-phosphorylated IRP-6; lane 4, phosphorylated IRP-7; lane 5, non-phosphorylated IRP-7; lane 6, phosphorylated IRP-1; lane 7, non-phosphorylated IRP-1; lane 8, phosphorylated IRP-5; lane 9, non-phosphorylated IRP-5).

from that of p85 of PI 3-kinase on IRS-1 (Skolnik *et al.*, 1993a).

Finally, to assess the effect of association of tyrosine-phosphorylated IRS-1 with dynamin GTPase activity in the Grb2–dynamin complex, we employed phosphorylated and non-phosphorylated synthetic IRS-1 peptides. IRP-1, -6 and -7 contain binding sites for p85, Syp and Grb2, respectively (Sun *et al.*, 1993) and have the ability to inhibit the *in vitro* association of tyrosine-phosphorylated IRS-1 with p85, Syp and Grb2 as GST fusion proteins, respectively. Purified rat brain dynamin has no detectable intrinsic GTPase activity when assayed in the presence of GST alone (Figure 6A, lane 1). Addition of the GST–Grb2 fusion protein alone or phosphorylated IRP-7, corresponding to a Grb2 binding site of IRS-1, alone slightly stimulated dynamin GTPase activity (Figure 6A, lanes 2 and 4). Addition of Grb2, which was pre-incubated with phosphorylated IRP-7, markedly stimulated dynamin GTPase activity (Figure 6A, lane 3). The amount of [^{32}P]GDP formed as a percentage of the amount of [α-^{32}P]GTP added initially to the reaction mixture in lanes 2, 3 and 4 were 20, 100 and 17%. Addition of non-phosphorylated IRP-7 alone failed to stimulate dynamin GTPase activity (data not shown). Purified rat brain dynamin may contain a small amount of Grb2 and this may explain the activation of dynamin GTPase activity caused by addition of phosphorylated IRP-7 alone. Next, the effects of peptides other than IRP-7 on dynamin GTPase activity were examined. While addition of Grb2 pre-incubated with IRP-7 stimulated dynamin GTPase activity (Figure 6B, lane 4), addition of Grb2 pre-incubated with the other phosphorylated and non-phosphorylated peptides did not significantly affect dynamin GTPase activity (Figure 6B, lanes 2, 3 and 5–9) compared with

dynamin GTPase activity in the presence of Grb2 alone (Figure 6B, lane 1).

In summary, we show here that dynamin is complexed with Grb2 in CHO-IR cells and insulin stimulation results in the formation of a complex between IRS-1 and Grb2–dynamin. Dynamin possesses a GTPase activity which has been reported to be regulated by phosphorylation or protein–protein interaction. Phosphorylation of dynamin by protein kinase C was reported to enhance its GTPase activity (Robinson *et al.*, 1993). However, we found that insulin did not induce significant phosphorylation of the dynamin in CHO-IR cells. Binding of microtubules and a subset of SH3 domains including Grb2–SH3 to dynamin have also been shown to stimulate GTPase activity, although SH3 domains were less effective than microtubules (Maeda *et al.*, 1992; Gout *et al.*, 1993; Herskovits *et al.*, 1993b). In this study, we have shown that the binding of phosphopeptides similar to the Grb2 binding site of IRS-1 to Grb2 enhances the dynamin GTPase activity of the Grb2–dynamin complex *in vitro*. Consistent with the observation that synthetic IRS-1 phosphopeptides containing the YMXM motif or tyrosine-phosphorylated IRS-1 stimulate PI 3-kinase activity (Backer *et al.*, 1992), our observation suggests that IRS-1 can regulate the function of its associated molecules *in vivo*.

There are five proline-rich peptide sequences that may be SH3 domain binding sites within dynamin (Gout *et al.*, 1993). These sequences occur in two clusters within the C-terminal 100 amino acids of dynamin and are conserved in both the human and rat proteins (Gout *et al.*, 1993). In the absence of the SH2 binding phosphopeptide, one of the two SH3 domains of Grb2 may bind to one of the five proline-rich peptide sequences of dynamin. Binding of the phosphopeptide to the SH2 domain of Grb2 may induce the binding of the other SH3 domain of Grb2 to another distinct proline-rich site of dynamin and enhance dynamin GTPase activity. Thus, it will be important to locate the SH3 domain binding sites in dynamin more precisely and to examine their involvement in the stimulation of dynamin GTPase activity.

Some clues about the cellular function of dynamin have come from studies on the *Drosophila* temperature-sensitive dynamin mutant *shibire*. This protein is thought to be required for endocytosis, in particular for pinching-off of coated vesicles from the plasma membrane (Kosaka and Ikeda, 1983; Chen *et al.*, 1991; van der Bliek and Meyerowitz, 1991). Transfection of mammalian cells with mutant forms of rat dynamin has recently been found to block receptor-mediated endocytosis (Herskovits *et al.*, 1993a; van der Bliek *et al.*, 1993), suggesting that the rat and *Drosophila* proteins have similar function *in vivo*. The next important step is to identify the involvement of the Grb2–dynamin complex in an insulin-induced endocytotic event, such as the internalization of the IR.

Materials and methods

Cell cultures and antibodies

CHO-IR cells were routinely maintained in Ham's F-12 medium supplemented with 10% fetal calf serum. The antibodies used were: rabbit antiserum against purified rat brain dynamin (Maeda *et al.*, 1992); polyclonal anti-Grb2 antibodies against GST fusion protein containing residues 15–217 of Grb2 (Matuoka *et al.*, 1992); monoclonal antibodies against p85α (Yonezawa *et al.*, 1992b); a monoclonal antibody against phosphotyrosine residues (py20; ICN, Costa Mesa, CA); polyclonal anti-mSos antibodies (UBI, Lake Placid, NY); a monoclonal antibody against rat IRS-1 (1D6) (Yonezawa *et al.*, 1994); a monoclonal antibody against the β-subunit of the human IR (2F3; a gift from Dr Kozui Shii, Hyogo Institute of Clinical Research, Akashi, Japan). The polyclonal anti-Grb2 antibodies were purified by pre-clearing the antisera with induced GST-expressing bacteria followed by affinity purification using the GST–Grb2 fusion protein bound to Affigel15 agarose beads (Bio-Rad). This antibody was appropriate for both immunoprecipitation and immunoblotting.

Proteins and peptides

The sources of the GST fusion proteins used in this study were as follows. Full-length (amino acids 1–217) and N- and C-terminal SH3 domains (amino acids 1–58 and 159–217, respectively) of Grb2 have been described previously (Gout *et al.*, 1993). The N-terminal SH2 domain of p85α was prepared as described previously (Yonezawa *et al.*, 1992b). Sequences coding for the SH3 domain of p85α were amplified by PCR from the cDNA of bovine p85α. PCR primers were designed with *Bam*HI and *Eco*RI restriction sites and in-frame stop codons. PCR-amplified sequences were cloned into the pGEX-2T (Pharmacia) expression vector using *Bam*HI and *Eco*RI. pGEX-2T constructs were transformed into *Escherichia coli* DH5α. Expression of GST fusion proteins was carried out as described previously (Yonezawa *et al.*, 1992b). Rat brain dynamin was purified as described (Gout *et al.*, 1993). Recombinant rat IRS-1 (details will be described elsewhere) and bovine p85α (Otsu *et al.*, 1991) were prepared from Sf9 cells infected with recombinant virus. Synthetic peptides corresponding to putative SH3 domain binding sequences derived from dynamin (P2, SPTPQRRAPAVPPARPGS) were synthesized, purified and coupled to Actigel resin as described previously (Gout *et al.*, 1993). The following synthetic IRS-1 peptides (designated IRP-1, -5, -6 and -7) containing the tyrosine phosphorylation sites of IRS-1: TDDGYMPMSPGV, ARLEYYENEKKW, NGLNYIDLDLVK and SPGEYVNIEFGS, corresponding to amino acids 604–615, 42–53, 1168–1179 and 891–902 of rat IRS-1, respectively, were used (Sun *et al.*, 1991). Phosphorylated and non-phosphorylated peptides were synthesized as described previously (Yonezawa *et al.*, 1992b) and purchased from Fujiya Bioscience (Hatano, Japan).

Immunoprecipitation, immunoblotting and cell labelling

Confluent 100 mm plates of CHO-IR cells were serum-starved in Ham's F-12 medium for 18 h at 37°C. Cells were then incubated with or without 10^{-7} M insulin for 2 min at 37°C, frozen with liquid nitrogen and stored at –80°C until lysis. Cells were then lysed in cold lysis buffer A [20 mM Tris (pH 7.6), 1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM CaCl2, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate] and immunoprecipitated with either rabbit antiserum against dynamin, polyclonal anti-Grb2 antibodies or control normal rabbit serum bound to protein A–Sepharose. The immunoprecipitates were washed twice with 50 mM HEPES (pH 7.6) buffered saline containing 0.1% Triton X-100 and electrophoresed on SDS–polyacrylamide gels, transferred to Immobilon-P membranes (Millipore), blocked in 3% gelatin, 10 mM Tris–HCl (pH 7.6), 154 mM NaCl and blotted with either rabbit antiserum against dynamin or a monoclonal antibody against phosphotyrosine residues. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG (Promega) followed by ECL detection (Amersham), according to the manufacturer's instructions.

For labelling studies, 60 mm plates of cells were washed and then incubated with 0.15 mCi of [^{35}S]methionine and [^{35}S]cysteine (Trans^{35}S-label, ICN) in methionine-free Dulbecco's modified Eagle's medium with 0.5% dialysed fetal calf serum for 8 h at 37°C. These cells were lysed and immunoprecipitated with either rabbit antiserum against Grb2 or control normal rabbit serum bound to protein A–Sepharose. The immunoprecipitates were washed three times with a high salt wash buffer [20 mM Na_2HPO_4 (pH 8.6), 0.5% Triton X-100, 0.1% SDS, 1 M NaCl, 0.1% BSA] and twice with the same buffer containing only 0.15 M NaCl and no albumin. The samples were then electrophoresed on SDS–polyacrylamide gels and analysed by autoradiography.

In vitro binding assay

To examine the ability of GST–SH3 fusion proteins to bind dynamin in CHO-IR cells, detergent lysates of CHO-IR cells were incubated for 4 h at 4°C with 1 μg of each fusion protein immobilized on glutathione–Sepharose beads. The beads were washed and proteins associated with

fusion proteins were immunoblotted with rabbit antiserum against dynamin as described above.

To reconstitute the association of Grb2–dynamin complex with tyrosine-phosphorylated IRS-1 *in vitro*, synthetic peptide P2, coupled to Actigel resin, was incubated with 2 μg of the GST–Grb2 (full-length) fusion protein. After extensive washing, recombinant baculovirus-expressed rat IRS-1 (which had been tyrosine-phosphorylated by human IRs immunoprecipitated with a monoclonal anti-IR antibody, 2F3, from insulin-treated CHO-IR cells) was added to the beads and immunoblotted with a monoclonal antibody against phosphotyrosine residues.

PI 3-kinase assay

The PI 3-kinase assay was carried out as described previously (Yonezawa *et al.*, 1992a).

Assay of dynamin GTPase activity

Dynamin GTPase activity was assayed in a 60 μl reaction mixture containing 0.5 μg of dynamin in 50 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM DTT, 130 μM GTP and 13 nM [α-^{32}P]GTP (3 μCi, 3000 Ci/mmol; Amersham) for 1 h at 37°C. One μg of GST–Grb2, pre-incubated with 500 μM phosphorylated or non-phosphorylated synthetic IRS-1 peptides for 2 h at 4°C, was added to the reaction mixture to assess the effect on dynamin GTPase activity (final concentration of peptides, 100 μM). Reactions were terminated by the addition of 4× stopping solution [80 mM Tris–HCl (pH 7.5), 80 mM EDTA, 8% SDS, 2 mM GDP, 2 mM GTP] and heating to 65°C for 10 min. The products of the reaction were resolved by thin layer chromatography on polyethyleneimine–cellulose plates in 1.6 M LiCl. The extent of GTP hydrolysis was assessed by quantitating GDP formation using a BAS 2000 Image Analyzer (Fuji film, Tokyo).

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