# The IRS-2 Gene on Murine Chromosome 8 Encodes a Unique Signaling Adapter for Insulin and Cytokine Action

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**Signal transduction by insulin and IGF-1, several interleukins (IL-2, IL-4, IL-9, IL-13), interferons, GH, and other cytokines involves IRS proteins, which link the receptors for these factors to signaling molecules with Src homology-2 domains (SH2-proteins). We recently reported the amino acid sequence of murine IRS-2; in order to examine a potential genetic role for this molecule in disease, we isolated the murine IRS-2 gene and compared the expression pattern of IRS-2 against IRS-1. Like IRS-1, IRS-2 is encoded by a single exon. Whereas IRS-1 is located on murine chomosome 1, IRS-2 is located on murine chromosome 8 near the insulin receptor. IRS-2 is expressed together with IRS-1 in many cells and tissues; however, IRS-2 predominates in murine hematopoietic cells where it may be essential for cytokine signaling; IRS-1 predominates in adipocytes and differentiated 3T3-L1 cells where it contributes to the normal insulin response. In 32D cells, IRS-1 and IRS-2 undergo differential tyrosine phosphorylation during insulin or IL-4 stimulation, as assessed indirectly by interaction with various recombinant SH2 domains. Thus, signaling specificity through the IRS proteins may be accomplished by specific expression patterns and distinct phosphorylation patterns during interaction with various activated receptors. (Molecular Endocrinology 11: 251–262, 1997)**

0888-8809/97/$3.00/0
Molecular Endocrinology


## INTRODUCTION

The identification of downstream elements controlling cellular growth and metabolism is a central issue in contemporary biology. Common themes are emerging to explain how these processes are linked to growth factor, hormone, or cytokine receptors with intrinsic or associated tyrosine kinase activity (1, 2). Ligand-induced receptor dimerization stimulates tyrosine autophosphorylation of growth factor receptors or various components in cytokine receptor complexes. In many cases, these phosphorylation sites selectively bind to the Src homology-2 (SH2) domain in the various enzymes or adapter molecules that mediate biological responses (2–5).

In a few cases, notably insulin and insulin-like growth factor 1 (IGF-1), receptor autophosphorylation correlates closely with increased kinase activity but poorly with the recruitment of most SH2-proteins to the receptor (6, 7). In contrast, tyrosine phosphorylation of the IRS proteins (IRS-1 and IRS-2) provides an interface between these receptors and various SH2-proteins (8, 9). IRS proteins are also phosphorylated by Jak kinases, which are activated by the receptors for various cytokines, including interleukins (IL-2, IL-4,

3IL-9, IL-13), interferons (IFN$\alpha$, IFN$\beta$ and IFN$\gamma$), and GH. During insulin stimulation the phosphorylation of multiple tyrosine residues in the COOH terminus of IRS-1 enables the binding of several SH2 proteins, including the PI-3 kinase-regulatory subunits (p85$\alpha$/p85$\beta$/p55$^{PIK}$), GRB-2, nck, c-*fyn*, and SHPTP2 (10–14). As a consequence of these and other interactions, IRS-1 mediates multiple downstream signals, including the direct activation of PI-3 kinase and SHPTP2, the indirect stimulation of mitogen-activated protein kinase and p70$^{s6k}$, and other events that regulate gene expression and stimulate protein synthesis, mitogenesis, and glucose transport (6, 15–20).

IRS-2 was difficult to identify using several standard approaches, including expression screening, RT-PCR, and low stringency cDNA or genomic screening. However, purification of 4PS, an insulin/IL-4 receptor substrate in FDC-P2 cells, allowed the cloning of a cDNA that encodes a protein with several structural and functional features in common with IRS-1 (21). IRS-2 appears to be especially important in mice lacking IRS-1, as IRS1$^{(-/-)}$ mice survive, reproduce, and display only mild insulin resistance (22, 23). Hepatocytes from these animals reveal increased tyrosine phosphorylation of IRS-2 and retain a significant responsiveness to insulin and IGF-1 (22, 23). However, muscle from the IRS1$^{(-/-)}$ mouse is significantly insulin resistant and does not display a compensatory increase in IRS-2 phosphorylation (24). Thus, the distinct expression of IRS-1 and IRS-2 may further contribute to their unique signaling potential and importance for survival.

In this paper we characterize and analyze the murine IRS-2 gene and investigate the potential for alternate signaling by IRS-1 and IRS-2 in different cell types and in response to different upstream signals. The IRS-2 gene is located on murine chromosome 8 in a location close to the insulin receptor; the coding region is contained in a single exon. Although IRS-2 possesses similar structural features to IRS-1, the tyrosine phosphorylation of IRS-1 and IRS-2 by insulin and IL-4 is qualitatively different.

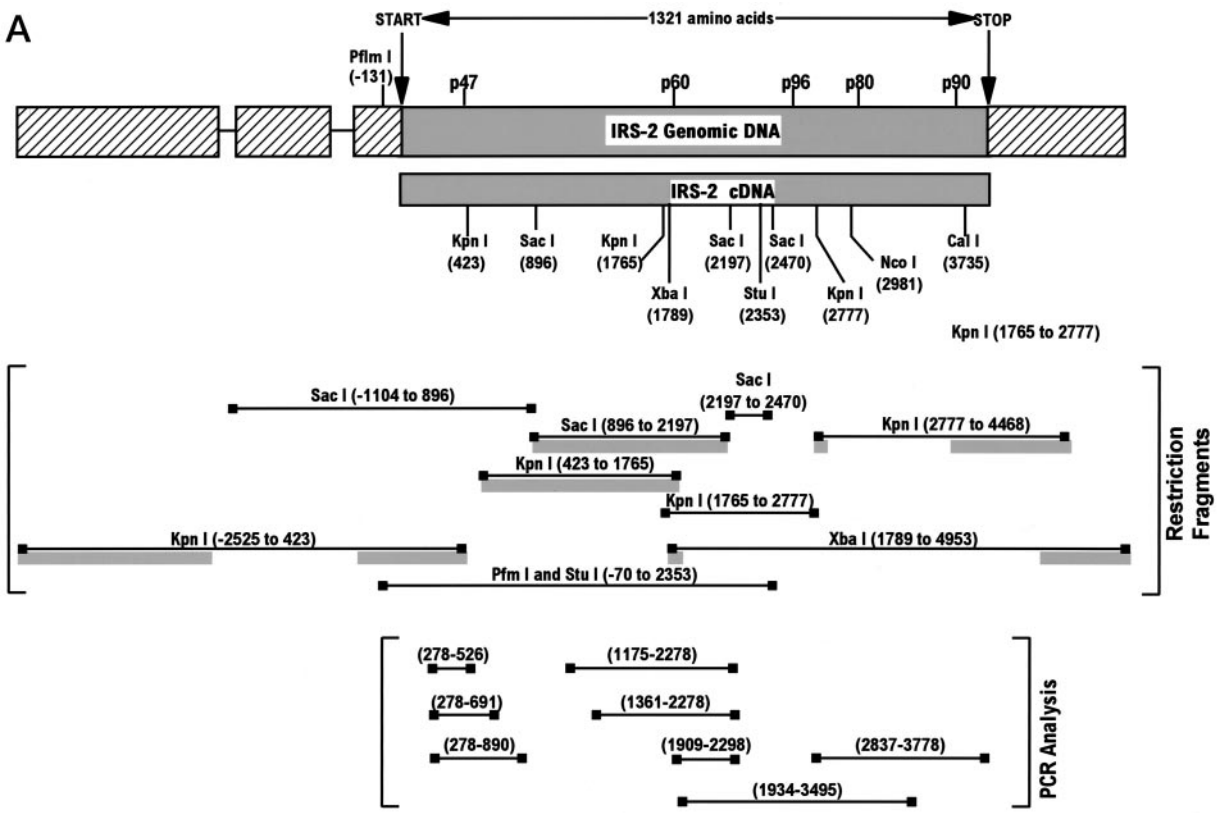


**Fig. 1.** The Molecular Cloning of IRS-2 Genomic DNA

Panel A; IRS-2 genomic DNA was obtained by screening the mouse genomic library with IRS-2 cDNA. The *solid black bars* indicate the restriction fragments hybridized with IRS-2 cDNA and PCR products. *Shaded bars* indicate the regions that have been sequenced. Start and stop codons and the location of 4PS peptides are indicated. As usual, positive numbers begin at the start codon and negative numbers indicate the sequence before the start codon into the 5′-untranslated region. Panel B; Genomic DNA and deduced protein sequence of mouse IRS-2. The putative 145-kDa open reading frame of IRS-2 is shown beginning with a Kozac start site and ending at an in-frame stop codon (*). The IRS-homology domains (IH1$^{PH}$ and IH2$^{PTB}$) are *shaded*; potential tyrosine phosphorylation sites are *highlighted as white characters on black* backgrounds.

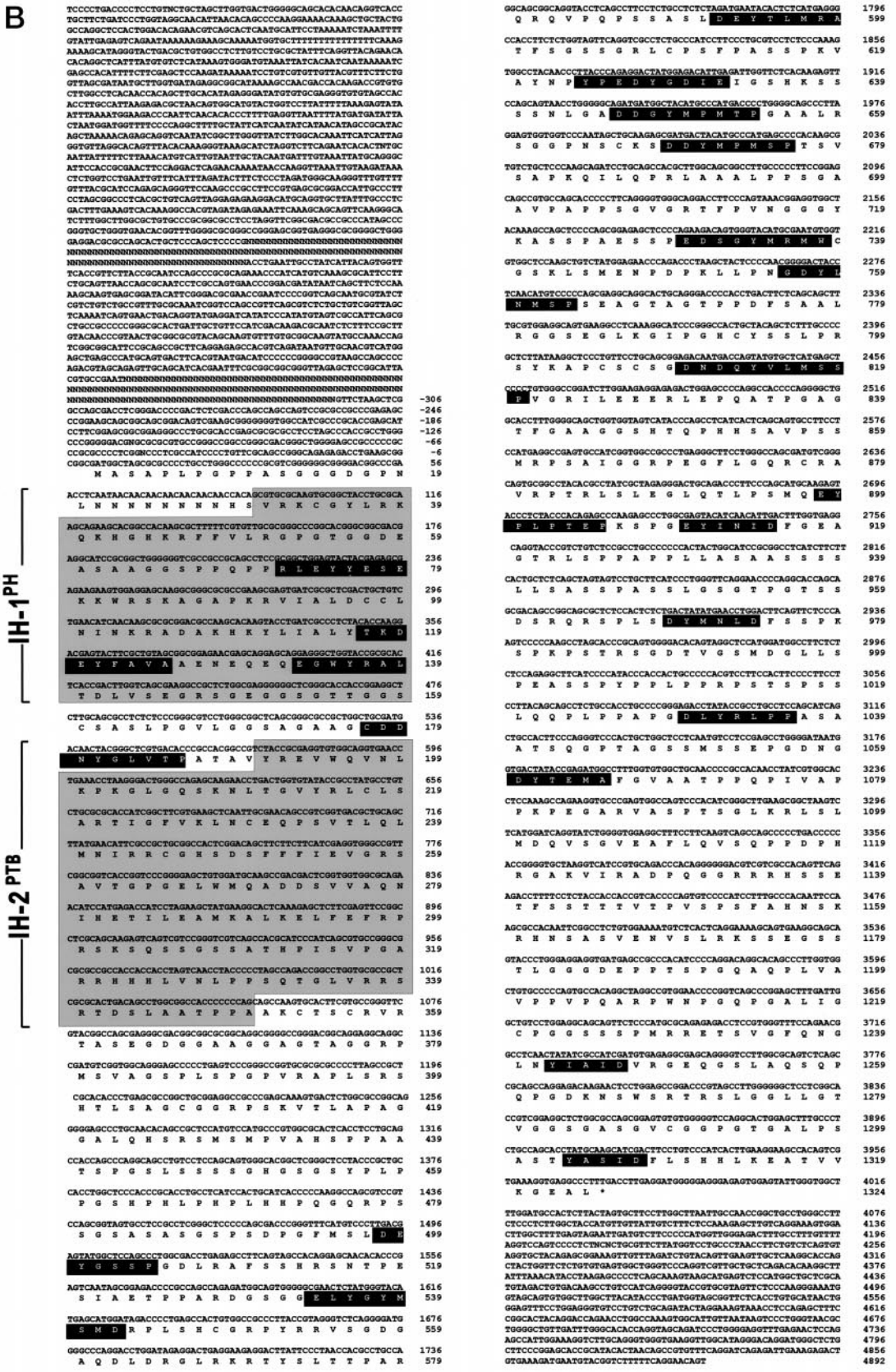


**Fig. 1.** *(Continued)*

## RESULTS

### The structure and Location of the Murine IRS-2 Gene

We recently described an optimized 45-mer nucleotide probe based on a partial amino acid sequence of 4PS that was used to isolate overlapping cDNA molecules encoding a consensus cDNA for IRS-2 (21). This oligonucleotide was also used to isolate a 17-kb genomic fragment (mG28) from a murine genomic library. Several restriction fragments of mG28 were isolated, subcloned, and sequenced (Fig. 1A). Restriction mapping and PCR analysis confirmed that the fragments obtained from the genomic DNA corresponded exactly to the partial cDNA sequences obtained previously from various murine libraries (Fig. 1A). Translation of the open reading frame obtained from the genomic sequence revealed 1324 amino acids, including three additional residues at the COOH terminus that were not previously described (Fig. 1B) (21). The 5′-untranslated region of the IRS-2 gene contains two GC-rich regions that proved difficult to sequence accurately and remain undefined (Fig. 1B).

Southern analysis of 129-mouse genomic DNA with a 5′-end IRS-2 probe (−2525 to 423) revealed a single *Afl*II fragment and two *Sac*I fragments (2 kb and 6 kb); *Sma*I digestion revealed two fragments of 0.72 kb and 3.7 kb, which hybridized with a 3′-end probe (2777 to 4468) (Fig. 2A). This pattern is consistent with the conclusion that IRS-2 is encoded by a single gene in the 129 mouse as diagramed in Fig. 1A.

We mapped the chromosomal location of the murine *Irs2* gene by following a *Bam*HI restriction fragment length polymorphism (RFLP) during interspecific backcross analysis on (C57BL/6J × *M. spretus*)$F_1$ × C57BL/6J progeny. The mapping results indicated that *Irs2* is located in the proximal region of murine chromosome 8, linked to the *Insr*, *Col4al*, and *Plat* loci. Although 90 mice were analyzed for every marker (shown in the segregation analysis, Fig. 2B), up to 181 mice were characterized for some pairs of markers; each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci suggests the following gene order: centromere→*Insr* (1/95)→*Irs2*

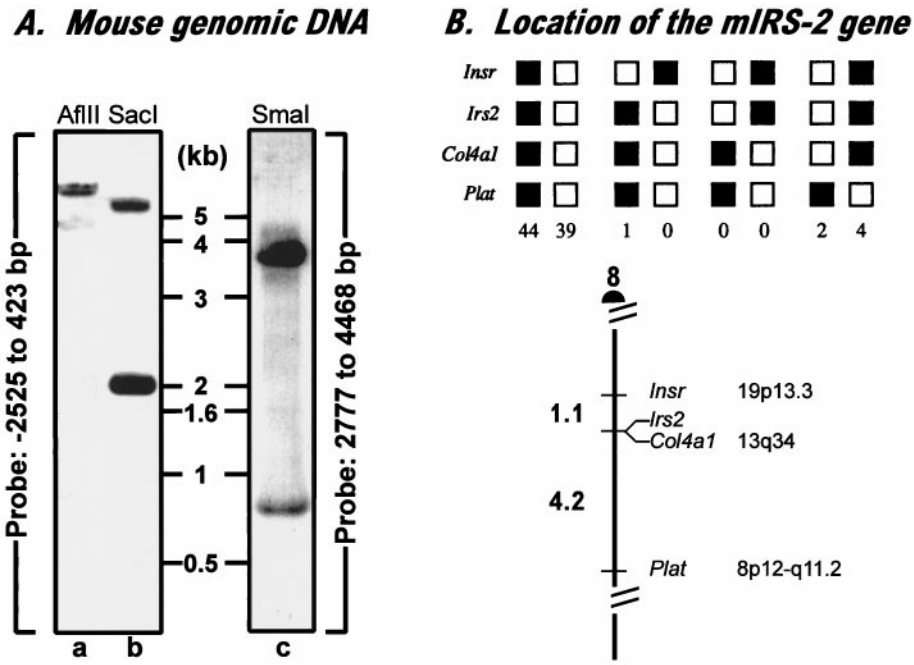


**Fig. 2.** Southern Blot and Chromosome Localization

Panel A, Mouse genomic DNA was obtained from liver and digested with the indicated restriction enzymes. The restriction fragments were blotted with IRS-2 cDNA as indicated in the figure. Panel B, Chromosomal localization of the mouse *Irs2* gene. *Irs2* was placed on mouse chromosome 8 by interspecific backcross analysis. The segregation patterns of *Irs2* and flanking genes in 90 backcross animals that were typed for all loci are shown at the *top* of the figure. For individual pairs of loci, more than 90 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the hybrid parent. *Shaded boxes* represent the C57BL/6J allele and *white boxes* represent the *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the *bottom* of each column. A partial linkage map of chromosome 8 is shown. Recombination distances between loci are shown in centimorgans on the *left* and the human chromosomal positions, where known, are shown on the *right*. References for the human map positions used in this study can be obtained from GDB, a computerized database of human linkage information maintained by the William H. Welch Medical Library of the Johns Hopkins University (Baltimore, MD).

(0/142)→*Col4al* (8/181)→*Plat* (Fig. 2B). The lack of recombinations detected between *Irs2* and *Col4al* in this number of animal suggests that the two loci are within 2.1 centiMorgans (cM) of each other at the 95% confidence limit. The recombination frequencies reported as the genetic distance in centiMorgans ± SE is 1.1 ± 1.1 between the *Insr* loci and *Irs2/Col4al*, and 4.4 ± 1.5 between *Irs2/Col4al* and *Plat* (Fig. 2B).

### Downstream Signaling Specificity of IRS-1 and IRS-2

The amino acid sequence identity in the COOH terminus of IRS-2 and IRS-1 is only 35%, which arises largely from similar tyrosine phosphorylation motifs surrounded by variable stretches of amino acid sequence (21). To explore possible signaling diversity in IRS-1 and IRS-2 at the level of tyrosine phosphorylation, we prepared a panel of GST-fusion proteins containing SH2-domains from various signaling molecules and tested their ability to bind to IRS-2 or IRS-1 expressed in $32D^{IR/IL4R}$ cells. After insulin or IL-4 stimulation, cell lysates were incubated with GST-SH2 fusion proteins and the associated IRS-2 and IRS-1 were measured by immunoblotting with αPY (25).

The $NH_2$-terminal SH2 domain of p85 bound strongly to both IRS-2 and IRS-1 during stimulation with insulin (Fig. 3); insulin and IL-4 stimulated similar amounts of IRS-2 binding to p85, while insulin stimulated more binding to IRS-1 than IL-4 did. Similar results were observed with the SH2 domain of c-*fyn*. However, the SH2 domains from Crk, phospholipase C, and GRB-2 revealed functional differences. Each SH2 domain bound IRS-1 more strongly than IRS-2 during insulin and IL-4 stimulation. Moreover, IRS-1 bound more tightly during insulin stimulation than during IL-4 stimulation, whereas IRS-2 bound equally during insulin and IL-4 (Fig. 3). The SH2 domains of Abl and SHP-2 associated only with IRS-1, and then only during insulin stimulation (Fig. 3). These results suggest that IRS-2 and IRS-1 may be phosphorylated differently by various receptors and engage a unique cohort of SH2 proteins.

### Distribution of IRS-1 and IRS-2 in Cells and Tissues

IRS-1 and IRS-2 may regulate unique signaling pathways due, in part, to their distinct cellular distribution in addition to the unique interactions with downstream signaling elements. To examine the effect of differentiation on IRS-1 and IRS-2 expression, three cell culture systems were investigated, including 3T3-L1 cells, P19 embryonic carcinoma cells, and Bal-17 lymphocytes. The 3T3-L1 adipocyte is frequently used to study the mechanism of insulin-stimulated glucose uptake, as several metabolic responses acquire insulin sensitivity during differentiation into adipocytes (26). Before differentiation of 3T3-L1 fibroblasts, IRS-1 and IRS-2 were barely detected by immunoblotting with αPY during insulin stimulation (Fig. 4A). However, after differentiation, the amount of IRS-1 increased dramat-

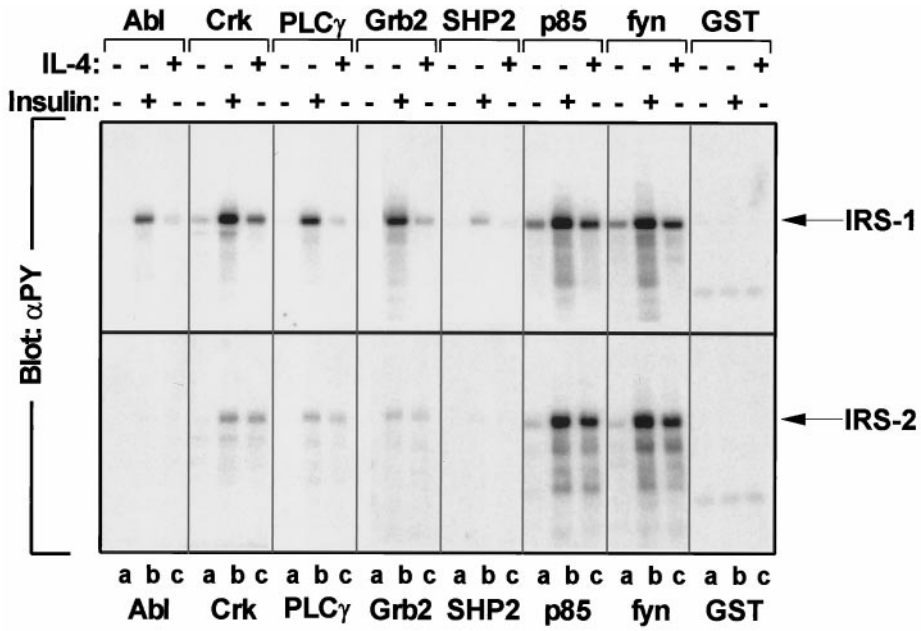


**Fig. 3.** Differential Binding of SH2 Domain Proteins to IRS-1 or IRS-2

$32D^{IR,IL4R}$/IRS-1 and $32D^{IR,IL4R}$/IRS-2 cells matched for expression of IRS-1, IRS-2, IR, and IL4R were incubated without or with 100 nM insulin for 1 min or 10 nM IL-4 for 10 min; these conditions were shown previously to give maximal phosphorylation. Cell extracts were precipitated with the indicated GST-fusion proteins as described in *Materials and Methods*, separated by SDS-PAGE, and immunoblotted with αPY. Phosphorylated IRS-1 and IRS-2 are indicated. These data are representative of two similar experiments.

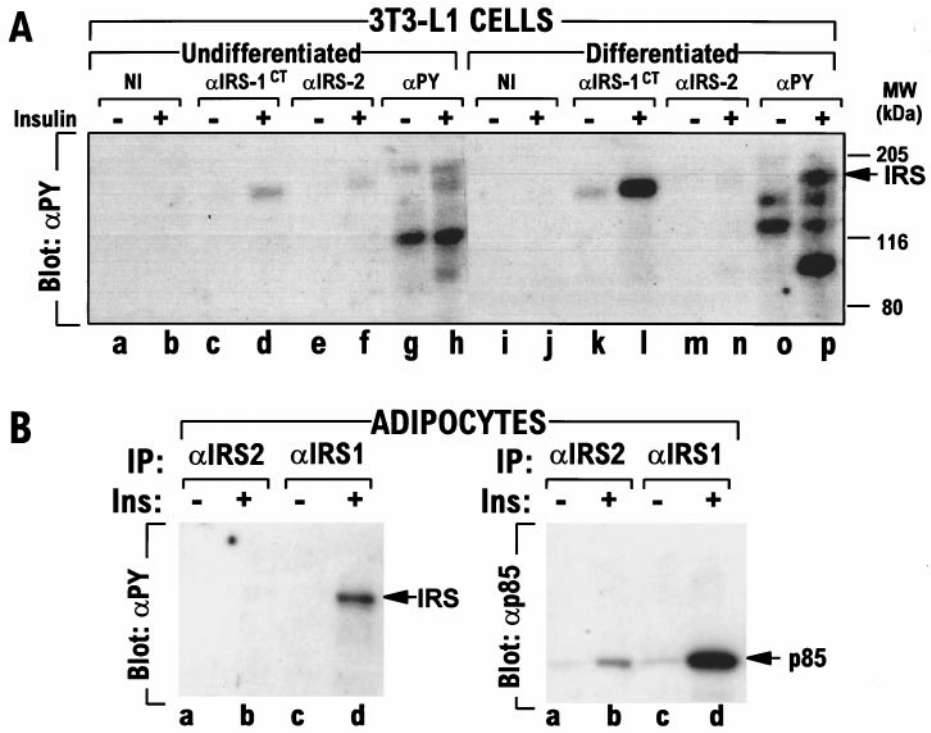


**Fig. 4.** IRS Proteins in 3T3-L1 Cells, Rat Adipocytes, and B Cells

Panel A, Undifferentiated 3T3-L1 fibroblasts or differentiated 3T3-L1 adipocytes were incubated with or without 100 nM insulin for 2 min and lysed. The indicated antibodies were used to prepare immunoprecipitates, and the protein were detected by immunoblotting with αPY. Freshly isolated rat adipocytes (panel B) were stimulated with insulin for 5 min, and proteins in the cell extracts were immunoprecipitated with specific antibodies against IRS-1 or IRS-2; tyrosine phosphorylation of these proteins or their association with p85 was determined by immunoblotting with αPY or αp85.

ically, and insulin robustly stimulated its tyrosine phosphorylation; however, IRS-2 was barely detected and its insulin-stimulated tyrosine phosphorylation was quite low (Fig. 4A). Consistent with this observation, IRS-1 was the predominant phosphorylated IRS protein in isolated rat adipocytes during insulin stimulation; however, a small amount of p85 was detected in αIRS2 immunoprecipitates after insulin stimulation, suggesting that a small amount of IRS-2 is expressed in this background (Fig. 4B). Whether this low expression of IRS-2 is sufficient to rescue insulin signaling in murine adipocytes from the IRS-1$^{(-/-)}$ mouse is unknown, but future experiments should reveal whether other pathways such as the tyrosine phosphorylation of p60 or Gab1 are involved (27, 28).

IGF-1 plays an important role in neuronal differentiation and survival (29). Retinoic acid-induced differentiation of P19 embryonic carcinoma cells provides a cell culture model of neuronal differentiation (30). Before induction with retinoic acid, IRS-1 and IRS-2 were expressed approximately equally in P19 cells, and both proteins were tyrosine phosphorylated and associated with p85 during IGF-1 stimulation. However, 2 days after retinoic acid removal, while the cells continue to differentiate, the IRS-2 expression fell whereas IRS-1 expression was unchanged (Fig. 5). This change in relative expression continued until IRS-1 predominated in the fully differentiated P19 neurons (6 days after retinoic acid removal). Consistent with these results, IGF-1 strongly mediated IRS-1 phosphorylation in the P19 neurons and IRS-1 associated with p85 (Fig. 5). It will be interesting to determine whether the reduced expression of IRS-2 is required for retinoic acid-induced differentiation in P19 cells.

Finally, we investigated the expression and function of IRS-1 and IRS-2 in differentiated, but proliferating, Bal-17 cells. Bal-17 cells are mature B lymphocytes that are ordinarily activated by cross-linking of the surface IgM with specific IgM antibodies (31). Insulin strongly stimulated IRS-2 tyrosine phosphorylation in these cells, whereas IRS-1 was barely detected (Fig. 6). Interestingly, cross-linking surface IgM with αIgM induced weak (relative to insulin) tyrosine phosphorylation of IRS-2 (Fig. 6, lanes g-i). The effect of αIgM was specific as nonimmune serum had no effect, suggesting that IRS-2 may function downstream of the B cell antigen receptor (Fig. 6, lanes a-c). We also observed the tyrosine phosphorylation of IRS-2 in isolated B cells during anti-IgM stimulation (data not shown).

In summary, IRS-2 was expressed in nearly all cells that we analyzed by RT-PCR or immunoblotting, including lymphoid and myeloid progenitor cells, B cells, carcinoma cells, fibroblasts, liver, skeletal muscle, and

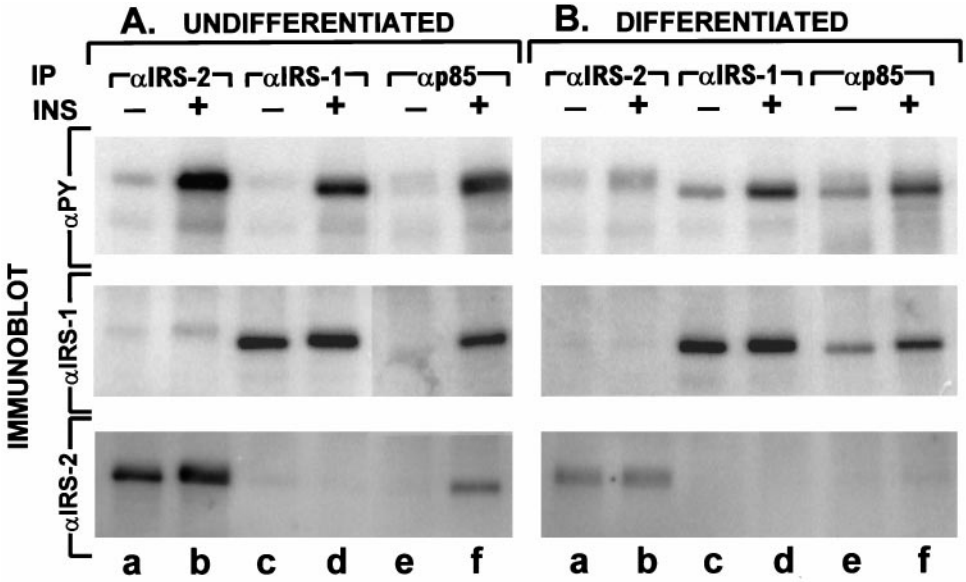


**Fig. 5.** Phosphorylation of IRS-1 and IRS-2 in P19 Embryonic Carcinoma Cells and Differentiated P19 Neurons

Undifferentiated (A) or neuronally differentiated p19 cells (B) were incubated in the absence or presence of 100 nM insulin for 2 min and lysed. Clarified lysates were incubated with αIRS-2 (a and b) αIRS-1 (c and d), or αp85 (e and f). Immune complexes were collected with protein A Sepharose, resolved by SDS-PAGE, and transferred to nitrocellulose for immunoblotting with αPY (*top panel*), αIRS-1 (*middle panel*), or αIRS-2 (*bottom panel*).

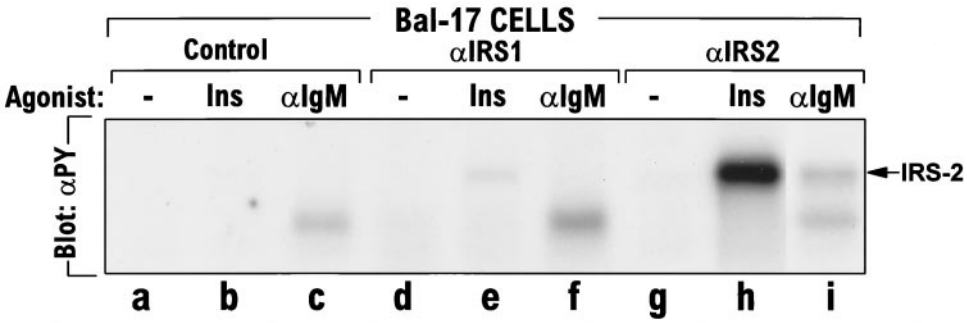


**Fig. 6.** Expression and Phosphorylation of IRS-1 and IRS-2 in Bal-17 Cells

Bal-17 cells were stimulated with 100 nM insulin or 15 μg/ml anti-IgM (αIgM) for 2 min. Cell extracts were immunoprecipitated with nonimmune serum (control), or specific antibodies against IRS-1 and IRS-2; tyrosine phosphorylation was detected by immunoblotting with αPY.

brain (Table 1). However, IRS-2 was conspicuously absent from a few cell lines of lymphoid origin, including Daudi and ABMC1 (B cell lines), and CT6 and EL4 (T cell lines). IRS-2 was barely detected in rat testis and surprisingly weak in 3T3-L1 cells and rat adipocytes (Table 1). By comparison, IRS-1 was also broadly expressed; however, it was absent from several lymphoid cell lines and all cells of the myeloid lineage that were tested (Table 1). Differential expression was also observed in several carcinoma cell lines: IRS-2 was highly expressed in three colon carcinoma cells (clone-A, CX-1, RKO), whereas only clone A coexpressed IRS-1; both IRS-1 and IRS-2 were expressed relatively highly in several mammary carcinoma cell lines where they may contribute to their prolonged survival (Table 1).

## DISCUSSION

The identification of IRS-2 and its alignment against IRS-1 established the modular structure and function of the IRS proteins (21). Additional proteins in this family are expected to contain at least one interaction domain, either a pleckstrin homology (PH) domain or a phosphotyrosine binding (PTB) domain, and a COOH terminus with multiple tyrosine phosphorylation sites. Thus, mammalian Gab-1 and *Drosophila* DOS, each possessing a single PH domain and a tail with tyrosine phosphorylation sites, fit this general paradigm and may be considered to be members of the IRS family (28). Teleologicaly, IRS proteins provide an evolutionary signaling advantage in several ways: 1) They pro-

**Table 1.** Relative Detection of IRS-1 and IRS-2 in Various Cell Lines and Tissues from Mice (m), Rats (r), Humans (h), or Hamsters (ham)

| Cell Line | Description | Growth Considerations | IRS-1 | | IRS-2 | |
|---|---|---|---|---|---|---|
| | | | RT-PCR | Immun. Blot | RT-PCR | Immun. Blot |
| 32D | Myeloid progenitor | IL-3 dependent | − | − | − | − |
| 32D$^{IRS-1}$ | Myeloid progenitor | IL-3 dependent | + | +++ | − | − |
| FDC-P1 | Myeloid progenitor | IL-3 dependent | − | − | + | +++ |
| FDC-P2 | Myeloid progenitor | IL-3 dependent | − | − | + | +++ |
| CFTL12 | Mast cell | IL-3 dependent | − | | + | |
| CFTL15 | Mast cell | IL-3 dependent | − | | + | |
| WEHI-3B | Monocyte | | − | | + | |
| P338D1 | Macrophage | | − | | + | |
| M1 | Myeloid progenitor | | − | | + | |
| FEMCL | Lymphoid progenitor (Fes) | | + | | + | |
| Feuc | Lymphoid progenitor (Fes) | | − | | + | |
| HAFTL1 | Lymphoid progenitor (ras) | | + | | + | |
| HSIC5 | Lymphoid progenitor (ras) | | − | | + | |
| JP4 | Lymphoid progenitor (src) | | + | | + | |
| JP7 | Lymphoid progenitor (src) | | + | | + | |
| A20.3 | preB Lymphocyte (Abl) | | − | | + | |
| LYH7 | B Lymphocyte | IL-3 dependent | − | | + | |
| WEHI-238 | B cell | | | ± | | ++ |
| M20/169/C5 | B Cell hybridoma (plasmacytoma) | | − | | + | |
| Daudi | B Cell (h) | | | + | | − |
| Bal-17 | B Cell (m) | | | ± | | ++ |
| B Cells | Freshly isolated murine | | | ± | | ++ |
| IGH-168-D11 | B cell hybridoma | | + | | + | |
| IM92-7 | B cell hybridoma | | + | | + | |
| AAC6 | pre B Lymphoid (Abl) | | + | | + | |
| ABMC1 | pre B Lymphoid (Abl) | | − | | − | |
| CT6 | T Cell (m) | IL-2 dependent | − | | − | |
| EL4 | T Cell (m) | | + | | − | |
| T Cells | Freshly isolated murine | | | ± | | ++ |
| B6 Sut | Multipotential progenitor | IL-3 dependent | − | | + | |
| MDA 231 | Mammary carcinoma (h) | Estrogen R. neg. | | + | | + |
| MDA 435-C | Mammary carcinoma (h) | Estrogen R. neg. | | + | | +++ |
| 21NT | Mammary carcinoma (h) | Estrogen R. neg. | | + | | + |
| MCF-7 | Mammary carcinoma (h) | Estrogen R. pos. | | +++ | | + |
| T47D | Mammary carcinoma (h) | Estrogen R. pos. | | +++ | | ++ |
| Clone A | Colon carcinoma (h) | Well differentiated | | ++++ | | +++ |
| CX-1 | Colon carcinoma (h) | Well differentiated | | − | | +++ |
| RKO | Colon carcinoma (h) | Poorly differentiated | | − | | +++ |
| Fao | Hepatoma (r) | | | ++ | | ++ |
| bCT-3 | Pancreatic beta cell (m) | | | ± | | ++ |
| PC12 | Rat pheocromocytoma | | | ± | | ++ |
| CHO | Ovarian carcinoma (ham) | | | + | | + |
| P19 | Embryonic carcinoma cells (m) | | | + | | ++ |
| P19 | Neuron (m) | Retinoic acid induced | | ++ | | + |
| NIH-3T3 | Fibroblast (m) | | + | + | | + |
| 3T3-L1 | Fibroblast (m) | | | + | | ± |
| 3T3-L1 | Adipocyte (m) | | | +++ | | ± |
| liver | Fresh homogenates (m) | | | + | | + |
| skeletal mus. | Fresh homogenates (m) | | | + | | + |
| Brain | Mouse | | | ++ | | ++ |
| Adipocyte | Epidymal (r) | | | + | | + |
| Testis | Mouse | | | + | | ± |

For comparison, the 32D, 32D$^{IRS1}$, FDC-P1, and FDC-P2 cells were analyzed by both RT-PCR and immunoblotting. For RT-PCR analysis, a minus indicates that no PCR product was detected relative to the negative control (32D cells), whereas a plus indicates that the expected PCR by comparison to the positive FDC-P2 cell control was obtained. Immunoblots were performed with $\alpha$IRS1$^{CT}$ and $\alpha$IRS2 as previously described (55). The detection of IRS-1 and IRS-2 was judged qualitatively relative to the negative 32D cells (−) or positive 32D$^{IRS1}$ (+++) and 32D$^{IRS2}$ (+++) cells.

vide a means for signal amplification by eliminating the stoichiometric constraints encountered by receptors that directly recruit SH2 proteins to their autophosphorylation sites. 2) IRS proteins may dissociate the intracellular signaling complex from the endocytic pathways of the activated receptor. 3) The ability of a single receptor to engage multiple IRS proteins (each with potentially different signaling characteristics) expands the repertoire of signaling pathways that can be regulated. 4) The shared use of IRS proteins by multiple receptors provides a mechanism to integrate several receptor systems through one signaling complex to generate a coordinated cellular response.

IRS-1 and IRS-2 appear to interact with a similar cohort of cellular membrane receptors, including insulin/IGF-1, several ILs, IFNs, and GH (9). During stimulation of these receptors with their cognate ligands, the IRS proteins undergo tyrosine phosphorylation and associate with signaling proteins that contain SH2-proteins. Although the overall amino acid sequence identity between IRS-2 and IRS-1 is 43%, the alignment shows regions of striking homology within the $NH_2$-terminal third of the molecule (21). Translation of the coding region of IRS-2 reveals at least three functional regions. Two highly conserved regions occur in the $NH_2$-terminal portion of IRS-1 and IRS-2: the first *I*RS-*H*omology region ($IH1^{PH}$) contains a PH domain; the second, $IH2^{PTB}$, contains a PTB domain (32). Recent experiments indicate that both regions couple IRS-1 to the insulin receptor in 32D cells; however, the $IH1^{PH}$ region provides the most efficient coupling (33). Another region (between residues 313 and 462 of IRS-1) was aligned recently to the PTB domain in Shc and designated the "SAIN" domain (34–36); however, this region is poorly conserved between IRS-1 and IRS-2 and does not function as a PTB domain (33).

By contrast, the COOH-terminal regions of IRS-1 and IRS-2 are poorly conserved, displaying only 35% identity (21). The middle of IRS-2 contains a unique region that interacts specifically with the phosphorylated regulatory loop of the insulin receptor (35). This region, which is absent from IRS-1, may alter the phosphorylation pattern of the COOH terminus by restricting the flexibility of IRS-2 in the catalytic domain. On the other hand, several tyrosine phosphorylation sites in IRS-2 align with similarly spaced sites in IRS-1 (21). In several cases, the amino acid sequence around the tyrosine residues are nearly identical in IRS-2 and IRS-1, including the $NH_2$-terminal acidic residues and the COOH-terminal hydrophobic residues. In at least half of these cases, however, either the relative position of the acidic or hydrophobic residues are different; either change is likely to alter the interaction of the phosphorylation site with upstream kinases or downstream SH2-proteins, effectively changing the signal. The comparative association of IRS-1 and IRS-2 with various SH2 domains observed in our experiments is consistent with these predictions.

The mIRS-2 gene lies near the type 1 procollagen 4a locus (*Col4al*), proximal to the centromere on mouse chromosome 8 (37, 38). We have compared our interspecific map of chromosome 8 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from the Mouse Genome Database, maintained by the Jackson Laboratory, Bar Harbor, ME). *Irs2* maps in a region that lacks mouse mutations that might be expected for an alteration in this locus (data not shown). By contrast, *Irs1* is located on mouse chromosome 1, and the human gene is on human chromosome 2q36-37 (39). A few point mutations have been found in the human IRS-1 gene, but their effect on function is unclear (40, 41). It will be important to determine whether mutations in the human IRS-2 gene contribute to insulin resistance of non-insulin-dependent diabetes mellitus or to immune system disorders. Toward this end, we have isolated a partial clone with a high degree of identity to murine IRS-2, which may be the human counterpart (M. F. White and D. Bernal, unpublished data).

The proximal region of mouse chromosome 8 shares regions of homology with human chromosomes 8p, 13q, and 19p. In particular, *Col4al*, the locus most closely linked with *Irs2*, has been placed on human 13q34, suggesting that *Irs2* may reside there as well. However, it is interesting that *Irs2* lies close to the murine insulin receptor. The *Drosophila* insulin receptor contains a COOH-terminal extension of 400 residues with similarity to the first half of the COOH-terminus of IRS-2 (42). It is possible that the evolutionarily early insulin receptor contained such a tail, and that during the evolution of higher organisms, this tail (which remains part of the *Drosophila* receptor) separated from the receptor and was reassembled into the IRS-2 gene. Based on the proximity and functional relation between the insulin receptor and IRS-2, *hIrs2* may be adjacent to the human insulin receptor on chromosome 19.

Broad distribution of IRS proteins and their interaction with various receptor systems suggests that they are essential for maintaining cell survival and growth and normal metabolic regulation (9, 11). However, our finding that phosphorylated IRS-1, but not IRS-2, increases during differentiation of 3T3-L1 fibroblasts into adipocytes suggest that IRS-1 and IRS-2 may not mediate identical signals. Because IRS-1 predominates in adipocytes, it may best mediate metabolic effects of insulin in this cell context, including the stimulation of glucose transport and inhibition of lipolysis. The predominance of IRS-2 in hematopoietic cells suggests that it may be preferred by cytokine receptors and be best adapted to mediate mitogenesis. This hypothesis is consistent with our previous finding that IRS-2 mediates a very sensitive mitogenic response to IL-4 (21). Thus, although IRS-1 and IRS-2 may be serving redundant roles in some circumstances, they perform physiologic functions in several instances based upon tissue distribution. Not only are IRS-1 and IRS-2 differentially expressed, but our analysis using various SH2 proteins to probe phosphory-

lation site characteristics suggests that IRS-1 and IRS-2 are phosphorylated differently and may recruit alternate downstream SH2-proteins. Furthermore, the stimulating factor (IL-4 or insulin) alters the association of SH2 proteins with IRS-1 and IRS-2. Similar differences may also occur during interaction with other IL, IFN, or GH receptors.

Thus, signaling by IRS-1 and IRS-2 varies by 1) tissue type, 2) growth factor, and 3) elements directed by the differing structures of the two IRS proteins. The ability to switch between IRS-1 or IRS-2 in cell context-dependent manner provides a unique mechanism for signal diversity that would be absent from classic receptors that engage SH2 proteins directly at their autophosphorylation sites. While it is interesting to note that the IRS-1$^{(-/-)}$ mouse displays disordered growth and metabolism, no immune system dysfunction has been detected, which is consistent with the general absence of IRS-1 from hematopoietic cells. However, based on the expression of IRS-1 in classical insulin target tissues, it is not surprising that IRS-2 cannot completely compensate for the absence of IRS-1 for carbohydrate metabolism (22, 23). The relative importance of IRS-2 and IRS-1 for growth and development and metabolic regulation awaits the generation of IRS-2$^{(-/-)}$ mouse and direct comparison and mating with the IRS-1$^{(-/-)}$ mouse. Moreover, the recent discovery that IRS-1, but not IRS-2, may mediate the inhibitory effect of tumor necrosis factor-$\alpha$ (TNF$\alpha$) on the insulin receptor suggests that the IRS proteins define and regulate the crossroads at which many diverse signaling systems converge and diverge (43).

## MATERIALS AND METHODS

### Cell Culture

All the cells were incubated at 37 °C in a humidified atmosphere composed of 5% $CO_2$ and 95% air, except 3T3-L1 cells, which were maintained in an incubator with 10% $CO_2$. IL-3-dependent FDC-P1, FDC-P2, and 32D cells were maintained in RPMI-1640 medium (GIBCO Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS (Sigma, St. Louis, MO) and 5% WEHI-3-conditioned RPMI-1640 medium as a source of IL-3 (44). Cell lines expressing the human insulin receptor, human IL-4 receptor (IL-4R$\alpha$), and IRS-1 have been described (6, 20). Bal-17 B lymphoma cells were maintained in RPMI-1640 medium supplemented with 10% FBS, L-glutamine (2 mM), and 2-mercaptoethanol (50 $\mu$M). The 3T3-L1 cells were maintained in DMEM high-glucose supplemented with 10% bovine calf serum; 2 days post-confluence, differentiation was induced by incubating the cells for 3 days in DMEM supplemented with 10% FBS, 5 $\mu$g/ml insulin, 1 $\mu$M dexamethasone.

p19 cultures were performed as previously described (30). Briefly, undifferentiated p19 cells were grown on tissue culture quality plastic in DMEM-F12 containing 10% FCS. Differentiation of p19 cells was induced by plating in the presence of 1 $\mu$M retinoic acid for 4 days on bacterial quality dishes (to which cells do not attach). At the end of the fourth day the aggregates were decanted, plated on tissue culture quality plastic, and allowed to differentiate for 4 days.

32D cells or cells expressing the human receptors for insulin and IL-4 (32D$^{IR/IL4R}$) were transfected with pCMV$^{his}$IRS-2 or pCMV$^{his}$IRS-1 by electroporation, as previously described (20, 45). Cells were selected by survival in 5 mM histidinol (Sigma), and expression of IRS-2 and IRS-1 was monitored by immunoblotting.

Epididymal adipocytes were isolated from Spraque-Dawley rats (280–300 g) using a modification of the original methods described by Rodbell (46). Fat was dissected and collected in modified Krebs Ringer bicarbonate (buffer A), supplemented with 10 mM HEPES (Sigma) 2.5% BSA (Sigma), and 200 nM adenosine (Sigma). The tissue was briefly minced, and then digested for 40 min in a 37 °C shaker bath set at 10 rpm/6 sec by adding 2 mg collagenase (Worthington Biochemical, Freehold, NJ) per gram of tissues (47). The digested tissue was passed through a 50-$\mu$m nylon screen and rinsed several times with buffer A to remove the collagenase, then rinsed several times with BSA-free buffer A. Equal volumes of adipocytes are treated without or with 80 nM insulin for 5 min with gentle rotation in a 37 °C water bath.

### Southern Analysis of Mouse Genomic DNA

Genomic DNA (10 $\mu$g) from mouse liver (48) was digested overnight with various restriction enzymes, resolved by electrophoresis, and transferred to Hybond N membranes (Amersham, Arlington Heights, IL) for Southern analysis. Two specific DNA probes, which contain the sequences corresponding to approximately −2525 to 423 bp, and 2777 to 4468 bp of IRS-2, were obtained by digesting mG28 with *Kpn*I. The *Kpn*I fragments were isolated and labeled with [$^{32}$P]phosphate as previously described (48). Hybridization was conducted overnight at 40 °C in 5 × saline sodium citrate (SSC), 40% formamide, 5× Denhardt's, 0.1% SDS, and 100 ng/ml of salmon sperm DNA. Final washing of the blots was at 65 °C in 0.5 × SSC containing 0.1% SDS.

### Interspecific Mouse Backcross Mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × *M. spretus*)$F_1$ females and C57BL/6J males as described (49). A total of 205 $N_2$ mice were used to map the *Irs2* locus. This mapping panel has been typed for more than 2000 loci that are distributed among all of the autosomes and the X chromosome (49). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed as described (50). An approximately 300-bp *Bam*HI/*Eco*RI mouse cDNA fragment was labeled with [$^{32}$P]dCTP using a nick translation kit (Boeringer Mannheim, Indianapolis, IN) and used as probe; the final wash stringency was 0.5× saline sodium citrate phosphate, 0.1% SDS, 65 °C. The probe detected 13 kb and 20 kb *Bam*HI fragments from C57B1/6J and *M. spretus* DNA, respectively. The 20-kb *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the linked insulin receptor (*Insr*) and tissue plaminogen activator (*Plat*) loci has previously been reported (37). The probe for type IV $\alpha$1 procollagen (*Col4al*) has not previously been reported; it was a 1.9-kb *Eco*RI fragment which detected 9.7 (C57BL/6J) and 8.6 (*M. spretus*) kb *Xba*I fragments. Recombination distances were calculated as described (51) using the SPRETUS MADNESS program. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

### Immunoprecipitation and Immunoblot Analysis

Quiesent cells were incubated for 1 min in the absence or presence of 100 nM insulin or 10 nM IL-4, and lysed in homogenization buffer. The lysates were incubated with poly-

clonal antibodies, and the immune complexes were collected on protein A and washed three times with homogenization buffer, denatured, separated by 7.5% SDS-PAGE, and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) for immunoblotting (52). The antibodies were prepared in rabbits (HRP Corp., Denver, PA) as previously described (10). The αIRS-2 was obtained using a GST-fusion protein containing residues 619-746 of mouse IRS-2 (25) as antigen; αIRS-1 was obtained using recombinant rat IRS-1 purified from Sf9 cells infected with a recombinant baculovirus as antigen (25); $\alpha$IRS-1$^{CT}$ (residues 1221–1234 of rat IRS-1) were made with synthetic peptides coupled to Keyhole limpet hemocyanin (53). αPY antibodies were rabbit polyclonal (54) or mouse monoclonal 4G10 (UBI, Lake Placid, NY). αp85 antibodies were purchased from UBI.

### Differential Binding of SH2 Domains with the IRS-Proteins

GST-fusion proteins containing nSH2$^{p85\alpha}$, SH2$^{fyn}$, SH2$^{Grb2}$, nSH2$^{PLC\gamma}$, nSH2$^{SHPTP2}$, SH2$^{abl}$, and SH2$^{Crk}$ were prepared as previously described (25). Cell lysates were prepared from unstimulated, insulin-stimulated, or IL-4-stimulated cells (32D$^{IR,IL4R}$, 32D$^{IR,IL4R}$/IRS-1 and 32D$^{IR,IL4R}$/IRS-2 cells) in homogenization buffer. The extracts were clarified by centrifugation at 100,000 × *g* for 1 h at 4 °C. The supernatants were incubated with the 1 μg GST fusion proteins containing SH2 domains as indicated at 4 °C for 1 h and precipitated with glutathione Sepharose at 4 °C for 1 h, washed twice with 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 250 μg/ml BSA, 0.2 mM vanadate, and 0.4 mM phenylmethylsulfonylfluoride, and boiled for 5 min in 100 μl of Laemmli sample buffer containing 0.1 M dithiothreitol. Samples were separated on 7.5% SDS-PAGE and analyzed by immunoblotting (25, 52).

## Acknowledgments

Many thanks to Mary Elizabeth Patti and Ron Kahn for helpful discussions and sharing unpublished data. R. Robinson, V. Bailey, J. Neveu, and D. Lizotte provided valuable expertise in peptide isolation and microsequencing. Bruce Mayer provided recombinant abl and crk SH2 domains; Ed Skolnik provided nck; βCT-3 cells were a gift from Chris Rhodes. Thanks to Dr Tom Charles for providing Bal-17 cells. We thank Mary Barnstead for excellent technical assistance.

Received August 1, 1996. Revision received October 23, 1996. Accepted November 6, 1996.

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This work was supported by NIH Grants DK-38712 and DK-43808 (M.F.W.) and by the National Cancer Institute, Department of Health and Human Services under contract with ABL (N.A.J.). X.J.S. is a recipient of the Juvenile Diabetes Foundation, and M.G.M., Jr. was partially supported by the Medical Scientist Training Program at Harvard Medical School.

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