

# SH2-B $\alpha$ is an insulin-receptor adapter protein and substrate that interacts with the activation loop of the insulin-receptor kinase

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We identified SH2-B $\alpha$  as an insulin-receptor-binding protein based on interaction screening in yeast hybrid systems and co-precipitation in cells. SH2-B $\alpha$  contains pleckstrin-homology ('PH') and Src homology 2 (SH2) domains and is closely related to APS (adapter protein with a PH domain and an SH2 domain) and lnk, adapter proteins first identified in lymphocytes. SH2-B $\alpha$  is ubiquitously expressed and is present in rat epididymal adipose tissue, liver and skeletal muscle, physiological sites of insulin action. On SDS/PAGE, SH2-B $\alpha$  migrates at a molecular mass of 98 kDa, although the predicted size of SH2-B $\alpha$  is 79.6 kDa. Insulin causes an electrophoretic mobility shift. SH2-B $\alpha$  can be immunoprecipitated using anti-(insulin receptor) antibody from insulin-stimulated cells. Anti-phosphotyrosine antibody or the

growth factor receptor-binding protein 2 (Grb2) SH2 domain precipitate SH2-B $\alpha$  after insulin stimulation, suggesting that SH2-B $\alpha$  is tyrosine-phosphorylated and may be a substrate for the insulin receptor. The SH2-B $\alpha$  SH2 domain did not interact with insulin-receptor substrate (IRS) proteins or epidermal-growth-factor receptor. Mutation of the juxtamembrane and C-terminus of the insulin receptor did not abolish the interaction with the SH2 domain. This was further confirmed using a panel of activation-loop single point mutants where mutation of Tyr<sup>1158</sup>, Tyr<sup>1162</sup> and Tyr<sup>1163</sup> abolished interaction. Thus SH2-B $\alpha$  is a likely component in the insulin-signalling pathway and may function as an alternative signalling protein by interacting with the activation loop of the insulin-receptor cytoplasmic domain.

## INTRODUCTION

The insulin receptor is a member of the class II family of receptor tyrosine kinases [1,2]. Ligand stimulation results in auto-phosphorylation on tyrosine residues. Phosphorylation of sites in the activation loop results in full activation of the tyrosine kinase, whereas phosphorylation in the juxtamembrane and C-terminal regions regulates substrate interaction or interaction with other signalling proteins, particularly those with Src homology 2 (SH2) domains [1–4]. Over a number of years there have been substantial advances made in identifying substrates and adapter proteins for these tyrosine kinases [3,4]. Substrates for the insulin receptor include the insulin-receptor substrate (IRS) family and Shc, both of which are phosphorylated by the insulin-receptor tyrosine kinase. Phosphorylation of these proteins allows the recruitment of other signalling proteins. Among tyrosine-kinase receptors, one significant difference between the insulin-receptor family and other tyrosine kinase receptors is that other receptors, e.g. epidermal-growth-factor and platelet-derived-growth-factor receptors, tend to recruit SH2-domain proteins directly [4]. However, there is recent evidence that insulin receptor itself can also recruit SH2-domain proteins directly [5,6]. In the case of the p85 subunit of phosphatidylinositol (PI) 3-kinase, this interaction with insulin receptor appears to be redundant, whereas this direct interaction may be more relevant in the case of growth factor receptor-binding protein-insulin receptor (Grb-IR)/Grb10 [7,12–17]. A variety of expression and interaction cloning methods have been used to identify novel interacting

proteins for receptor-tyrosine-kinase cytoplasmic domains. In the case of the insulin receptor, the yeast two-hybrid system has identified a number of novel interactions [7–17]. The identification of novel protein–protein interactions with the insulin receptor is of importance, since not all of insulin's effects can be explained by existing signal-transduction pathways. Our goal was to identify tyrosine-phosphorylation-dependent interactions. The insulin receptor contains three tyrosine residues in the activation loop of its tyrosine kinase and these are phosphorylated sequentially in the activation cascade initiated by insulin stimulation [1,2,18]. Phosphorylation of these residues serves several functions. Firstly, site-directed-mutagenesis studies revealed that phosphorylation of all three residues is essential for maximal activation of the tyrosine kinase [18]. Phosphorylation of these sites allows conformational changes that promote access of substrates to the active site [18]. Secondly, these residues may function in protein–protein interactions, particularly with SH2 domains [7,15,16]. We embarked on the present study to try to identify proteins that interact with the activation loop only after phosphorylation. We therefore chose to use a modified yeast two-hybrid interaction cloning system, a three-hybrid system, employing a dual promoter plasmid [19], to search for proteins that interact with the phosphorylated activation loop. Using this approach we identified a protein–protein interaction of the insulin-receptor activation loop with an SH2 domain adapter protein, SH2-B. SH2-B was first identified as an Fc $\epsilon$ R1 immunoreceptor tyrosine-based activation motif (ITAM)-interacting protein [20]. During preparation of this manuscript, Wang and

Abbreviations used: SH2, Src homology 2; Shc, Src homology-containing and collagen-related; PH, pleckstrin homology; IRS, insulin-receptor substrate; Grb-IR, growth factor receptor-binding protein-(insulin receptor); GST, glutathione S-transferase; FCS, fetal-calf serum; CHO, Chinese-hamster ovary; EGF, epidermal growth factor; DTT, dithiothreitol; HRP, horseradish peroxidase; PLC, phospholipase C; JAK, Janus kinase; ITAM, immunoreceptor tyrosine-based activation motif; APS, adapter protein with a PH domain and an SH2 domain; PY, phosphotyrosine; X-gal, 5-bromo-4-chloroindol-3-yl  $\beta$ -D-galactopyranoside.

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Riedel independently reported the identification of SH2-B as an insulin-receptor interacting protein [21].

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## EXPERIMENTAL

### Antibodies

Monoclonal antibody against insulin receptor (83-14) was provided by Professor Kenneth Sidde (Department of Clinical Biochemistry, University of Cambridge, Cambridge, U.K.), monoclonal anti-phosphotyrosine (anti-PY) antibody (PY20) was from Transduction Laboratories (Lexington, KY, U.S.A.). Rabbit antisera against a glutathione S-transferase (GST) fusion protein containing SH2-B $\alpha$  SH2 domain and C-terminus were produced by Regal group (UK) (Chapel Lane, Great Bookham, Surrey, U.K.). Secondary antibodies conjugated to horseradish peroxidase (HRP) and anti-LexA antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. These were tested against the fusion-protein-cleaved GST and against yeast expressing the cDNA clone.

### Yeast hybrid interaction screen

Manipulations of *Escherichia coli*, *Saccharomyces cerevisiae*, DNA and proteins were performed essentially as described in [8–17,20,22]. Interaction screens were performed as described using plasmid pTS51 derived from pHybZeoLexA (Invitrogen, Carlsbad, CA, U.S.A.). Details of subcloning strategies are available on request from T.S.P. pTS51 contains insulin-receptor residues 1116–1214 [23] fused to the LexA DNA-binding domain. PCR primers were designed to generate a fragment of the insulin-receptor residues 1116–1214 containing the activation loop of the insulin receptor with Phe substitutions for Tyr<sup>1122</sup> and Tyr<sup>1210</sup>. A separately encoded insulin-receptor cytoplasmic domain under control of a second promoter (triosephosphate isomerase) was subcloned into the pHybZeoLexA plasmid to generate pTS51. Expression and autophosphorylation of the kinase and trans-phosphorylation of the activation-loop fragment were confirmed by immunoblotting with anti-LexA, anti-PY antibodies and anti-insulin receptor antibodies.

A human skeletal-muscle cDNA library in pGAD424 (Clontech Laboratories, Palo Alto, CA, U.S.A.) was screened by standard two-hybrid method in the yeast strain L40. Approx. 500000 clones were screened for His prototrophy. These were then tested for  $\beta$ -galactosidase activity using a filter assay [8–17]. After exclusion of false positives, five interacting clones were identified and, on restriction and PCR analysis, these appeared to contain the same insert. The plasmids were rescued by transformation of yeast miniprep DNA into DH5 $\alpha$ . Clones were subjected to automated DNA sequencing (ABI373a). Dideoxy sequencing revealed > 95% identify with rat SH2-B spanning amino acid residues 501–722, including the SH2 domain. The dbEST database was interrogated using the BLAST program and several expressed sequence tags were identified. One tag, Image clone 48269, was selected for further study.

Standard two-hybrid interaction studies were performed as described in [8]. LexA plasmids containing a kinase-inactive and C-terminal deletion mutant [8–12] were provided by Dr. Gus Gustafson (University of Maryland and Metabolex, Inc., 3876 Bay Center Place, Hayward, CA, U.S.A.).

### Tissue extracts

Tissue extracts from Wistar rats were prepared by freezing the tissues in liquid nitrogen followed by homogenization using a Polytron homogenizer in buffer A [20 mM Tris/HCl (pH 7.6)/100 mM NaCl/1 mM MgCl<sub>2</sub>/10% glycerol/1 mM dithiothreitol (DTT)/1 mM Na<sub>3</sub>VO<sub>4</sub>/1 mM NaF/0.2 mM PMSF/2  $\mu$ g/ml aprotinin/20  $\mu$ M leupeptin/10  $\mu$ g/ml pepstatin/1% Triton X-100]. Insoluble material was removed by centrifugation and the soluble supernatants were used for immunoblotting.

### Cell culture

Chinese-hamster ovary (CHO) cells overexpressing wild-type insulin receptor (CHO.T) [kindly provided by Dr. Gus Gustafson (University of Maryland) and Jeremy Tavaré (Department of Biochemistry, University of Bristol, Bristol, U.K.)] and CHO cells overexpressing insulin receptor with the deletion of the NPEY domain containing tyrosine 972 (CHO $\Delta$ NPEY) (originally from Dr. Paulos Berhanu, University of Colorado, Denver, CO, U.S.A.) were obtained from Dr. Theodore Ciaraldi (Division of Endocrinology and Metabolism, University of California, San Diego, CA, U.S.A.). These were cultured in Ham's F12 medium supplemented with 10% fetal-calf serum (FCS), antibiotics and G-418. COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and antibiotics. CHO cells expressing activation-loop mutants were as previously described [25,26]. These contained single point mutations of Tyr<sup>1158</sup>, Tyr<sup>1162</sup> and Tyr<sup>1163</sup> to Phe.

### In vitro interaction

Src, phospholipase C $\gamma$  (PLC $\gamma$ ) N-terminal SH2-domain, Grb2 and Src-homology-containing and collagen-related (Shc) SH2-domain GST fusion proteins were obtained from Dr. Jerrold Olefsky (Division of Endocrinology and Metabolism, University of California, San Diego, CA, U.S.A.) and were purified from bacteria by affinity chromatography with glutathione immobilized on beaded agarose (Sigma). Insulin and epidermal-growth-factor (EGF) receptors were extracted from CHO.T and COS-7 cells respectively. Serum-starved cells were stimulated with insulin or EGF and lysed in buffer A. Following centrifugation to remove insoluble materials, the soluble supernatants were incubated with purified immobilized GST SH2 domain fusion proteins for 3 h at 4 °C and subjected to extensive washing with Wash buffer containing 20 mM HEPES, pH 7.5, 100 mM NaCl and 0.1% Triton-X 100. Bound proteins were eluted with Laemmli sample buffer, separated by SDS/PAGE, transferred to PVDF membrane and immunoblotted with antibody against PY.

### Immunoprecipitations and immunoblotting

Serum-starved cells were stimulated with insulin for 10 min and lysed using lysis buffer containing 20 mM Tris/HCl, pH 7.6, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 0.2 mM PMSF, 2  $\mu$ g/ml aprotinin, 20  $\mu$ M leupeptin, 10  $\mu$ g/ml pepstatin and 0.1% Triton X-100. The lysates were incubated with the indicated antibody for 4 h at 4 °C. The immunoprecipitates were immobilized on Protein G-agarose and washed sequentially with Wash buffer and PBS. Bound proteins were separated by SDS/PAGE, followed by immunoblotting with polyclonal antibody against SH2-B $\alpha$  (no. 18547) and HRP-conjugated secondary antibody. Bound antibody was detected using SuperSignal chemiluminescence substrate (Pierce).

### Subcellular fractionation

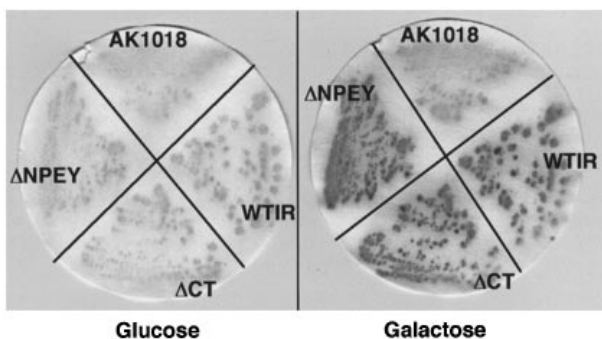
CHO.T cells were serum-starved for 24 h, stimulated with 100 nM insulin for 10 min and then rapidly frozen in liquid nitrogen. Cells were lysed in buffer containing 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10% glycerol, 1 mM DTT, 0.1 mM orthovanadate, 0.2 mM PMSF, 2  $\mu$ g/ml aprotinin, 20  $\mu$ M leupeptin and 10  $\mu$ g/ml pepstatin. Nuclei and unbroken cells were removed by a low-speed spin (1500 g) for 5 min. The remaining lysate was centrifuged at 26 000 g for 60 min at 4 °C. The supernatant was removed as the cytosol fraction and the pellet was resuspended in an equal volume of lysis buffer (membrane fraction). Equal volumes of each fraction were analysed by SDS/7.5%-PAGE and immunoblotting as described above.

## RESULTS

### Interaction of the insulin receptor activation loop with SH2-B $\alpha$

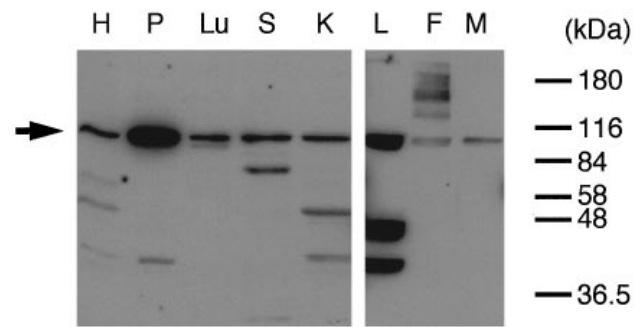
We used the phosphorylated activation loop of the insulin-receptor cytoplasmic domain as a bait in a modified two-hybrid system to identify interacting proteins in a human skeletal-muscle library. One clone containing an SH2 domain was obtained and appeared to encode the human homologue of rat SH2-B [20]. We designated this clone human SH2-B $\alpha$ , because recently an alternative spliced variant form, SH2-B $\beta$ , has been described [21] and our identified clone did not contain the insert which the  $\beta$  form is reported to have.

The SH2 domain was tested against the kinase active (wild-type insulin receptor) (WTIR) and several mutants including the kinase-dead (AK1018) cytoplasmic domain and mutants lacking the juxtamembrane ( $\Delta$ NPEY) and C-terminal ( $\Delta$ CT) phosphorylation sites in a standard two-hybrid assay for the expression of  $\beta$ -galactosidase and using X-gal as a substrate (Figure 1). This was done to verify the interaction with the activation loop and to confirm that a kinase-active cytoplasmic domain was required. The results confirm that neither the juxtamembrane tyrosine phosphorylation sites nor the C-terminal phosphorylation sites



**Figure 1** Interaction with SH2-B $\alpha$  SH2 domain in the two-hybrid system with different mutants

The SH2-B $\alpha$  SH2 domain was subcloned into the B42 activation domain plasmid pJG4-5 and then transformed into the yeast strain EGY48 along with LexA fusions of the wild-type (WTIR) and kinase-inactive (AK1018) (Lys<sup>1030</sup> → Ala mutation) insulin-receptor cytoplasmic domain and mutants containing deletions of the juxtamembrane ( $\Delta$ NPEY) and C-terminal ( $\Delta$ CT) autophosphorylation sites as described in the Experimental section. The resulting yeast transformants were replica-plated on to circular filters. The filters were grown on yeast drop-out medium containing glucose and galactose to induce expression of the SH2B $\alpha$  plasmid and then assayed for  $\beta$ -galactosidase activity as described in the Experimental section.



**Figure 2** Expression of SH2-B $\alpha$  in different tissues

Tissue extracts from rat tissues were prepared and subjected to immunoblotting using anti-SH2-B $\alpha$  serum 18547 as described in the Experimental section. Blots were developed using HRP-coupled antibodies and chemiluminescence. Abbreviations: H, heart; P, pancreas; Lu, lung; S, spleen; K, kidney; L, liver; F, fat; M, skeletal muscle.

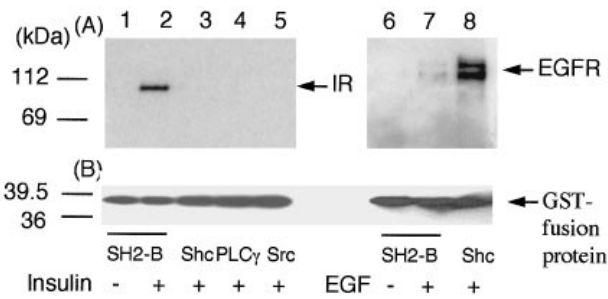
are required for interaction and that a kinase-active cytoplasmic domain is required for the interaction, since there was no interaction with the kinase-dead mutant.

### Expression of SH2-B $\alpha$ in rat tissues (Figure 2)

The expression pattern of SH2-B has previously been examined in several tissues using Northern blotting and transcripts were found to be present in liver and skeletal muscle [20]. To investigate SH2-B $\alpha$  protein expression in several tissues, particularly in the ones which are physiological sites of insulin action, we generated antisera against the human homologue of SH2-B $\alpha$  using a GST fusion protein containing the SH2 domain and the C-terminus of the protein. An anti-SH2-B antibody-reactive signal was detected in all rat tissues studied and this protein expression pattern may be ubiquitous. SH2-B $\alpha$  appears to migrate with a molecular mass of 97–100 kDa in most tissues in SDS/PAGE gels (Figure 2). Additional bands are seen that may represent degradation products or alternatively spliced forms. Recently, an alternatively spliced form, SH2-B $\beta$ , was described [21]. This  $\beta$  form has an identical SH2 domain but a distinct C-terminus and is smaller than the  $\alpha$  form. We would have expected our antisera to react with SH2-B $\beta$ , but we did not find any evidence of this. This is presumably because the major epitopes of our antisera are probably located in the C-terminus and the C-termini of the two isoforms are distinct.

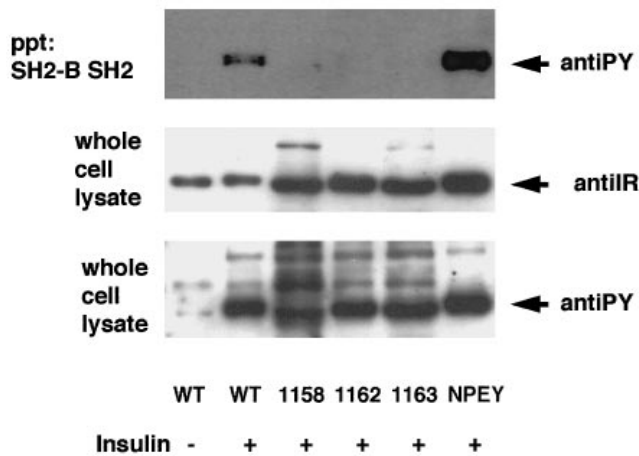
### Specificity of the SH2-B $\alpha$ SH2 domain for the insulin receptor (Figure 3)

To study the specificity of the interaction we tested GST-SH2-B SH2 fusion protein for its association with insulin receptor in cell extract. We compared the binding of the Src, Shc and N-terminal PLC $\gamma$  SH2 domains to the insulin receptor with that of the SH2-B $\alpha$  SH2 domain. Detergent extracts of insulin receptor in CHO.T-cell lysates were incubated with equivalent amounts of the GST-SH2 fusion protein as indicated. The amount of the respective GST fusion protein was quantified using anti-(GST protein) serum. Interaction with the insulin-receptor cytoplasmic domain in cell lysates was only seen with the SH2-B $\alpha$  SH2 domain but not the Shc, Src and PLC $\gamma$  SH2 domains (Figure 3).



**Figure 3** Specific interaction of the SH2-B $\alpha$  SH2 domain with insulin receptor

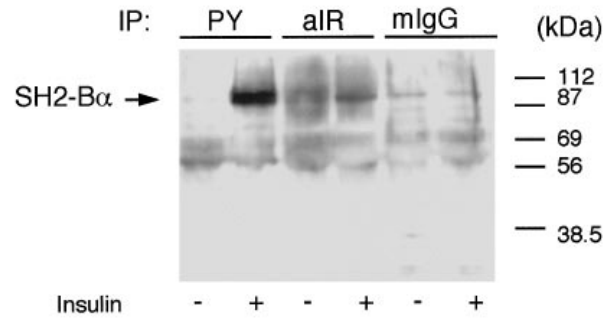
(A, lanes 1–5) CHO.T were stimulated with 100 nM insulin as indicated for 10 min. GST fusion proteins of the SH2-B $\alpha$ , Shc, PLC $\gamma$  and Src SH2 domains purified from bacteria were incubated with the CHO.T lysates followed by precipitation using glutathione–agarose. Bound receptor was separated by SDS/7.5% PAGE and analysed by Western blot using anti-PY antibodies (PY20). (A, lanes 6–8) COS-7 cells were stimulated with 10 nM EGF for 10 min. The cell lysates were incubated with purified SH2-B $\alpha$  and Shc SH2 domains followed by precipitation using glutathione–agarose. Bound receptor was analysed by Western blot using PY20. (B) Blots were subsequently stripped and re-probed using anti-GST antibodies.



**Figure 4** Interaction of the SH2-B $\alpha$  SH2 domain with various insulin-receptor mutants

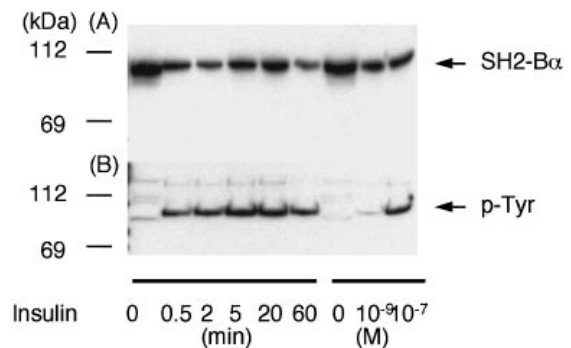
GST fusion proteins of the SH2-B $\alpha$  SH2 domain purified from bacteria were incubated with cell lysates derived from CHO cells expressing various insulin-receptor mutants, followed by precipitation using glutathione–agarose. Bound receptors were separated by SDS/7.5% PAGE and immunoblotted with anti-PY antibodies ('antiPY', upper panel). Whole-cell lysates were immunoblotted using anti-(insulin receptor) antibodies ('antiIR', middle panel) and anti-PY antibodies ('antiPY', lower panel). WT, wild-type; 1158, Y1158F (Tyr<sup>1158</sup> → Phe); 1162, Y1162F; 1163, Y1163F; NPEY, deletion mutant of the juxtamembrane phosphorylation site (Tyr<sup>972</sup>).

We did not find any interaction between the SH2-B $\alpha$  SH2 domain and IRS protein. We also examined the binding of the SH2-B $\alpha$  SH2 domain with the EGF receptor in comparison with the Shc SH2 domain. Detergent extracts of stimulated EGF receptors in COS-7 lysates were mixed with equivalent amounts of GST SH2 fusion protein as indicated. Whilst we detected a good interaction between the Shc SH2 domain and EGF receptor as previously reported [24], the SH2-B $\alpha$  SH2 domain did not interact with the EGF receptor *in vitro* (Figure 3).



**Figure 5** Association of SH2-B $\alpha$  with the insulin receptor after insulin stimulation *in vivo*

Serum-starved CHO.T cells were treated with or without 100 nM insulin for 10 min. Proteins were immunoprecipitated using PY20, anti-(insulin receptor) antibodies, mouse IgG coupled with Protein G–agarose. Immunoprecipitates were separated by SDS/PAGE and analysed by immunoblotting using anti-SH2-B $\alpha$  antisera.



**Figure 6** Mobility shift: time course and dose response

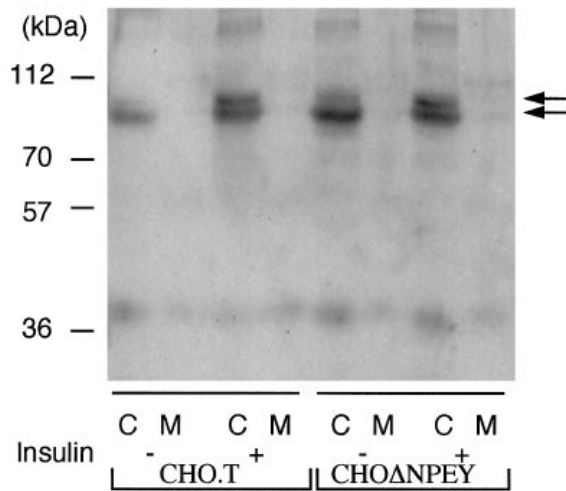
CHO.T cells were stimulated with 100 nM insulin for indicated periods, or with indicated concentration of insulin for 10 min. After being washed twice with cold PBS, cells were lysed. Indicated whole cell lysates ( $1 \times 10^5$  cells/sample) were separated by SDS/10% PAGE and probed with anti-SH2-B $\alpha$  antisera (A). Blots were subsequently stripped and re-probed using PY20 (B).

#### Interaction of the SH2 domain–GST fusion protein with activation-loop mutants (Figure 4)

We tested the binding of several insulin-receptor mutants expressed in CHO cells to confirm the binding to the activation loop. The single point mutants retain insulin-stimulated autophosphorylation at detectable levels. In contrast with the wild-type receptor and the juxtamembrane deletion, mutation of Tyr<sup>1158</sup>, Tyr<sup>1162</sup> or Tyr<sup>1163</sup> abolished the interaction of the SH2 domain with the insulin receptor. In the mutant cell lines the presence of phosphorylated receptor was confirmed by immunoblotting of whole-cell lysates using anti-(insulin receptor) antibody or anti-PY antibody.

#### Co-precipitation of SH2-B $\alpha$ with the insulin receptor after insulin stimulation *in vivo* (Figure 5)

We then determined, using immunoprecipitation, whether cellular SH2-B $\alpha$  associates with the insulin receptor (Figure 5). Serum-starved CHO.T cells were stimulated with insulin and the lysates were immunoprecipitated using either an anti-(insulin



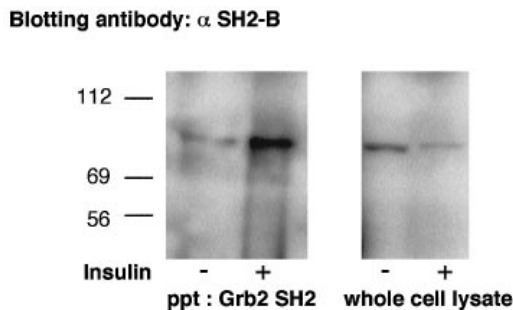
**Figure 7 Subcellular fractionation**

CHO.T and CHO $\Delta$ NPEY cells were stimulated with 100 nM insulin for 10 min. Proteins (10  $\mu$ g) from cytosolic (C) and membrane (M) fractions of the cells were separated by SDS/7.5%-PAGE and detected by Western blot using anti-SH2-B $\alpha$  antisera.

receptor) antibody (83-14) or an anti-PY antibody. Stimulation of cells with insulin resulted in the association of SH2-B $\alpha$  with the insulin receptor. In addition, anti-PY antibodies precipitated SH2-B $\alpha$  from insulin-stimulated cells. We consistently found that more SH2-B $\alpha$  was precipitated using anti-PY antibodies than by 83-14 and the SH2B $\alpha$  precipitated by PY20 tended to migrate as a doublet.

#### Insulin-stimulated mobility shift (Figure 6)

Whole-cell lysates from insulin-stimulated cells were blotted with the anti-SH2-B $\alpha$  antisera (Figure 6A) or anti-PY (Fig. 6B) antibody. The mobility shift with insulin occurs fairly rapidly and is evident at 0.5 min up to the 60 min time point we



**Figure 8 Association of SH2-B $\alpha$  with Grb2 SH2 domain after insulin stimulation**

Serum-starved CHO.T cells were treated with or without 100 nM insulin for 10 min and lysed. The lysates were incubated with GST Grb2 SH2 domain for 2 h at 4  $^{\circ}$ C. Proteins were precipitated using glutathione beads, separated by SDS/PAGE and probed with anti-SH2-B $\alpha$  antisera (left panel). The right-hand panel shows an aliquot (one-tenth) of the whole cell lysate used for this precipitation experiment.

examined (Figure 6A). The mobility shift of SH2-B $\alpha$  is dose-dependent and sensitive to insulin stimulation. In fact, the mobility shift is observed at 1 nM insulin when there is barely detectable receptor autophosphorylation by anti-PY immunoblotting (Figure 6B).

#### Subcellular fractionation (Figure 7)

Lysates of basal and stimulated CHO.T cells were subjected to subcellular fractionation to determine the localization of SH2-B $\alpha$  and also whether it changed under conditions of insulin stimulation (Figure 7). SH2-B $\alpha$  appears to be confined to the cytosolic fraction under basal conditions. Surprisingly, the cytosolic fraction also revealed a more marked change in electrophoretic mobility than that seen in the whole-cell lysates (Figure 5). We obtained the same result from the experiment using CHO $\Delta$ NPEY cells (Figure 7).

#### Interaction of SH2-B with the Grb2 SH2 domain (Figure 8)

The C-terminal phosphorylation site of APS has been shown to be a binding site for the Grb2 SH2 domain [27]. This tyrosine residue is conserved in SH2-B. Precipitations were performed using a GST fusion of the Grb2 SH2 domain and the precipitates were immunoblotted using anti-SH2-B antiserum. A greater amount of SH2-B $\alpha$  was consistently detected in the Grb2 SH2 domain precipitations after insulin stimulation (Figure 8). Using an aliquot of the same sample, the whole-cell lysates were probed with anti SH2-B antiserum and this demonstrated that the increased association with the Grb2 Sh2 domain is accompanied by a mobility shift as well as a decrease in the antibody-reactive signal.

#### DISCUSSION

We isolated a fragment of SH2-B in a yeast hybrid library screen using the activation loop of the insulin receptor as a bait. SH2-B, specifically the  $\alpha$ -isoform, was first identified as an Fc $\epsilon$ R1-interacting protein [20]. However, the function of this protein has not been established, although it appears to be similar to the recently identified APS and lnk proteins found in B-lymphocytes [27,28]. While this manuscript was in preparation, Wang and Riedel [21] reported the identification of SH2-B as an insulin-receptor-interacting protein. This was identified using the insulin-receptor cytoplasmic domain as a bait to screen a mouse embryo library. There are two important discrepancies between our results and theirs. Firstly, they demonstrated that the SH2-B SH2 domain interacts with the juxtamembrane and C-terminal tyrosine phosphorylation sites of the insulin-receptor cytoplasmic domain. Secondly, they did not find any evidence for the phosphorylation of SH2-B in response to insulin. We provide evidence for the interaction of SH2-B $\alpha$  with the activation loop and show that, after insulin stimulation, SH2-B is precipitable with anti-PY antibodies and the Grb2 Sh2 domain and also undergoes a mobility shift which is probably due to phosphorylation. The reasons for these discrepancies are not clear and they may be due to methodological and reagent differences. For example, SH2-B phosphorylation may be highly labile and subject to rapid dephosphorylation intracellularly and this may explain a failure to detect phosphorylation. Phosphopeptides were used by these authors to map the site and this may produce results at variance with other methods. Grb7/Grb10 is a particular example [15–17]. In this instance, using phosphopeptides, the binding site was mapped to the C-terminus [7,17] and this result conflicts with the assignment derived from other

methods, including site-directed mutagenesis [16]. Although Wang and Riedel used cells expressing insulin-receptor mutants, it is not clear why a single mutation at the C-terminus of the cytoplasmic domain affects binding to the juxtamembrane and vice versa [21].

Recently, a splice variant of SH2-B was identified in a two-hybrid screen using the Janus kinase (JAK) 2 as a bait [22]. This variant has a 100-nucleotide insertion at the 3' end of the SH2 domain and has been designated SH2-B $\beta$ . SH2-B $\beta$  interacts with and is phosphorylated by, JAK2. Insulin stimulation in mouse 3T3-F442A cells did not result in phosphorylation of SH2-B $\beta$ . It is possible that only SH2-B $\alpha$  is a substrate for the insulin receptor and alternative splicing may result in a loss of the C-terminal phosphorylation sites. Our antibodies do not appear to react with SH2-B $\beta$ , probably because all of the antigenic epitopes are in the C-terminus. Interestingly, differentially migrating forms of SH2-B $\beta$  were also observed in response to growth hormone or agents that altered phosphorylation [22].

Although both SH2-B and APS have been identified in an immunological context, they may function ubiquitously as signalling proteins in a number of tissues and thus their functions may not be strictly confined to mast cells and B-lymphocytes. SH2-B $\alpha$  is ubiquitously expressed and may be a universal signalling protein in the same way that Grb2 is, although the precise signalling pathway that it initiates is not known. SH2-B $\alpha$  may recruit additional signalling proteins, including phosphatidylinositol 3-kinase and Grb2, in a similar manner to lnk and APS [27,28]. Examples of similar universal signalling proteins include Grb2 and Shc, which play a role in signal transduction in a number of different cell types and receptor systems [24].

Here we present several distinct lines of evidence to implicate SH2-B $\alpha$  as a potential participant in insulin signalling. Firstly, SH2-B $\alpha$  is present in insulin-sensitive tissues. The predicted size of SH2-B $\alpha$  is 79.6 kDa, but on SDS/PAGE it migrates with a molecular mass of 98 kDa. This is similar to the insulin receptor  $\beta$ -subunit and could explain why it has not been detected as a substrate previously. The cause of this discrepancy is not clear, but may be due to serine/threonine phosphorylation and there are a number of predicted sites for serine/threonine phosphorylation. Furthermore, this difference is also observed with the related protein APS [27], as well as the splice variant SH2-B $\beta$  [22].

Secondly, the co-precipitation experiments demonstrate that SH2-B $\alpha$  associates with the insulin receptor *in vivo*. We consistently detect more SH2-B in anti-PY immunoprecipitates than in insulin-receptor immunoprecipitates, even though 83-14 is an efficient and quantitative precipitating antibody. Anti-PY antibodies also precipitate a higher-molecular-mass form of SH2-B $\alpha$ . Taken together, this strongly suggests that the precipitation with PY20 is direct rather than due to co-precipitation with the insulin receptor. Thirdly, we have also found that insulin stimulates a mobility shift of SH2-B $\alpha$  in a rapid and dose-dependent manner and this is more readily apparent after separation of the cytosolic fraction. This mobility shift may be due to a combined tyrosine and serine/threonine phosphorylation following insulin stimulation. Subcellular fractionation experiments reveal that SH2-B $\alpha$  is present in the cytosol. We could not detect any SH2-B $\alpha$  in the membrane fraction after insulin stimulation under our fractionation conditions and at the time points examined. There are several putative sites for tyrosine phosphorylation in the C-terminus and the related protein, APS, is also a substrate for tyrosine phosphorylation in B-lymphocyte and associates with the Grb2 SH2 domain [27]. The potential C-terminal tyrosine phosphorylation site is conserved in SH2-B $\alpha$ . We reasoned that

insulin may stimulate phosphorylation of this site and therefore this PY may interact with the Grb2 SH2 domain. We tested this directly *in vitro* and found that, after insulin stimulation, the Grb2 SH2 domain precipitated SH2-B. This additional evidence provides further support that SH2-B $\alpha$  is subject to insulin-stimulated tyrosine phosphorylation. An alternative explanation is that the Grb2 SH2 domain precipitation is indirect, although it is difficult to envisage how this may occur through Shc or IRS-1.

This is another example of an SH2-domain protein interacting directly with the activation loop, as has been observed for Grb-IR/Grb10 [7,15,16]. Interaction of other SH2 domains with the insulin-receptor cytoplasmic domain has been demonstrated previously, although the exact roles of these is unclear. In the case of Syp and phosphatidylinositol 3-kinase p85, insulin receptor binding is redundant, as these can interact directly with IRS proteins [3–6].

The interaction of SH2-B $\alpha$  with the activation loop of the insulin receptor is intriguing in view of several key findings of previous workers. For example, Wilden et al. [25,26] demonstrated evidence for signalling divergence at the level of Tyr<sup>1158</sup> in relation to DNA synthesis and glycogen synthesis. The specific interaction of this residue with a signalling protein may underlie this divergence. Furthermore, the recent elucidation by Hubbard [18] of the crystal structure of the activated insulin receptor complexed with its substrate demonstrates that phosphorylated Tyr<sup>1158</sup> is relatively exposed and well placed to interact with other signalling proteins. Thus further studies of the role of SH2-B $\alpha$  insulin signalling are clearly warranted to determine if the divergent signalling observed can be explained by site-specific interaction of SH2-domain-containing signalling proteins. Furthermore, this approach will prove useful in identifying other protein–protein interactions with the activation loop.

The interaction of SH2-B $\alpha$  with the insulin-receptor cytoplasmic domain may generate some specificity for receptor signalling relative to the EGF receptor, since the EGF receptor does not interact with the SH2-B $\alpha$  SH2 domain. The exact binding site has not been mapped, although Tyr<sup>1158</sup> appears to be present in a Tyr-Glu-Thr-Asp motif and this is similar to the motif present in the ITAM sequence in Fc $\epsilon$ R1 [20].

Although, the precise function of SH2-B $\alpha$  is unclear, some clues may be inferred from the functions of the APS protein. SH2-B $\alpha$  may act to recruit additional signalling molecules, either through tyrosine phosphorylation or through its proline-rich region and PH domains. In addition, SH2-B $\alpha$  may regulate the tyrosine kinase activity of the insulin by interacting with the activation-loop phosphorylation sites, either as a feedback inhibitory protein in a similar manner to Grb-IR [14] or may anchor the activation loop in the active conformation [18] so as to sustain kinase activation. All of these possibilities need to be addressed in future experiments.

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