Nck associates with the SH2 domain-docking protein IRS-1 in insulin-stimulated cells

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ABSTRACT  Nck, an oncogenic protein composed of one SH2 and three SH3 domains, is a common target for various cell surface receptors. Nck is thought to function as an adaptor protein to couple cell surface receptors to downstream effector molecules that regulate cellular responses induced by receptor activation. In this report, we show that Nck forms a stable complex in vivo with IRS-1 in insulin-stimulated cells. The interaction between IRS-1 and Nck is mediated by the binding of the SH2 domain of Nck to tyrosine-phosphorylated IRS-1. Although Nck associates with IRS-1, Nck phosphorylation is not affected by insulin stimulation. Furthermore, in vitro and in vivo studies show that the SH2 domains of Nck, GRB2, and p85 bind distinct phosphotyrosine residues in IRS-1. After insulin stimulation all three signaling molecules can be found complexed to a single IRS-1 molecule. These findings provide further evidence that, in response to insulin stimulation, IRS-1 acts as an SH2 docking protein that coordinates the regulation of various different signaling pathways activated by the insulin receptor.

Many growth factors mediate their effects, such as cell growth and differentiation, by interacting with transmembrane receptors that contain tyrosine kinase activity. A critical early event following ligand binding is activation of the tyrosine kinase catalytic domain (1-3). The receptor itself is one of the major intracellular targets for the tyrosine kinase activity and becomes autophosphorylated. One function of receptor autophosphorylation is to up-regulate the activity of the tyrosine kinase catalytic domain (4, 5). However, for most tyrosine kinase receptors, autophosphorylation functions to create high-affinity binding sites for signaling molecules that contain src homology (SH) 2 domains (1-3). SH2 domains are modular, noncatalytic domains of about 100 amino acids that are found in various distinct signaling molecules and function by binding a phosphotyrosine moiety in the context of short amino acid sequences (1, 6, 7). Since target molecules must bind to activated receptors to activate signaling pathways, autophosphorylation of specific tyrosine residues on a given receptor determines which signaling pathways are recruited by the receptor.

Although ligand binding to the insulin receptor activates the receptor tyrosine kinase and leads to receptor autophosphorylation, the insulin receptor interacts poorly, if at all, with known SH2 domain-containing target proteins (6, 8). Instead, the insulin receptor utilizes an alternative strategy to activate signaling molecules that contain SH2 domains. Insulin or IGF-1 stimulation results in tyrosine phosphorylation of a 185-kDa intermediate protein, insulin receptor substrate 1 (IRS-1), which functions as a docking protein for SH2 domain-containing signaling molecules. Tyrosine-phosphorylated IRS-1 binds the SH2 domains of p85-associated phosphatidylinositol 3'-kinase (PI-3 kinase), resulting in PI-3 kinase activation (9-11). Recently, it was shown that GRB2 couples the Ras guanine nucleotide releasing factor (GRF), Sos, to IRS-1 (12-14). The finding that GRB2 overexpression enhances insulin-induced activation of Ras and Map kinases suggests that binding of GRB2 to IRS-1 may directly link the insulin receptor to Ras signaling pathways (13).

We wanted to test whether IRS-1 interacts with other SH2 domain-containing signaling proteins and thereby provides additional links between the insulin receptor and other signaling pathways. One candidate protein is Nck, a ubiquitously expressed protein composed of one SH2 and three SH3 domains (15). Several recent findings have implicated Nck as a potentially important signaling molecule. First, Nck is an oncoprotein; cellular overexpression of Nck transforms NIH 3T3 and 3Y1 rat fibroblasts (16, 17). In addition, Nck is phosphorylated in response to activation of various cytoplasmic and receptor tyrosine kinases and Nck has been shown to bind, via its SH2 domain, the activated platelet-derived growth factor and epidermal growth factor receptors (16-19). It is thought that Nck might regulate signaling pathways by functioning as an adaptor protein, coupling the activated receptor to an as yet-to-be defined catalytic molecule whose activity is modulated by binding of Nck to the activated receptor.

In this report, we show that insulin stimulation results in the formation of a stable complex between tyrosine-phosphorylated IRS-1 and the SH2 domain of Nck. Although Nck binds IRS-1 in insulin-stimulated cells, Nck is not a substrate either for the insulin receptor or for the kinases activated downstream from the insulin receptor. Moreover, we show that IRS-1 binds Nck through a different phosphotyrosine-containing motif than that required for binding p85 and GRB2 and that after insulin stimulation a single IRS-1 molecule can simultaneously bind Nck, GRB2, and p85 in vivo. These findings support the idea that IRS-1 forms the core for a large signaling complex through which the insulin receptor regulates various distinct signaling pathways.

MATERIALS AND METHODS

Cell Lines and Antibodies. CHO/IR cells are Chinese hamster ovary cells that overexpress the insulin receptor (10). Cells were grown in F12 medium supplemented with 10% fetal bovine serum. Rabbit polyclonal anti-Nck antibody was raised against a full-length Nck-glutathione S-transferase (GST) fusion protein (16). IRS-1 antibodies (PEP 80), anti-GRB2 antibodies (86 and 50), and anti-p85 and anti-phosphotyrosine antibodies have been described (12).

Immunoprecipitation and Immunoblotting. After overnight starvation in F12 medium containing 0.5% bovine serum albumin, CHO/IR cells were stimulated for 2 or 5 min with

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100 nM bovine insulin. Cell lysis, immunoprecipitation, and immunoblotting were performed as described (20).

**IRS-1 Produced in Insect Cells.** Baculovirus-expressed IRS-1 was collected from SF9 cells and purified as described (10, 11). Purified IRS-1bac was then phosphorylated with [γ-32P]ATP using wheat germ agglutinin purified insulin receptor (10, 11).

**Peptide Synthesis and Purification.** Peptides were prepared by conventional methods using a Milligen/Biosearch 9600 synthesizer (21). Phosphopeptides were prepared using FmocTyr(OP(OCH2)2). All peptides were purified by HPLC and subjected to amino acid analysis.

**GST Fusion Proteins and in Vitro Binding Assays.** Oligonucleotides flanking the regions of interest and containing appropriate restriction sites were synthesized and PCR was used to amplify the appropriate DNA fragments (12). The amplified products were cloned into pGEX3X and the fusion proteins were purified by affinity chromatography using immobilized glutathione agarose beads. The following fusion proteins were produced: full-length Nck, the SH2 domain of Nck, the N and C SH2 domains of p85, and full-length GRB2.

To assess the binding of IRS-1 to GST fusion proteins in vitro, 0.5 μg of GST fusion protein was immobilized on glutathione agarose beads and incubated with 32P-labeled IRS-1bac in buffer containing 50 mM Tris-HCl (pH 7.8), 250 mM NaCl, 10 mM dithiothreitol, and 0.5% Triton X-100 (14). After a 30-min incubation at 4°C, the beads were washed with HNTG (20 mM Hepes, pH 7.5/10% glycerol/0.1% Triton X-100/150 mM NaCl), and after boiling in sample buffer, the proteins were separated on an 8% SDS/polyacrylamide gel. Bound IRS-1 was visualized by autoradiography.

Peptide inhibition studies were done in an identical manner with the exception that 100 nM of Nck protein was preincubated with peptides for 30 min at 4°C prior to the addition of labeled IRS-1. The amount of bound IRS-1 was quantitated using a PhosphoImager, with 100% binding defined as the amount of IRS-1 bound in the absence of competing peptide.

**PI-3 Kinase Assay.** PI-3 kinase was assayed from immunoprecipitated proteins as described (22).

**Metabolic Labelling of Cells and Phosphoamino Acid Analysis.** Serum-starved CHO/IR cells were labeled with 32P as described (16). After washing the cells three times with phosphate-free medium, the cells were labeled for 3 hr in medium containing 32P at 1 μCl/ml (1 Ci = 37 GBq). Labeled cells were then stimulated with 100 nM insulin for 10 min at room temperature, lysed, and immunoprecipitated with antibodies to Nck. The immunoprecipitated proteins were separated on a 10% SDS gel and phosphorylated Nck and IRS-1 were excised from the gel and subjected to phosphoamino acid analysis (16).

**RESULTS**

**Nck Associates with IRS-1 in Vivo.** To determine whether Nck associates with IRS-1 in cells, CHO/IR cells were stimulated with insulin, and the association of IRS-1 with Nck was assessed by determining whether the two proteins coimmunoprecipitated. In unstimulated cells, there is neither detectable tyrosine phosphorylation of IRS-1 nor association between IRS-1 and Nck (Fig. 1, lanes 2, 6, and 8). However, upon growth factor stimulation, IRS-1 becomes tyrosine phosphorylated (Fig. 1, lane 9) and Nck immunoprecipitates contain tyrosine-phosphorylated IRS-1 (Fig. 1, lanes 3 and 7).

**Nck Associates with IRS-1 Through its SH2 Domain.** Previous studies have shown that SH2 domains mediate the binding of signaling molecules, such as p85 and GRB2, to IRS-1 (9–12). To confirm that the SH2 domain of Nck is also responsible for the association between Nck and IRS-1, bacterial fusion proteins containing full-length Nck as well as

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**Fig. 1.** Association of Nck with IRS-1 in cells. CHO/IR cells were unstimulated or stimulated with 100 nM insulin for 2 min, lysed, and immunoprecipitated with antibodies to Nck. The immunoprecipitated proteins were separated by SDS/PAGE (10%), transferred to nitrocellulose, and immunoblotted with antibodies as indicated. One-tenth of the Nck immunoprecipitate (ip) was immunoblotted with antibodies to Nck (lanes 4 and 5). The remainder of the Nck immunoprecipitate was divided in two and immunoblotted with either anti-IRS-1 antibodies (lanes 2 and 3) or anti-phosphotyrosine antibodies (lanes 6 and 7). Lane 1 is a control for IRS-1 and contains purified IRS-1bac. Molecular masses are indicated in kDa.

**Fig. 2.** In vitro binding of IRS-1 to Nck-GST fusion proteins. Various GST fusion proteins were incubated with 32P-labeled IRS-1bac for 1 hr at 4°C. After washing, proteins were separated by SDS/PAGE (8%) and bound IRS-1 was visualized by autoradiography. The GST fusion proteins used are GST alone (GST), full-length Nck (GST-Nck), the SH2 domain of Nck (GST-Nck SH2), the three SH3 domains of Nck (GST-Nck SH3), and full-length GRB2 (GST-GRB2).
stimulation results in phosphorylation of Nck, Nck was immunoprecipitated from orthophosphate-labeled cell lysates. Phosphorylation of Nck was determined by SDS/PAGE followed by autoradiography. We were unable to detect a change in Nck phosphorylation after insulin stimulation (Fig. 3, lanes 3 and 4). Phosphoamino acid analysis of Nck from control and insulin-stimulated cells demonstrated that Nck was phosphorylated predominantly on serine residues, although a very small amount of tyrosine phosphorylation was also detected (Fig. 3, panels 2 and 3). There was, however, no detectable change in Nck phosphorylation after insulin stimulation. The failure to detect an increase in Nck phosphorylation was not secondary to poor stimulation of the cells with insulin; phosphorylated IRS-1 coimmunoprecipitated with Nck in the stimulated cell lysates. Corroborating these findings, we were unable to detect tyrosine-phosphorylated Nck before or after insulin stimulation, using antibodies to phosphotyrosine (Fig. 1, lanes 6 and 7). These results suggest that the regulation of Nck by insulin is not mediated by phosphorylation of Nck.

IRS-1 Simultaneously Binds Several SH2 Domain-Containing Proteins. We have recently demonstrated that a single IRS-1 molecule can bind the SH2 domains of p85 and GRB2 and that the SH2 domains of each molecule recognize distinct phosphotyrosines on IRS-1 (12). To investigate whether IRS-1 can simultaneously bind Nck, GRB2, and p85, we assessed whether GRB2, p85, and PI-3 kinase activity coimmunoprecipitates, using antibodies to Nck. Insulin stimulation resulted in a marked increase in the amount of GRB2, p85, and PI3 kinase activity that coimmunoprecipitated with antibodies to Nck (Fig. 4). The interaction between Nck, p85, and GRB2 appears to be mediated by the binding of the SH2 domains of each molecule to tyrosine-phosphorylated IRS-1.

Nck Binds a Phosphotyrosine Residue on IRS-1 That Is Distinct from Those Bound by p85 and GRB2. The finding that IRS-1 can simultaneously interact with GRB2, p85, and Nck suggests that the SH2 domain of Nck recognizes a phosphotyrosine residue on IRS-1 different from those recognized by the SH2 domains of p85 and GRB2. We have previously shown that the SH2 domain of GRB2 binds tyrosine 895 of IRS-1 through a YVNI motif and that the SH2 domains of p85 bind IRS-1 through several YMXM and YXXM motifs (10, 12). To determine the binding site of Nck to IRS-1, phosphopeptides corresponding to known tyrosine phosphorylation sites on IRS-1 were synthesized and the ability of these phosphopeptides to inhibit the binding of IRS-1 to Nck was assessed. In designing peptides for competition studies, we used the information, obtained using degenerate phosphopeptide libraries, that the SH2 domain of Nck binds a synthetic phosphopeptide containing the sequence pYDEP (23). The SH2 domain of Nck most frequently selected an aspartic acid residue at a position +1 from the phosphotyrosine. Since rat IRS-1 contains only a single tyrosine residue (Y147), followed by an aspartic acid, we determined whether a phosphopeptide corresponding to the sequence around Y147 inhibited the binding of IRS-1 to Nck. Fig. 5A shows

![Fig. 3](image-url)  
**Fig. 3.** Phosphorylation of Nck is unchanged after insulin stimulation. (A) After labeling CHO/IR cells with $^{32}P_i$, the cells were either unstimulated or stimulated with 100 nM insulin, and Nck was immunoprecipitated from the labeled cell lysates. Nck immunoprecipitates were analyzed by SDS/PAGE (10%) and autoradiography (lanes 3 and 4). As a control, unlabelled cell lysates were electrophoresed on the same SDS gel as the labeled cell lysate, transferred to nitrocellulose, and immunoblotted with antibodies to phosphotyrosine (lanes 1 and 2). Molecular masses are indicated in kDa. (B) $^{32}P_i$-labeled bands corresponding to IRS-1 and Nck were excised from the gel and subjected to phosphoamino acid analysis. Phosphoamino acid analysis of IRS-1 (panel 1) and Nck from unstimulated (panel 2) and insulin-stimulated (panel 3) cells is shown. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

![Fig. 4](image-url)  
**Fig. 4.** IRS-1 simultaneously binds Nck, GRB2, and p85 in insulin-stimulated cells. CHO/IR cells were either unstimulated or stimulated with 100 nM insulin for five min and lysed, and cell lysates were immunoprecipitated with antibodies (IP Ab) to Nck, p85, or GRB2. The immunoprecipitated proteins were separated by SDS/PAGE (10%) and immunoblotted with antibodies to p85 (A) or GRB2 (B). (C) Anti-Nck immunoprecipitates were subjected to a PI-3 kinase assay. PIP, phosphatidylinositol phosphate.
that a phosphopeptide corresponding to the sequence around tyrosine 147 of rat IRS-1 consistently inhibited the binding of IRS-1 to Nck by >60%. This inhibition was specific; binding of IRS-1 to Nck was not inhibited by either a phosphopeptide corresponding to the binding site of GRB2 to IRS-1 (pY895) or a phosphopeptide (pY727) that has been shown to inhibit the binding of p85 to IRS-1 (Fig. 5 A and C). In addition, various other phosphopeptides corresponding to known tyrosine phosphorylation sites in IRS-1 did not inhibit Nck binding to IRS-1 (Fig. 5C).

The pY147 peptide specifically inhibits the binding of Nck to IRS-1. Under conditions in which the binding of IRS-1 to Nck was inhibited, the pY147 peptide did not competitively inhibit the binding of IRS-1 to fusion proteins containing either the p85 or the GRB2 SH2 domains (Fig. 5B). However, phosphopeptides corresponding to binding sites on IRS-1 for GRB2 (pY895) and p85 (pY727) inhibited the binding of IRS-1 to GRB2 and p85, respectively.

**DISCUSSION**

Recent investigations indicate that SH2 domain-containing proteins without catalytic activity, such as GRB2 and p85, regulate signal transduction pathways (1–3, 6, 10, 13, 24–28). GRB2 and p85 are tightly associated with catalytic molecules and act as adaptors linking these molecules to phosphotyrosine-containing proteins. The interaction of the adaptor proteins either with the autophosphorylated receptor or with intermediate phosphotyrosine-containing proteins, such as IRS-1, has been shown to have important regulatory consequences (1–3). Based on these findings, a great effort has been undertaken to identify proteins with SH2 domains that respond to growth factor stimulation by forming stable complexes with phosphotyrosine-containing molecules. One protein of interest, Nck, is similar to GRB2 in that it is composed virtually entirely of SH2 and SH3 domains. In this report we demonstrate that Nck, via its SH2 domain, forms a stable complex with tyrosine-phosphorylated IRS-1 in insulin-stimulated cells.

The importance of IRS-1 in regulating signaling pathways that are stimulated by the insulin and IGF-1 receptors has been established by indirect and direct evidence. Mutations in the juxtamembrane region of the insulin receptor that eliminate tyrosine 960 result in a mutant unable to tyrosine phosphorylate IRS-1 (29). Although the tyrosine kinase is active, these mutated receptors are unable to stimulate DNA or glycogen synthesis (29, 30). More recently, IRS-1 has been shown to directly regulate PI-3 kinase; binding of p85 to IRS-1 leads to activation of the 110-kDa PI-3 kinase catalytic domain (10). We have recently shown that tyrosine-phosphorylated IRS-1 also binds GRB2 and that this interaction couples the Ras GRF Sos, bound to GRB2’s SH3 domains, to IRS-1 (12, 13). The finding that GRB2 overexpression potentiates the activation of Ras signaling pathways by insulin suggests that binding of GRB2 to IRS-1 may function to activate Sos (13).

It is envisioned that the binding of Nck to IRS-1 may regulate an as-yet-to-be-defined signaling pathway. The finding that cellular overexpression of Nck is transforming implies that Nck can activate mitogenic signaling pathways (16, 17). However, in contrast to p85 and GRB2, for which the catalytic component is known, the catalytic component that associates with Nck is unknown and thus Nck’s role in growth factor signaling remains unclear. Recently it has been shown that SH3 domains regulate protein–protein interactions by binding short proline-rich motifs present in effector molecules (31–34). The signaling pathways regulated by adaptor proteins are most likely to be defined by the identity of the specific catalytic molecule(s) coupled to each SH3 domain. For example, the SH3 domains of GRB2 bind a proline-rich sequence present in the effector molecule Sos (33, 34), and, as predicted, GRB2 activates Ras in vivo (13, 25, 28). The SH3 domains of Nck are likely to bind a putative effector protein that is coupled to IRS-1 and other tyrosine-phosphorylated proteins through Nck’s SH2 domain.

Though it is of particular interest to identify the effector molecules that interact with Nck, it will also be important to determine how the activities of these molecules are regulated by the interaction of Nck with IRS-1. Previous studies have shown that Nck is a target for various tyrosine and serine/threonine kinases (16–19). However, we did not detect a change in Nck phosphorylation in response to insulin stimulation. Interestingly, p85 and GRB2 also fail to undergo tyrosine phosphorylation after insulin stimulation (10, 12). Though the tyrosine 960 result in a mutant insusceptible to the action of these adaptor molecules by phosphorylation; rather, it is likely that the interaction between Nck and IRS-1 is analogous to the regulation of PI-3 kinase by p85 and that binding of Nck to IRS-1 alone may modulate the activity of downstream effector molecules. Alternatively, binding of Nck to IRS-1 may serve to relocalize an effector molecule within the cell to a site where it can act. For example, the interaction of GRB2 with the activated epidermal growth factor receptor or
with IRS-1 in insulin-stimulated cells may function to reposition Sos adjacent to Ras, in the plasma membrane (13, 35).

Since the creation of specific SH2 binding sites by a receptor tyrosine kinase determines which signaling pathways are activated by a given receptor (1–3, 6, 7), the identification of SH2 binding motifs is critical to our understanding of signaling specificity. We have found that the SH2 domain of Nck recognizes a binding motif on IRS-1 that is clearly distinguishable from the motif recognized by p85 and GRB2 (12). This is consistent with our observation that a single IRS-1 molecule can simultaneously interact with p85, GRB2, and Nck. Our competition experiments indicate that Y147 of rat IRS-1 represents at least one potential high-affinity binding site for Nck to IRS-1. Tyrosine 147 of rat IRS-1 is followed by an aspartic acid in the +1 position; this aspartic acid was the most frequently selected residue in experiments using a phosphopeptide library to identify sequence-specific high-affinity binding motifs for the SH2 domain of Nck, suggesting that an aspartic acid at the +1 position is important for high-affinity binding of the SH2 domain of Nck to phosphotyrosine. However, it is unlikely that this motif is the only one to interact with the SH2 domain of Nck. This motif is not conserved in human IRS-1, yet Nck binds tyrosine-phosphorylated IRS-1 with higher affinity in vitro (M.G.M. and M.F.W., unpublished observation). Furthermore, Nck has been shown to bind a very different sequence in the platelet-derived growth factor receptor (36); this sequence is not found in rat or human IRS-1.

The ability of a single SH2 domain to bind distinct motifs has been reported previously (37). The SH2 domain of Src binds to the tyrosine-phosphorylated juxtamembrane region of the platelet-derived growth factor receptor and also to a sequence in the C terminus of Src; these two sequences are only weakly similar (37). Screening of degenerate phosphopeptide libraries identified yet another sequence, pYEEL, that bound Src’s SH2 domain (23), and crystallographic analysis revealed that all three amino acids after the phosphotyrosine make specific contacts with the SH2 domain of Src (38). Although the SH2 domain of Src may bind different motifs with different affinities (23), further clarification of the structural basis for the interaction of an SH2 domain with many different motifs will require determining the structure, by x-ray crystallography, of the SH2 domain complexed with the different tyrosine-phosphorylated peptides.

Our finding that IRS-1 binds Nck reinforces the concept that, in contrast with most receptor tyrosine kinases, which interact directly with SH2 domain-containing signaling molecules, the insulin and IGF-1 receptors employ IRS-1 as an intermediate SH2 domain-docking protein. Recent studies have shown that Nck is a target for various cell surface receptors. Our demonstration that Nck is also recruited to the insulin signaling pathway supports the idea that Nck is an essential component of a common pathway used by many receptors. Identifying the activity that Nck couples to IRS-1 is likely to lead to the elucidation of the exact functions regulated by Nck in insulin-stimulated cells.

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