Src homology 3 domain-dependent interaction of Nck-2 with insulin receptor substrate-1

Yizeng TU*, Liang LIANG†, Stuart J. FRANK†‡ and Chuanyue WU*[1]

*Department of Pathology, University of Pittsburgh, 3550 Terrace Street, Pittsburgh, PA 15261, U.S.A., †Department of Medicine, Division of Endocrinology and Metabolism, University of Alabama at Birmingham, Birmingham, AL 35294-0019, U.S.A., and ‡Veterans Administration Medical Center, Birmingham, AL 35294, U.S.A.

Insulin receptor substrate-1 (IRS-1) is a multi-domain protein that mediates signal transduction from receptors for insulin and other growth factors to a variety of downstream molecules through both tyrosine-phosphorylation-dependent and -independent interactions. While the tyrosine-phosphorylation-dependent interactions mediated by IRS-1 have been well characterized, the molecular basis underlying the tyrosine-phosphorylation-independent IRS-1 interactions is largely unknown. We previously detected, in an *in vitro* binding assay, interactions of Nck-2 Src homology (SH) 3 domains with IRS-1. We show here that IRS-1 associates with Nck-2 *in vivo*. Additionally, we have investigated the molecular basis underlying the IRS-1–Nck-2 complex formation. We have found that (i) mutations at the highly conserved tryptophan within the Nck-2 SH3 domains markedly reduced the association with IRS-1, (ii) interactions mediated by multiple SH3 domains enhance the complex formation of Nck-2 with IRS-1, (iii) deletion of either the phosphotyrosine-binding/Shc and IRS-1 NPXY-binding (PTB/SAIN) domains or the Pre-C-terminal domain of IRS-1, but not the pleckstrin homology (PH) domain, reduced the Nck-2 binding, (iv) PTB/SAIN domains or the Pre-C-terminal domain alone is capable of interacting with Nck-2, and (v) the IRS-1–Nck-2 interaction occurs in the absence of other proteins and therefore is direct. These results establish that IRS-1 is a *bona fide* target of the Nck-2 SH3 domains and reveal that IRS-1 forms a complex with Nck-2 via direct interactions mediated by multiple domains from both binding partners.

Key words: IRS-1, protein–protein interaction, SH3.

INTRODUCTION

Insulin receptor substrate-1 (IRS-1) is a large (1235 amino acids in rat) docking protein that is involved in transducing signals from receptors for insulin and other growth factors, hormones and cytokines to a variety of downstream signalling molecules [1,2]. Upon insulin stimulation, IRS-1 is recruited to activated insulin receptors and phosphorylated at multiple tyrosine sites, which serve as binding sites for a number of Src homology (SH) 2 domain-containing signalling proteins including the p85 subunit of phosphoinositide 3-kinase (PI 3-kinase), Grb-2, Fyn and SHP2. The engagement of IRS-1 with downstream SH2-containing signalling proteins through the phosphotyrosine sites clearly plays important roles in insulin signalling. Nevertheless, recent studies suggest that IRS-1 can also mediate at least certain aspects of insulin signalling in the absence of interactions between IRS-1 phosphotyrosine sites and SH2 domains. For example, IRS-1 mutants in which all 18 potential tyrosine-phosphorylation sites are replaced by phenylalanine can mediate to a certain extent insulin-stimulated mitogenesis [3]. Thus in addition to interacting with SH2 domains via phosphotyrosine-containing sites, IRS-1 may also serve as a docking protein for other protein-binding domains that are independent of tyrosine phosphorylation.

IRS-1 consists of several structurally and functionally distinctive domains [1,2]. At the N-terminus of IRS-1 lies a pleckstrin homology (PH) domain that is involved in coupling IRS-1 to the insulin receptor [4,5]. Immediately C-terminal to the PH domain are the phosphotyrosine-binding (PTB) and Shc and IRS-1 NPXY-binding (SAIN) domains that interact directly with the phosphorylated NPXY motif in the activated insulin receptor [6,7]. Further downstream is the Pre-C-terminal (Pre-C) domain that contains several tyrosine residues, which, upon insulin-induced phosphorylation, are known to provide docking sites for several SH2 domains including those of p85 and Grb2 [1,2]. The C-terminal domain contains several additional tyrosine residues that can be phosphorylated by activated insulin receptors and provide binding sites for other SH2-containing proteins including SHP2 [1,2].

Nck-2 (also known as Nckβ [8] or Grb4 [9]) is a newly identified, ubiquitously expressed adaptor protein comprising primarily three N-terminal SH3 domains and one C-terminal SH2 domain [10]. Nck-2 is implicated in cellular signal transduction regulating cell growth, morphology and gene expression [8–10]. At the molecular level, Nck-2 can interact with components of both the growth-factor signalling pathways and the cell-adhesion signalling pathways. For example, Nck-2 binds to PINCH (particularly interesting new Cys-His protein), a LIM-containing binding partner of integrin-linked kinase (ILK), as well as ligand-activated receptors for platelet-derived growth factor and epidermal growth factor [10]. We previously detected, in an *in vitro* binding assay, interactions of Nck-2 SH3 domains with IRS-1 [10]. The current study is aimed to determine (i) whether Nck-2 and IRS-1 form a complex *in vivo* and (ii) the

Abbreviations used: FBS, fetal bovine serum; GST, glutathione S-transferase; ILK, integrin-linked kinase; IRS-1, insulin receptor substrate-1; PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; PTB, phosphotyrosine-binding; SAIN, Shc and IRS-1 NPXY-binding; SH, Src homology; Pre-C, Pre-C-terminal; AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; MEM, minimal essential medium; MBP, maltose-binding protein; PINCH, particularly interesting new Cys-His protein; CHO-IR-IRS-1, Chinese hamster ovary cells expressing insulin receptor and IRS-1.

[1] To whom correspondence should be addressed (e-mail carywu@imap.pitt.edu).

molecular basis underlying the complex formation between Nck-2 and IRS-1.

EXPERIMENTAL

Cells, antibodies and other reagents

HEK-293 cells were from American Type Culture Collection (Rockville, MD, U.S.A.). CHO-IR-IRS-1 (Chinese hamster ovary cells expressing insulin receptor and IRS-1) cells were kindly provided by Dr Thomas A. Gustafson (Metabolex, Hayward, CA, U.S.A.). Media for cell culture were from Gibco-BRL (Life Technologies, Grand Island, NY, U.S.A.) or Mediatech/Cellgro® (Herndon, VA, U.S.A.). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT, U.S.A.). Mouse monoclonal anti-Nck-2 antibody (mAb 8.8) and rabbit polyclonal anti-GST-Nck-2 antibodies (where GST is glutathione S-transferase) were produced as described previously [10]. Rabbit polyclonal anti-IRS-1 antibody (C-20) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse monoclonal anti-FLAG antibody M5 and anti-FLAG antibody M2-conjugated agarose beads (anti-FLAG M2 affinity gel) were from Kodak and Sigma. Restriction enzymes, DNA-modifying enzymes, DNA molecular-mass markers and dNTPs were purchased from Promega. Synthetic oligonucleotides were prepared by Gibco-BRL.

Co-immunoprecipitation

CHO-IR-IRS-1 cells were maintained in McCoy's 5A medium containing 10 % FBS and 100 μg/ml hygromycin (Thomas A. Gustafson, personal communication). A FLAG-Nck-2 expression vector (pFLAG-Nck-2) was generated by inserting the full-length Nck-2 cDNA into the *Hin*dIII/*Sal*I sites of a mammalian expression vector pFLAG-CMV-2 (Kodak). The cells were transfected with the pFLAG-Nck-2 vector or a control vector pFLAG-CMV-2 that lacks the Nck-2 sequence, using LipofectAMINE reagent (Life Technologies). The expression of FLAG-Nck-2 in the FLAG-Nck-2 transfectants, but not the control transfectants, was confirmed by Western blotting with an anti-FLAG antibody. The FLAG-Nck-2-expressing cells and the control cells were starved in serum-free McCoy's 5A medium for 18 h and then either harvested or stimulated with 5 μg/ml insulin for 5 min and then harvested. To confirm that the insulin treatment stimulated tyrosine phosphorylation of IRS-1, the cell lysates were probed with a monoclonal anti-phosphotyrosine antibody (PY-20, 0.5 μg/ml) by Western blotting. The cells were lysed with 1 % (v/v) Triton X-100 in 50 mM Hepes buffer (pH 7.1) containing 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 100 mM NaF, 10 μM leupeptin, 0.2 mM AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride], 2 μg/ml aprotinin and 1 μM pepstatin (lysis buffer). The cell lysates (625 μg) were incubated with monoclonal anti-FLAG antibody M2-conjugated agarose beads (anti-FLAG M2 affinity gel; 13 μl) at 4 °C overnight. The beads were then washed four times with the lysis buffer and mixed with SDS reducing sample buffer (10 μl). IRS-1 and FLAG-Nck-2 co-precipitated with the beads were detected by Western blotting with a rabbit polyclonal anti-IRS-1 antibody and a mouse monoclonal anti-FLAG antibody (M5), respectively.

Human MCF-7 breast cancer cells were cultured in the Improved Minimal Essential Medium (MEM) supplemented with 10 % FBS, 1 × non-essential amino acid solution and 1 mM MEM sodium pyruvate solution (Life Technologies). The interaction between endogenous Nck-2 and IRS-1 was analysed by immunoprecipitation with anti-IRS-1 antibody (Santa Cruz Biotechnology) and UltraLink immobilized Protein G (Pierce, Rockford, IL, U.S.A.) based on a protocol provided by the manufacturer (Pierce). Briefly, human MCF-7 breast cancer cells were lysed with an immunoprecipitation buffer (50 mM sodium acetate, pH 5.0/500 mM NaCl/1 % Nonidet P-40/0.1 % SDS/0.02 % sodium azide) containing protease inhibitors (10 μM leupeptin/0.2 mM AEBSF/2 μg/ml aprotinin/1 μM pepstatin). The cell lysates (175 μg) were incubated with rabbit anti-IRS-1 IgG (1 μg) or an irrelevant rabbit IgG (1 μg) at 4 °C for 2 h or longer, followed by incubation with UltraLink immobilized Protein G (15 μl of 50 % slurry) at 4 °C for 1 h. The UltraLink immobilized Protein G beads were washed five times with washing buffer (50 mM sodium acetate, pH 5.0/500 mM NaCl/0.1 % Triton X-100/10 μM leupeptin/0.2 mM AEBSF/2 μg/ml aprotinin/1 μM pepstatin). Proteins bound to the beads were released by boiling in SDS/PAGE sample buffer (15 μl) for 3 min. Nck-2 and IRS-1 in the immunoprecipitates were detected by Western blotting with a mouse monoclonal anti-Nck-2 antibody 8.8 and a rabbit polyclonal anti-IRS-1 antibody, respectively. To test whether the Nck-2–IRS-1 interaction occurs in the absence of insulin stimulation, MCF-7 cells were serum-starved in the Improved MEM supplemented with 0.1 % FBS, 1 × non-essential amino acid solution and 1 mM MEM sodium pyruvate solution for 20 h and then harvested. The interaction between endogenous Nck-2 and IRS-1 was analysed by immunoprecipitation as described above.

Site-directed mutagenesis

A QuikChange™ site-directed mutagenesis system (Stratagene) was used to change the highly conserved tryptophan residues (amino acids 148 and 234) in the second and third SH3 domains of Nck-2 to lysine. The point mutation was confirmed by DNA sequencing using a Sequenase version 2.0 kit (United States Biochemicals).

Production of recombinant GST-Nck-2 and GST-IRS-1 fusion proteins

To generate GST-Nck-2 fusion proteins, Nck-2 cDNA sequences (as specified in each experiment) were amplified by PCR and inserted into the *Eco*RI/*Xho*I site of a pGEX-5x-1 vector (Pharmacia). The generation of cDNA plasmids encoding GST-IRS-1(PH) (including rat IRS-1 residues 21–203), GST-IRS-1(PH/PTB) (residues 21–400), GST-IRS-1(PTB/SAIN) (residues 108–516) and GST-IRS-1(Pre-C) (residues 516–896; provided generously by Thomas A. Gustafson) has been described in [6]. To generate the plasmid encoding GST-IRS-1(C-ter), the rat cDNA region encoding residues 899–1235 was ligated into the pGEX 2TRS plasmid using the *Eco*RI and *Kpn*I sites [11]. Correct assembly of this cDNA fragment was verified by dideoxy sequencing. A cDNA construct encoding a GST-fusion protein containing the SH3 domain of $p130^{Cas}$ (pGEX2T/CasSH3) was kindly provided by Dr Jun-lin Guan (Cornell University, Ithaca, NY, U.S.A.). The recombinant vectors were then used to transform *Escherichia coli* cells. The expression of the GST fusion proteins was induced with isopropyl β-D-thiogalactoside and induced proteins were isolated with glutathione–Sepharose 4B beads.

Expression of IRS-1 mutants in mammalian cells

32D cells expressing IRS-1ΔPH (deletion of residues 6–155), IRS-1ΔPTB/SAIN (deletion of residues 140–578), IRS-1ΔPre-C (deletion of residues 578–895) and IRS-1(wt) (wild type) were

generated by electroporation of 32D-rGHR cells [11,12] [2×10^7/ml in complete medium; 250 V, 960 mF in a GenePulser (Bio-Rad) electroporator] with pSX eukaryotic expression vectors encoding the corresponding IRS-1 mutants (mutant cDNAs kindly provided by Dr Jacalyn H. Pierce, National Cancer Institute, Bethesda, MD, U.S.A.) and a vector (pCMV) that encodes the histidinol-resistance marker. Selection of the transfectants was carried out in RPMI 1640 culture medium containing histidinol (2 mM, Sigma).

The isolation and preparation of the rat IRS-1 cDNA and the cDNA encoding IRS-1ΔPH have been described in [13,14]. IRS-1ΔPTB/SAIN and IRS-1ΔPre-C were prepared by PCR using the rat IRS-1 cDNA as a template (the sequences of the oligodeoxynucleotide primers used are available from the authors upon request). The presence of the intended mutation and the lack of unwanted mutations in the regions subjected to PCR were verified by dideoxy DNA sequencing. The wild-type and mutant IRS-1 cDNAs were each ligated into the pSX plasmid at the *Sac*I and *Dra*I/*Sma*I sites.

GST fusion protein pull-down assays

HEK-293 cells were cultured in Eagle's MEM (Mediatech, Herndon, VA, U.S.A.) supplemented with 10% FBS. The cells were transfected with the pFLAG-Nck-2 vector and a pEGFP-C2 vector (Clontech) that bears a chemical (neomycin) selection marker using LipofectAMINE reagent. After transfection (48 h), the transfectants were selected with Eagle's MEM supplemented with 10% FBS and 1 mg/ml geneticin. The geneticin-resistant cells were cloned and the expression of FLAG-Nck-2 was confirmed by Western blotting with a mouse monoclonal anti-FLAG antibody (M5). The HEK-293 cells expressing FLAG-Nck-2 were maintained in Eagle's MEM supplemented with 10% FBS and geneticin. 32D cells expressing the rabbit growth hormone receptor and the wild-type or mutated forms of IRS-1 were cultured in RPMI 1640 medium containing 10% fetal calf serum and 2 mM histidinol. Cells were lysed with 1% Triton X-100 in 20 mM Tris/HCl, pH 7.5/150 mM NaCl/5 mM EDTA/1 mM Na_3VO_4/2 mM AEBSF/5 μg/ml pepstatin A/10 μg/ml aprotinin/10 μg/ml leupeptin, and centrifuged at 20800 ***g*** for 30 min at 4 °C. Protein concentrations of the cell lysates were determined with a Coomassie Plus Protein Assay Reagent (Pierce) and then used for GST fusion protein pull-down assays.

For GST fusion protein pull-down assays, the cell lysates (as specified in each experiment) were pre-incubated with glutathione–Sepharose beads (50% slurry) for 1 h at 4 °C, followed by centrifugation at 15300 ***g*** for 5 min. The pre-cleared cell lysates were then incubated with equal amounts (as specified in each experiment) of GST (as a control) or GST fusion proteins containing the wild-type or mutated forms of Nck-2 or IRS-1 on a rocking platform for 2 h at 4 °C. The GST and GST fusion proteins were then precipitated with glutathione–Sepharose beads. After incubation for 2 h at 4 °C the beads were washed with the lysis buffer three times and finally washed twice with 50 mM Tris/HCl, pH 7.5, containing 1 mM Na_3VO_4. The precipitated proteins were separated by SDS/PAGE and analysed by Western blotting with rabbit polyclonal anti-IRS-1 antibodies (1 μg/ml), rabbit polyclonal anti-GST-Nck-2 antiserum (1:10000 dilution), mouse monoclonal anti-Nck-2 antibody (0.5 μg/ml) or mouse monoclonal anti-FLAG antibody (M5; 3.3 μg/ml) as specified in each experiment. The primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibodies and the SuperSignal® chemiluminescent substrate (Pierce). The relative amounts of IRS-1 pulled down with the GST fusion proteins containing the wild-type or mutated forms of Nck-2 were estimated by densitometric analysis as described previously [11]. Briefly, densitometry of immunoblots was performed using a solid-state video camera (Sony-77, Sony Corp.) and a 28 mm MicroNikkor lens over a lightbox of variable intensity (Northern Light Precision 890, Imaging Research, Toronto, Canada). Quantification was performed using a Macintosh II-based image-analysis program (Image 1.61, developed by W. S. Rasband, Research Services Branch, National Institute of Medical Health, Bethesda, MD, U.S.A.) [11]. Percentage of binding was calculated as the amount of IRS-1 associated with each Nck-2 mutant/the amount of IRS-1 associated with the wild-type Nck-2.

Direct Nck-2–IRS-1 interaction

To generate a MBP (maltose-binding protein) fusion protein containing the full-length Nck-2, the full-length human Nck-2 cDNA was inserted into the *Eco*RI/*Sal*I site of the pMAL-C2 vector (New England BioLabs). The MBP-Nck-2 fusion protein was expressed in *E. coli* (DH5α) and purified by affinity chromatography using amylose–agarose beads (New England BioLabs). Direct interactions between Nck-2 and IRS-1 domains were analysed by incubation of purified MBP-Nck-2 fusion protein (0.5 ml of 1 μg/ml in 20 mM Hepes, pH 7.1/100 mM NaCl/1 mM EDTA/1.5 mM $MgCl_2$/10% glycerol/0.5% Triton X-100/1 mM PMSF/10 μg/ml aprotinin) with equal amounts (10 μg) of GST fusion proteins containing various domains of IRS-1, or GST alone as a control, that were immobilized on glutathione–Sepharose beads for 2 h at 4 °C. At the end of incubation, the beads were washed with the lysis buffer three times and finally washed twice with 50 mM Tris/HCl, pH 7.5. MBP-Nck-2 bound to the GST-IRS fusion proteins was detected by Western blotting with a monoclonal anti-Nck-2 antibody (mAB 8.8), a horseradish peroxidase-conjugated anti-mouse IgG antibody and the SuperSignal® chemiluminescent substrate (Pierce).

RESULTS

IRS-1 and Nck-2 form a complex *in vivo*

The association of IRS-1 with Nck-2 in mammalian cells was analysed by co-immunoprecipitation experiments. Because the currently available anti-Nck-2 antibodies do not work well in immunoprecipitation assays [8], we expressed a FLAG-tagged Nck-2 in CHO-IR-IRS-1 cells by DNA transfection. Control transfectants were generated by transfecting the cells with a vector (pFLAG-CMV-2) that lacks Nck-2 sequence. FLAG-Nck-2 was immunoprecipitated from the FLAG-Nck-2 transfectants (Figure 1A, lanes 1 and 2), but not the control transfectants (Figure 1A, lanes 3 and 4), with a monoclonal anti-FLAG antibody. Analysis of the anti-FLAG immune complex revealed that IRS-1 was readily co-precipitated with FLAG-Nck-2 (Figure 1B, lanes 1 and 2). No IRS-1 was precipitated from the control cells (Figure 1B, lanes 3 and 4), confirming that IRS-1 could not be precipitated by the anti-FLAG antibody in the absence of FLAG-Nck-2. Similar amounts of the Nck-2–IRS-1 complex were detected in the cells that were serum-starved (Figures 1A and 1B, lanes 2) and in those stimulated with insulin (Figures 1A and 1B, lanes 1), which, as expected, dramatically increased the tyrosine phosphorylation of IRS-1 (Figure 1C, lanes 1 and 3). These results suggest that IRS-1 forms a specific complex with FLAG-Nck-2 in mammalian cells in a tyrosine-phosphorylation-independent manner.

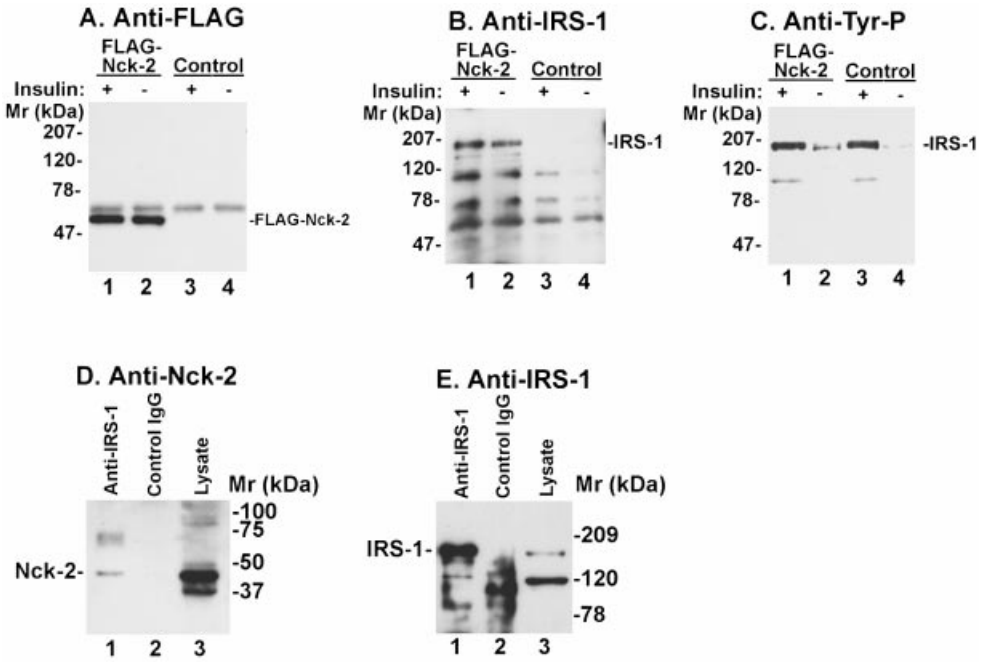

Figure 1 IRS-1 and Nck-2 form a complex *in vivo*

(**A**–**C**) Co-immunoprecipitation of IRS-1 with FLAG-Nck-2. CHO-IR-IRS-1 cells that were transfected with a FLAG-Nck-2 vector (lanes 1 and 2) or a control vector (lanes 3 and 4) were serum-starved (lanes 2 and 4), or serum-starved and then stimulated with 5 μg/ml insulin for 5 min (lanes 1 and 3). FLAG-Nck-2 was immunoprecipitated from the cell lysates with monoclonal anti-FLAG antibody M2 as described in the Experimental section. FLAG-Nck-2 and associated IRS-1 in the immunoprecipitates were detected by Western blotting with a mouse monoclonal anti-FLAG antibody (**A**) and a rabbit polyclonal anti-IRS-1 antibody (**B**). In control experiments (**C**), lysates (12 μg/lane) of the cells that were serum-starved (lanes 2 and 4), or serum-starved and then stimulated with 5 μg/ml insulin for 5 min (lanes 1 and 3), were probed with a monoclonal anti-phosphotyrosine antibody (PY-20) by Western blotting to confirm that the insulin treatment stimulated the tyrosine phosphorylation of IRS-1. (**D** and **E**) Interaction between endogenous Nck-2 and IRS-1. Human MCF-7 breast cancer cells were cultured in the Improved MEM supplemented with 10% FBS, 1 × non-essential amino acid solution and 1 mM MEM sodium pyruvate solution and then lysed as described in the Experimental section. Lysates of human MCF-7 cells were incubated with a rabbit anti-IRS-1 antibody (lanes 1) or an irrelevant rabbit IgG (lanes 2) as a control. The immune complexes were precipitated with UltraLink immobilized Protein G. Nck-2 and IRS-1 were detected by Western blotting with a mouse monoclonal anti-Nck-2 antibody 8.8 (**A**) and a rabbit polyclonal anti-IRS-1 antibody (**B**). Lanes 3 were loaded with 7.5 μg of MCF-7 cell lysates.

To test whether endogenous Nck-2 interacts with IRS-1 in mammalian cells, we precipitated IRS-1 proteins from lysates of human MCF-7 cells with an anti-IRS-1 antibody. Immunoblotting analyses of the anti-IRS-1 precipitates with anti-Nck-2 and anti-IRS-1 antibodies showed that Nck-2 (Figure 1D, lane 1) was co-immunoprecipitated with IRS-1 (Figure 1E, lane 1). By contrast, no Nck-2 (Figure 1D, lane 2) was detected in control IgG precipitates lacking IRS-1 (Figure 1E, lane 2). In additional experiments, we have analysed the binding of Nck-2 to IRS-1 in serum-starved MCF-7 cells. Consistent with the results obtained with the FLAG-tagged Nck-2, the endogenous Nck-2 was also co-immunoprecipitated with IRS-1 in the absence of insulin stimulation (results not shown), indicating that the binding of Nck-2 to IRS-1 is not dependent upon insulin-stimulated tyrosine phosphorylation.

A W → K point mutation inhibits the SH3-domain-mediated IRS-1 binding

We next sought to investigate the molecular basis underlying the Nck-2–IRS-1 complex formation. The ability of IRS-1 to interact with the wild-type and mutant forms of Nck-2 (Figure 2A) was analysed in GST fusion protein pull-down assays. As expected, IRS-1 efficiently associated with GST fusion proteins containing the full-length Nck-2 (Figure 2D, lane 3). Nck-2 fragments containing either the third SH3 domain (Figure 2D, lane 5) or the second SH3 domain (Figure 2D, lane 6), but not the Nck-2 fragment lacking the SH3 domains (Figure 2D, lane 4), were also capable of forming a complex with IRS-1, although the efficiency was noticeably lower than that of the full-length Nck-2, indicating that interactions mediated by multiple SH3 domains enhance the complex formation between Nck-2 and IRS-1. In contrast to GST fusion protein containing the second or third SH3 domains of Nck-2, GST-fusion protein containing $p130^{Cas}$ SH3 domain was unable to bind to IRS-1 (results not shown), confirming the specificity of the binding. Additionally, no significant interaction between the first SH3 domain of Nck-2 and IRS-1 was detected (Figure 2D, lane 7). A Nck-2 fragment lacking the first SH3 domain (Figure 2D, lane 2) associated with IRS-1 almost as efficiently as the full-length Nck-2 (Figure 2D, lane 3). Taken together, these results suggest that the second and third SH3 domains, but not the first SH3 domain, contribute to the complex formation between Nck-2 and IRS-1.

Both the second and third SH3 domains of Nck-2, like all other SH3 domains, contain a highly conserved tryptophan residue located immediately C-terminal to the n-Src loop. Previous studies have shown that replacement of this highly conserved tryptophan with a lysine inhibits protein-binding activities of several SH3 domains (e.g. the SH3 domains of Abl and Crk) [15–17]. To test whether the association of Nck-2 with IRS-1 is truly mediated by the SH3 domains, we substituted the highly conserved tryptophan residues within the Nck-2 SH3 domains with lysine (Figure 2A). The ability of the SH3 point mutants to associate with IRS-1 was analysed in a GST fusion protein pull-down assay. To do this, we expressed and purified the GST-SH3

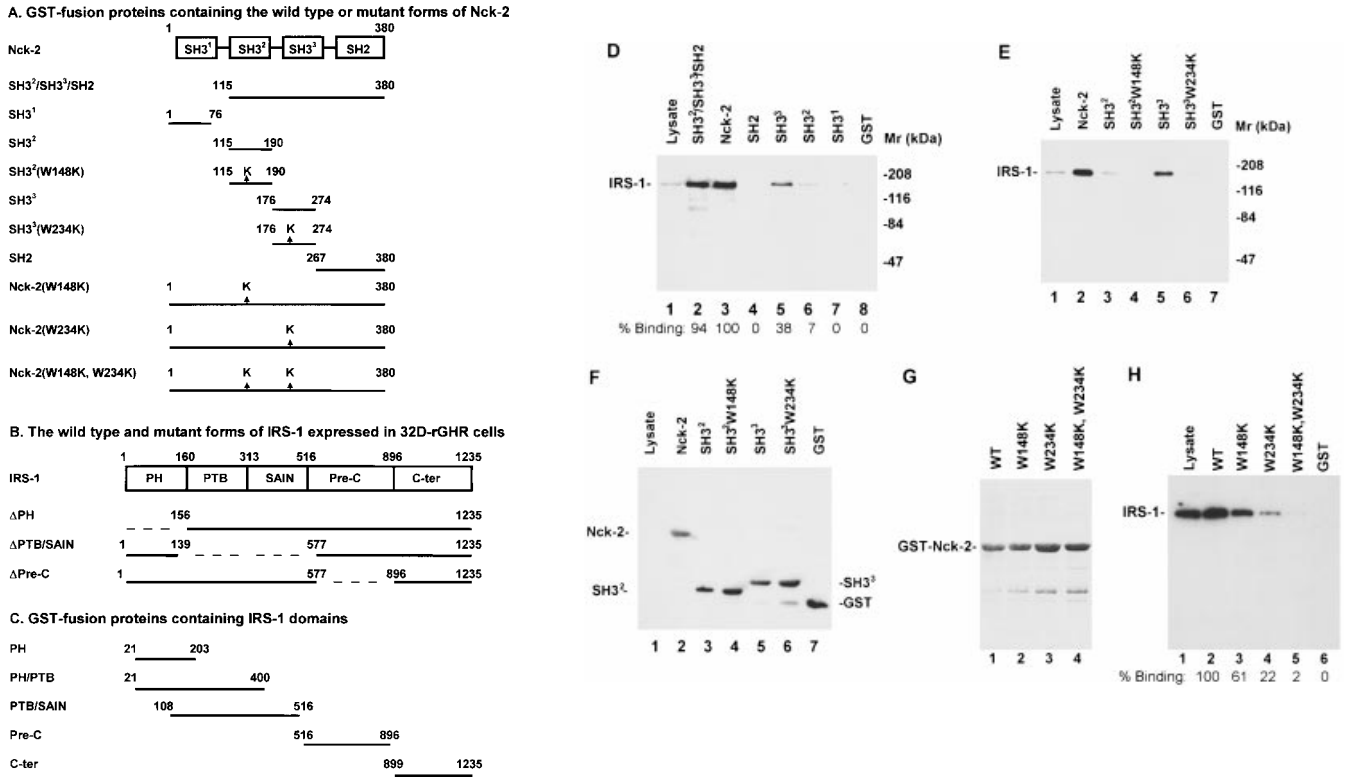

Figure 2 Mutational analyses of the Nck-2–IRS-1 interaction

(**A**–**C**) Schematic representation of the wild-type and mutated forms of Nck-2 and IRS-1. The wild-type and mutant forms of Nck-2 and IRS-1 are expressed in *E. coli* as GST fusion proteins (**A** and **C**) or in mammalian 32D-rGHR cells (**B**) as described in the Experimental section. Nck-2 and IRS-1 residues included in each of the mutants are represented by solid lines. Deleted regions are depicted by broken lines. W → K point mutations within the second and/or third SH3 domains of Nck-2 are indicated by arrows. (**D**–**F**) A W → K point mutation inhibits the SH3-mediated IRS-1-binding activity. (**D**) Lysates (16.7 μg) of 32D cells expressing IRS-1 were incubated with equal amounts (6.7 μg) of GST fusion proteins containing the full-length Nck-2 (lane 3), the second and third SH3 domains and the SH2 domain (lane 2, residues 115–380), the SH2 domain (lane 4, 267–380), the third SH3 domain (lane 5, 176–274), the second SH3 domain (lane 6, 115–190), the first SH3 domain (lane 7, 1–76) or GST alone as a control (lane 8), for 2 h at 4 °C. The GST and GST fusion proteins were precipitated and the associated IRS-1 was detected by Western blotting with an anti-IRS-1 antibody as described in the Experimental section. Lane 1 was loaded with 0.7 μg of cell lysates. Relative amounts of IRS-1 bound to the full-length Nck-2 and the Nck-2 SH3 domains were estimated by densitometric analysis as described in the Experimental section. (**E** and **F**) The highly conserved tryptophans within the second SH3 domain (residue 148) and the third SH3 domain (residue 234) of Nck-2 were replaced with lysine as described in the Experimental section. The association of IRS-1 with GST fusion proteins containing the wild-type Nck-2 (lanes 2), the wild-type second SH3 domain (lanes 3), the W → K mutant of the second SH3 domain (lanes 4), the wild-type third SH3 domain (lanes 5) or the W → K mutant of the third SH3 domain (lanes 6) was analysed by a GST pull-down assay. IRS-1 associated with the GST-Nck-2 fusion proteins was detected by Western blotting with an anti-IRS-1 antibody (**E**). The membrane was stripped and re-probed by Western blotting with an anti-GST-Nck-2 antibody (**F**). (**G** and **H**) Contributions of the second and third SH3 domains to the Nck-2–IRS-1 complex formation. (**G**) GST fusion proteins containing the wild-type (WT) Nck-2 (lane 1) or mutant forms of Nck-2 in which residue W148 (lane 2), W234 (lane 3) or both W148 and W234 (lane 4) were substituted with K were analysed by SDS/PAGE and stained with Coomassie Brilliant Blue. (**H**) To analyse the IRS-1-binding activities, HEK-293 cell lysates (200 μg) were incubated with equal amounts (6.7 μg) of GST fusion proteins containing the wild-type Nck-2 (lane 2), Nck-2 point mutant W148K (lane 3), W234K (lane 4), Nck-2 double mutant (W148K, W234K; lane 5) or GST lacking Nck-2 sequence as a negative control (lane 6). The GST fusion proteins and GST were precipitated with glutathione–Sepharose beads and the associated IRS-1 was detected by Western blotting with an anti-IRS-1 antibody. Relative amounts of IRS-1 bound to the wild-type and mutant forms of Nck-2 were estimated by densitometric analysis as described in the Experimental section. Percentage binding is the amount of IRS-1 associated with each Nck-2 mutant/the amount of IRS-1 associated with the wild-type Nck-2. Lane 1 was loaded with 10 μg of cell lysates.

fusion proteins containing the W → K point mutation. The GST fusion proteins containing the SH3 W → K point mutants, and those containing the wild-type SH3 domains as positive controls, were incubated with the cell lysates. The GST fusion proteins and associated proteins were then precipitated with glutathione–Sepharose beads. Analyses of the GST fusion protein precipitates by Western blotting with an anti-IRS-1 antibody revealed that the W → K point mutation significantly reduced the amount of IRS-1 bound to the Nck-2 SH3 domains (Figure 2E, lanes 3–6). Stripping and re-probing the same membrane with an anti-GST-Nck-2 antibody showed that abundant SH3 point mutants were present in the precipitates (Figure 2F), indicating that the decrease of the amount of IRS-1 in the precipitates is indeed caused by an impaired ability of the SH3 mutants to associate with IRS-1. We conclude from these experiments that the highly conserved tryptophan residues located within the second and third SH3 domains of Nck-2 are critical for the IRS-1-binding activity.

Contributions of the second and third SH3 domains to the Nck-2–IRS-1 complex formation

To assess the contributions of the second and third SH3 domains to the complex formation between the full-length Nck-2 and IRS-1, we generated full-length recombinant Nck-2 proteins containing the W → K point mutation at position 148 (Figure 2G, lane 2), position 234 (Figure 2G, lane 3) or both (Figure 2G, lane 4). A reduction in the IRS-1 binding activity was observed when either the second SH3 domain or the third SH3 domain was mutated (Figure 2H, lanes 2–4). Noticeably, the IRS-1-binding activity of the W234K mutant (Figure 2H, lane 4) was significantly lower than that of the W148K mutant (Figure 2H,

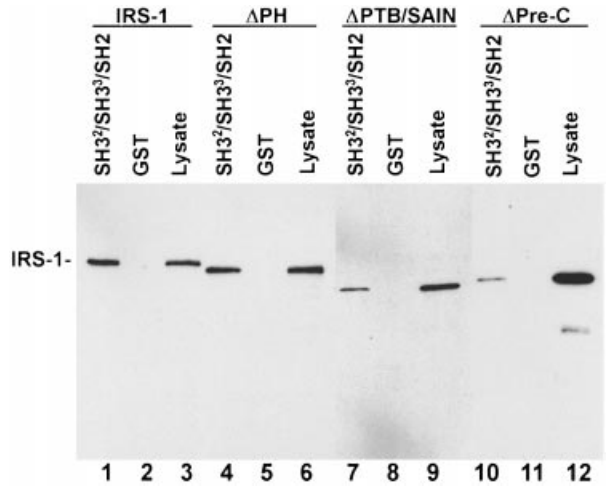

Figure 3 Multiple IRS-1 domains mediate the interaction with Nck-2

Lysates (11 μg) of 32D cells expressing IRS-1 (lanes 1 and 2) or IRS-1 mutants in which residues 6–155 (ΔPH, lanes 4 and 5), residues 140–576 (ΔPTB/SAIN, lanes 7 and 8) or residues 579–895 (ΔPre-C, lanes 10 and 11) were deleted were incubated with equal amounts (5 μg) of GST-SH3^2/SH3^3/SH2 (lanes 1, 4, 7 and 10) or GST (lanes 2, 5, 8 and 11), for 2 h at 4 °C. The GST and GST fusion proteins were precipitated and the associated IRS-1 was detected by Western blotting with an anti-IRS-1 antibody as described in the Experimental section. Lanes 3, 6, 9 and 12 were loaded with lysates of 32D cells expressing the wild-type or mutated forms of IRS-1 as indicated (2 μg/lane).

lane 3). Mutations at both positions (148 and 234) almost completely eliminated the IRS-1-binding activity (Figure 2H, lane 5). These results demonstrate that the third SH3 domain and, to a lesser extent, the second SH3 domain, are used during complex formation between the full-length Nck-2 and IRS-1.

PTB/SAIN and Pre-C domains of IRS-1 are involved in the association with Nck-2 SH3 domains

IRS-1 comprises several structurally and functionally distinct domains including the PH, PTB, SAIN, Pre-C and C-terminal domains (Figure 2B). To identify the domains of IRS-1 that are involved in the association with Nck-2, we expressed IRS-1 mutants in which one or more of the domains (the PH domain, PTB/SAIN or Pre-C domain) were deleted (Figure 2B, ΔPH domain, ΔPTB/SAIN and ΔPre-C) in the IRS-1-deficient 32D-rGHR cells. The expression of each of the IRS-1 mutants in the transfectants was confirmed by Western blotting with an antibody recognizing the C-terminal domain of IRS-1 (Figure 3, lanes 6, 9 and 12). The cells expressing the domain-deletion mutants were then used to analyse the association with Nck-2. Deletion of the PH domain did not affect the ability of IRS-1 to associate with GST-SH3^2/SH3^3/SH2 (Figure 3, lane 4) or GST-Nck-2 (results not shown). By contrast, deletion of the PTB/SAIN (Figure 3, lane 7) or Pre-C (Figure 3, lane 10) domains substantially reduced, although did not completely eliminate, the association with GST-SH3^2/SH3^3/SH2 (Figure 3) or GST-Nck-2 (results not shown). In control experiments, no binding was detected between GST and the wild-type or mutant forms of IRS-1 (Figure 3, lanes 2, 5, 8 and 11), confirming the specificity of the binding assay. These results suggest that both the PTB/SAIN domains and the Pre-C domain, but not the PH domain, contribute to the association with Nck-2.

Because Nck-2 contains two major IRS-1-association sites that are located in two separate SH3 domains (one in the second SH3 domain and the other in the third SH3 domain), we next analysed the effects of the PTB/SAIN or Pre-C deletion on association of IRS-1 with each of the individual Nck-2 SH3 domains. Consistent with the results shown earlier (Figures 2 and 3), the third SH3 domain, and to a less extent the second SH3 domain, associated with the PH-deletion IRS-1 mutant (results not shown). Deletion of the PTB/SAIN or Pre-C decreased but did not completely eliminate the association with either the third or the second SH3 domain (results not shown). No significant association between the first SH3 domain of Nck-2 and any of the IRS-1 deletion mutants was detected. In additional experiments, none of the IRS-1 mutants associated with the SH2 domain of Nck-2, and no band was detected in the precipitates when the lysates of cells expressing the IRS-1 mutants were substituted with that of the IRS-1-deficient control cells (results not shown), further confirming the specificity of the precipitation and detection assays. Taken together, these results suggest that either the second or the third SH3 domains of Nck-2 can recognize at least two IRS-1 sites; one localizes in the PTB/SAIN domains and the other in the Pre-C domain.

PTB/SAIN and Pre-C domains of IRS-1 are capable of interacting with Nck-2

To prove that there indeed exist at least two separate Nck-2-association sites on IRS-1, one in the PTB/SAIN domains and the other in the Pre-C domain, we used a series of GST fusion proteins that contain different IRS-1 domains (Figure 2C, PH, PH/PTB, PTB/SAIN, Pre-C and C-terminal). Together they cover the entire coding region of IRS-1 (Figure 2B). The ability of each of the IRS-1 fragments to associate with Nck-2 was tested in a GST fusion protein pull-down assay using cells expressing a FLAG-tagged Nck-2. The results showed that GST fusion proteins containing either the PTB/SAIN (Figure 4A, lane 4) or the Pre-C domain (Figure 4A, lane 7) were capable of associating with the FLAG-tagged Nck-2. By contrast, GST alone (Figure 4A, lane 2) or GST fusion proteins containing the PH domain (Figure 4A, lane 5), the PH and PTB domains (Figure 4B, lane 6) or the C-terminal domain (Figure 4B, lane 3) failed to associate with the FLAG-tagged Nck-2. These results demonstrate that IRS-1 contains two major Nck-2-association sites, one in the PTB/SAIN domains (most probably within the SAIN domain) and the other in the Pre-C domain.

Nck-2 binds directly to the PTB/SAIN and Pre-C domains of IRS-1

The association of IRS-1 with Nck-2 could result from direct interactions between the two proteins, or alternatively from indirect associations mediated through other intracellular proteins. To distinguish these two possibilities, we tested whether purified bacterially expressed recombinant MBP-Nck-2 fusion protein could interact directly with GST fusion proteins containing various IRS-1 domains in a GST fusion protein pull-down assay. The results showed that GST fusion proteins containing either the PTB/SAIN (Figure 4B, lane 4) or Pre-C (Figure 4B, lane 7) domains were capable of interacting with the purified MAP-Nck-2. By contrast, no Nck-2 binding was detected with GST (Figure 4B, lane 2) or GST fusion proteins containing the PH (Figure 4B, lane 5), PH/PTB (Figure 4B, lane 6) or C-terminal (Figure 4B, lane 3) domains. These results are highly consistent with those obtained with the mammalian cell lysates (Figure 4A) and indicate that there exist two direct Nck-2-binding sites on IRS-1, one in the PTB/SAIN domains (most probably the SAIN domain) and the other in the Pre-C domain.

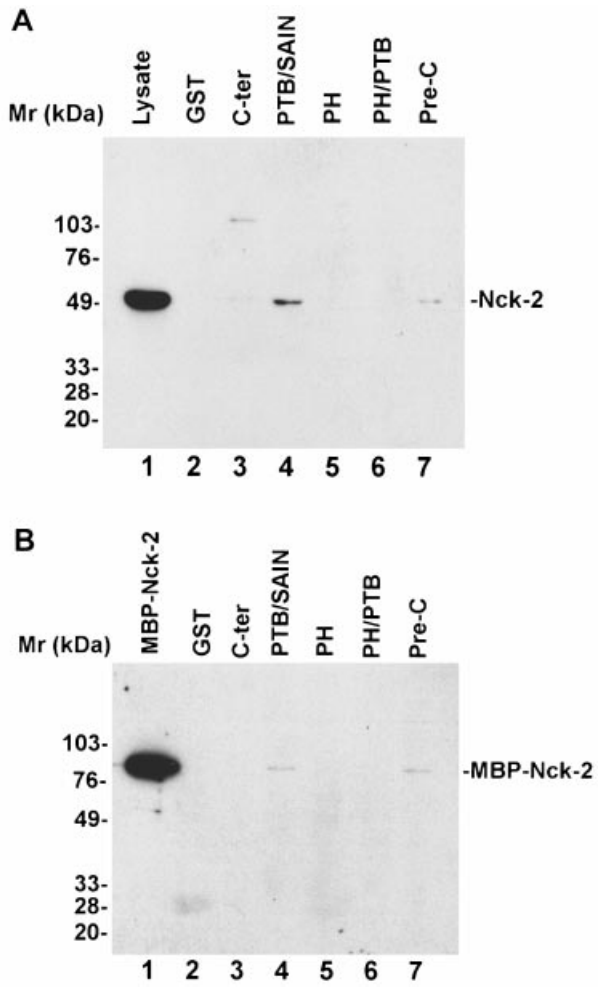

Figure 4 PTB/SAIN and the Pre-C domains of IRS-1 bind directly to Nck-2

(**A**) The PTB/SAIN or the Pre-C domain of IRS-1 is sufficient for interacting with Nck-2. Lysates (200 μg) of the HEK-293 cells expressing FLAG-Nck-2 were incubated with equal amounts (10 μg) of GST fusion proteins containing the C-terminal domain (residues 899–1235, lane 3), the PTB and SAIN domains (residues 108–516, lane 4), the PH domain (residues 21–203, lane 5), the PH and PTB domains (residues 21–400, lane 6) and the Pre-C domain (residues 516–896, lane 7) of IRS-1, or GST alone as a control (lane 2), for 2 h at 4 °C. The GST fusion proteins and GST were precipitated and the associated FLAG-tagged Nck-2 was detected by Western blotting with a monoclonal anti-FLAG antibody (M5). Lane 1 was loaded with 10 μg of cell lysates. (**B**) Direct interactions between Nck-2 and IRS-1. Purified MBP-Nck-2 was incubated with equal amounts of GST (lane 2) or GST fusion proteins containing the domains listed for (**A**). The GST fusion proteins and GST were precipitated with glutathione–Sepharose beads and associated MBP-Nck-2 was detected by Western blotting with a monoclonal anti-Nck-2 antibody (mAB 8.8). Lane 1 was loaded with 5 ng of MBP-Nck-2.

DISCUSSION

IRS-1 is an important intracellular signalling protein downstream of receptors for insulin and other growth factors, hormones and cytokines. The primary function of IRS-1 in signal transduction is to mediate protein–protein interactions [1,2]. Recent studies showing that IRS-1 is capable of mediating signal transduction in the absence of tyrosine phosphorylation have led to a greater appreciation of the importance of tyrosine-phosphorylation-independent protein–protein interactions mediated by IRS-1 [3]. Several proteins, including simian virus 40 large T antigen [18], 14-3-3 [19], JAK2 [11] and Nck-2 [10], can associate with IRS-1 in a tyrosine-phosphorylation-independent manner. However, unlike the tyrosine-phosphorylation-dependent IRS-1 interactions, the molecular basis underlying the tyrosine-phosphorylation-independent IRS-1 interactions is much less well understood. This study was undertaken to determine whether the tyrosine-phosphorylation-independent Nck-2–IRS-1 interaction, which we recently detected in an *in vitro* binding assay [10], occurs *in vivo*, and furthermore, to dissect it to gain insight into the molecular basis underlying the tyrosine-phosphorylation-independent complex formation between IRS-1 and Nck-2. The results reported in this paper demonstrate that IRS-1 can indeed form a complex with Nck-2 *in vivo* in a tyrosine-phosphorylation-independent manner. Furthermore, our results show that at least two domains from each of the binding partners (the PTB/SAIN and the Pre-C domains of IRS-1, and the second and third SH3 domains of Nck-2) contribute to the association between IRS-1 and Nck-2. The involvement of multiple domains allows higher efficiency for the formation of the Nck-2–IRS-1 complex, as interactions mediated by a single SH3 domain are relatively weak (with dissociation constants typically in the range of 5–100 μM) [20]. The participation of multiple domains could also, in principle, provide a more versatile mechanism for cells to fine-tune the association between IRS-1 and Nck-2 during signal transduction.

Our results indicate that Nck-2 binding is an intrinsic activity of two separate IRS-1 domains (the PTB/SAIN and the Pre-C domains). Because substitution of the highly conserved tryptophan within the second or third SH3 domains of Nck-2 (residues 148 or 234) with a lysine significantly reduced the IRS-1 binding, the Nck-2-binding sites within the PTB/SAIN and Pre-C domains of IRS-1 most probably consist of proline-rich sequences. Indeed, there exist several potential SH3-recognition sites with consensus PXXP sequences in the PTB/SAIN and Pre-C domains of IRS-1. The recognition of the proline-rich sequences within the PTB/SAIN and Pre-C domains by the Nck-2 SH3 domains appears to be quite specific, as the C-terminal domain of IRS-1 also contains several proline-rich sequences but they are apparently incapable of interacting with the Nck-2 SH3 domains. The specificity of the interactions between IRS-1 and Nck-2 was further manifested by the fact that the SH3 domain of $p130^{Cas}$ or the first SH3 domain of Nck-2, unlike the second and third SH3 domains of Nck-2, does not interact with any of the IRS-1 domains. Additionally, our preliminary experiments suggest that Nck-2 does not form a complex with IRS-4 in mammalian cells (Y. Tu and C. Wu, unpublished work), indicating that the SH3-mediated interaction of Nck-2 with IRS-1 is highly specific.

We have recently demonstrated that residues 176–274 of Nck-2, which constitute the third SH3 domain, also contain a binding site for the LIM-only protein PINCH [10]. It is worth noting, however, that substitution of the highly conserved W (residue 234) within this region with K does not inhibit the Nck-2–PINCH interaction [10]. Thus although this region of Nck-2 is recognized by both IRS-1 and PINCH, these two binding sites are apparently distinct. Nck-2, through the interaction with PINCH, is capable of forming a complex with ILK [21]. ILK is an intracellular serine/threonine kinase initially identified based on an interaction with the β1 integrin cytoplasmic domain in a yeast two-hybrid screen [22]. ILK associates with PINCH (and consequently Nck-2) through the N-terminal ANK (ankyrin) repeat domain [21], which is likely to be involved in the regulation of ILK function [23,24]. Recent studies have shown that the kinase activity of ILK is regulated not only by cell adhesion to fibronectin (and thus the integrins) but also by insulin in a PI 3-kinase-dependent manner [25]. Moreover, ILK can directly phosphorylate PKB

(protein kinase B)/AKT at serine-473, one of the two phosphorylation sites involved in activation of PKB/AKT, and can regulate GSK-3 (glycogen synthase kinase 3) activity [25]. Thus ILK probably functions as an important downstream effector of insulin and PI 3-kinase signalling. Given the well-documented function of IRS-1 in insulin and PI 3-kinase signalling, it is attractive to hypothesize that the association of IRS-1 with Nck-2 is involved in regulating ILK activity in insulin signal transduction, possibly by facilitating the assembly of signalling complexes. Additionally, the association of IRS-1 with Nck-2 could potentially bring IRS-1 to the vicinity of Nck-2-associated serine/threonine kinases such as ILK and therefore modulate serine/threonine phosphorylation of IRS-1, a process implicated in attenuation of insulin signalling [26–36]. Clearly, information on the molecular basis underlying the interactions between IRS-1 and Nck-2 described in this paper should greatly facilitate future studies aimed at testing these hypotheses.

We thank Dr Thomas A. Gustafson (Metabolex, Hayward, CA, U.S.A.), Dr Jacalyn H. Pierce (National Cancer Institute, Bethesda, MD, U.S.A.) and Dr Jun-lin Guan (Cornell University, Ithaca, NY, U.S.A.) for kindly providing reagents. This work was supported by National Institutes of Health grants DK54639 (to C. W.), DK46395 (to S. J. F.), and T32 HL07631 (to L. L.), and a research project grant no. 98-220-01-CSM from the American Cancer Society (to C. W.).

REFERENCES

1 White, M. F. (1998) The IRS-signalling system: a network of docking proteins that mediate insulin action. Mol. Cell. Biochem. **182**, 3–11
2 Ogawa, W., Matozaki, T. and Kasuga, M. (1998) Role of binding proteins to IRS-1 in insulin signalling. Mol. Cell. Biochem. **182**, 13–22
3 Myers, Jr, M. G., Zhang, Y., Aldaz, G. A., Grammer, T., Glasheen, E. M., Yenush, L., Wang, L. M., Sun, X. J., Blenis, J., Pierce, J. H. and White, M. F. (1996) YMXM motifs and signaling by an insulin receptor substrate 1 molecule without tyrosine phosphorylation sites. Mol. Cell. Biol. **16**, 4147–4155
4 Voliovitch, H., Schindler, D. G., Hadari, Y. R., Taylor, S. I., Accili, D. and Zick, Y. (1995) Tyrosine phosphorylation of insulin receptor substrate-1 *in vivo* depends upon the presence of its pleckstrin homology region. J. Biol. Chem. **270**, 18083–18087
5 Yenush, L., Makati, K. J., Smith-Hall, J., Ishibashi, O., Myers, Jr, M. G. and White, M. F. (1996) The pleckstrin homology domain is the principal link between the insulin receptor and IRS-1. J. Biol. Chem. **271**, 24300–24306
6 Gustafson, T. A., He, W., Craparo, A., Schaub, C. D. and O'Neill, T. J. (1995) Phosphotyrosine-dependent interaction of SHC and insulin receptor substrate 1 with the NPEY motif of the insulin receptor via a novel non-SH2 domain. Mol. Cell. Biol. **15**, 2500–2508
7 Wolf, G., Trub, T., Ottinger, E., Groninga, L., Lynch, A., White, M. F., Miyazaki, M., Lee, J. and Shoelson, S. E. (1995) PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities. J. Biol. Chem. **270**, 27407–27410
8 Chen, M., She, H., Davis, E. M., Spicer, C. M., Kim, L., Ren, R., Le Beau, M. M. and Li, W. (1998) Identification of Nck family genes, chromosomal localization, expression, and signaling specificity. J. Biol. Chem. **273**, 25171–25178
9 Braverman, L. E. and Quilliam, L. A. (1999) Identification of Grb4/Nckbeta, a src homology 2 and 3 domain-containing adapter protein having similar binding and biological properties to Nck. J. Biol. Chem. **274**, 5542–5549
10 Tu, Y., Li, F. and Wu, C. (1998) Nck-2, a novel Src homology 2/3-containing adaptor protein that interacts with the LIM-only protein PINCH and components of growth factor receptor kinase signaling pathways. Mol. Biol. Cell **9**, 3367–3382
11 Liang, L., Zhou, T., Jiang, J., Pierce, J. H., Gustafson, T. A. and Frank, S. J. (1999) Insulin receptor substrate-1 enhances growth hormone-induced proliferation. Endocrinology **140**, 1972–1983
12 Kim, S. O., Jiang, J., Yi, W., Feng, G. S. and Frank, S. J. (1998) Involvement of the Src homology 2-containing tyrosine phosphatase SHP-2 in growth hormone signaling. J. Biol. Chem. **273**, 2344–2354
13 Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J. and White, M. F. (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature (London) **352**, 73–77
14 Myers, Jr, M. G., Grammer, T. C., Brooks, J., Glasheen, E. M., Wang, L. M., Sun, X. J., Blenis, J., Pierce, J. H. and White, M. F. (1995) The pleckstrin homology domain in insulin receptor substrate-1 sensitizes insulin signaling. J. Biol. Chem. **270**, 11715–11718
15 Tanaka, M., Gupta, R. and Mayer, B. J. (1995) Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. Mol. Cell. Biol. **15**, 6829–6837
16 Tanaka, M., Lu, W., Gupta, R. and Mayer, B. J. (1997) Expression of mutated Nck SH2/SH3 adaptor respecifies mesodermal cell fate in *Xenopus laevis* development. Proc. Natl. Acad. Sci. U.S.A. **94**, 4493–4498
17 Rao, Y. and Zipursky, S. L. (1998) Domain requirements for the Dock adapter protein in growth-cone signaling. Proc. Natl. Acad. Sci. U.S.A. **95**, 2077–2082
18 Fei, Z. L., D'Ambrosio, C., Li, S., Surmacz, E. and Baserga, R. (1995) Association of insulin receptor substrate 1 with simian virus 40 large T antigen. Mol. Cell. Biol. **15**, 4232–4239
19 Craparo, A., Freund, R. and Gustafson, T. A. (1997) 14-13-3 (epsilon) interacts with the insulin-like growth factor I receptor and insulin receptor substrate I in a phosphoserine-dependent manner. J. Biol. Chem. **272**, 11663–11669
20 Chen, J. K., Lane, W. S., Brauer, A. W., Tanaka, A. and Schreiber, S. L. (1993) Biased combinatorial libraries – novel ligands for the SH3 domain of phosphatidylinositol 3-kinase. J. Am. Chem. Soc. **115**, 12591–12592
21 Tu, Y., Li, F., Goicoechea, S. and Wu, C. (1999) The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. Mol. Cell. Biol. **19**, 2425–2434
22 Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C. and Dedhar, S. (1996) Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature (London) **379**, 91–96
23 Li, F., Zhang, Y. and Wu, C. (1999) Integrin-linked kinase is localized to cell-matrix focal adhesions but not cell-cell adhesion sites and the focal adhesion localization of integrin-linked kinase is regulated by the PINCH-binding ANK repeats. J. Cell Sci. **112**, 4589–4599
24 Wu, C. (1999) Integrin-linked kinase and PINCH: partners in regulation of cell-extracellular matrix interaction and signal transduction. J. Cell Sci. **112**, 4485–4489
25 Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J. and Dedhar, S. (1998) Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc. Natl. Acad. Sci. U.S.A. **95**, 11211–11216
26 Folli, F., Saad, M. J., Velloso, L., Hansen, H., Carandente, O., Feener, E. P. and Kahn, C. R. (1999) Crosstalk between insulin and angiotensin II signalling systems. Exp. Clin. Endocrinol. Diabetes **107**, 133–139
27 Paz, K., Liu, Y. F., Shorer, H., Hemi, R., LeRoith, D., Quan, M., Kanety, H., Seger, R. and Zick, Y. (1999) Phosphorylation of insulin receptor substrate-1 (IRS-1) by protein kinase B positively regulates IRS-1 function. J. Biol. Chem. **274**, 28816–28822
28 Cengel, K. A. and Freund, G. G. (1999) JAK1-dependent phosphorylation of insulin receptor substrate-1 (IRS-1) is inhibited by IRS-1 serine phosphorylation. J. Biol. Chem. **274**, 27969–27974
29 Qiao, L. Y., Goldberg, J. L., Russell, J. C. and Sun, X. J. (1999) Identification of enhanced serine kinase activity in insulin resistance. J. Biol. Chem. **274**, 10625–10632
30 De Fea, K. and Roth, R. A. (1997) Protein kinase C modulation of insulin receptor substrate-1 tyrosine phosphorylation requires serine 612. Biochemistry **36**, 12939–12947
31 Eldar-Finkelman, H. and Krebs, E. G. (1997) Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. Proc. Natl. Acad. Sci. U.S.A. **94**, 9660–9664
32 Peraldi, P., Hotamisligil, G. S., Buurman, W. A., White, M. F. and Spiegelman, B. M. (1996) Tumor necrosis factor (TNF)-alpha inhibits insulin signaling through stimulation of the p55 TNF receptor and activation of sphingomyelinase. J. Biol. Chem. **271**, 13018–13022
33 Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F. and Spiegelman, B. M. (1996) IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science **271**, 665–668
34 Kanety, H., Feinstein, R., Papa, M. Z., Hemi, R. and Karasik, A. (1995) Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. J. Biol. Chem. **270**, 23780–23784
35 Laakso, M., Malkki, M., Kekalainen, P., Kuusisto, J. and Deeb, S. S. (1994) Insulin receptor substrate-1 variants in non-insulin-dependent diabetes. J. Clin. Invest. **94**, 1141–1146
36 Tanti, J. F., Gremeaux, T., van Obberghen, E. and Le Marchand-Brustel, Y. (1994) Serine/threonine phosphorylation of insulin receptor substrate 1 modulates insulin receptor signaling. J. Biol. Chem. **269**, 6051–6057

Received 22 June 2000/20 November 2000; accepted 15 December 2000