

Association of Insulin Receptor Substrate Proteins with Bcl-2 and Their Effects on Its Phosphorylation and Antiapoptotic Function

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Insulin receptor substrate (IRS) proteins are docking proteins that couple growth factor receptors to various effector molecules, including phosphoinositide-3 kinase, Grb-2, Syp, and Nck. Here we show that IRS-1 associates with the loop domain of Bcl-2 and synergistically up-regulates antiapoptotic function of Bcl-2. IRS-2 but not IRS-3 binds to Bcl-2, and IRS-1 associates with Bcl-XL but not with Bax or Bik. Overexpression of IRS-1 suppresses phosphorylation of Bcl-2 induced by stimulation with insulin, and the hypophosphorylation may lead to its enhanced antiapoptotic activity. The binding site for Bcl-2 is located on the carboxyl half-domain of IRS-1. IRS-3, which lacks the corresponding region, dominant-negatively abrogates the survival effects of IRS-1 and Bcl-2. For the antiapoptotic activity of IRS-1, binding to Bcl-2 is more critical than activating phosphoinositide-3 kinase. Our results indicate that IRS proteins transmit signals from the insulin receptor to Bcl-2, thus regulating cell survival probably through regulating phosphorylation of Bcl-2.

INTRODUCTION

Apoptosis is a form of programmed cell death, characterized by chromatin condensation, cytoplasmic blebbing, and DNA fragmentation. During development, programmed cell death plays critical roles in sculpting parts of body, deleting unwanted structures and misplaced, nonfunctional, or harmful cells in animal tissues (Jacobson *et al.*, 1997). Depletion of growth factors is one of the common causes of apoptosis, which probably plays a critical role in controlling cell number and eliminating misplaced cells. Growth factor receptors including receptor tyrosine kinases (RTKs) are considered to generate cell growth signals and cell survival signals to support continuous growth of cells. Although the precise mechanisms of how RTKs generate antiapoptotic

signals are not well understood, ligand stimulation of RTKs leads to activation of intracellular signaling cascades including the Ras-MAPK pathway and the phosphoinositide-3 kinase (PI3K) pathway that are considered to possess antiapoptotic activity (Datta *et al.*, 1997; Klesse and Parada, 1998; Kurada and White, 1998; Walker *et al.*, 1998).

In addition to them, several recent studies have shown that RTKs support cell survival by regulating the function of the Bcl-2 family proteins via phosphorylation. There are two types of the Bcl-2 family proteins, namely antiapoptotic members such as Bcl-2 (Cleary *et al.*, 1986) and Bcl-X_L (Boise *et al.*, 1993) and proapoptotic members, including Bad (Yang *et al.*, 1995), Bax (Oltvai *et al.*, 1993), and Bik (Boyd *et al.*, 1995). Survival of cells was considered to be regulated by a ratio of expressed amounts of antiapoptotic Bcl-2 family proteins to those of proapoptotic member proteins (Oltvai *et al.*, 1993). Furthermore, the function of Bcl-2 family proteins could be regulated by phosphorylation. For example, activation of RTKs leads to phosphorylation of Bad, a proapoptotic Bcl-2 family member, which results in suppression of apoptosis. The protein kinases that phosphorylate Bad are reported to be c-Akt (Datta *et al.*, 1997; del Peso *et al.*, 1997) and protein kinase A (Harada *et al.*, 1999). Furthermore,

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Abbreviations used: CHO, Chinese hamster ovary; FACS, fluorescence-activated cell sorting; HA, hemagglutinin; IGF, insulin-like growth factor; IL, interleukin; IRES, internal ribosomal entry site; IRMA, immunoradiometric assay; IRS, insulin receptor substrate; LF, loop fragment; PI3K, phosphoinositide-3 kinase; RTK, receptor tyrosine kinase.

several growth factors induce serine/threonine phosphorylation of Bcl-2 (Ito *et al.*, 1997), which modifies the antiapoptotic effects of Bcl-2. Paradoxically, however, because dephosphorylation rather than phosphorylation up-regulates the antiapoptotic activity of Bcl-2 (Haldar *et al.*, 1995; Chang *et al.*, 1997; Maundrell *et al.*, 1997), growth factors that induce phosphorylation of Bcl-2 would be expected to cause apoptotic cell death. Taken together, we hypothesize that there should be a mechanism that regulates phosphorylation of Bcl-2 when RTKs and their signaling pathways are activated. Therefore, we searched for regulatory molecules of Bcl-2 that are present in the signaling cascades of RTKs.

MATERIALS AND METHODS

Antibodies and Cell Culture

For immunoblotting, immunoprecipitation, and immunostaining, we used anti-Bcl-2 antibodies, clones 124 (for immunoblotting and immunostaining; Dako, Glostrup, Denmark), N-19 (for immunoprecipitation and immunoblotting), and C100 (for immunoprecipitation and immunoblotting; Santa Cruz Biotechnology, Santa Cruz, CA), anti-IRS-1 antibodies C-20 (for immunoblotting; Santa Cruz) and J36 (for immunoprecipitation; Yamamoto-Honda *et al.*, 1996), anti-IRS-2 antibody (Upstate Biotechnology, Lake Placid, NY), anti-insulin receptor β chain antibody C-19 (Santa Cruz), anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology), and anti-FLAG M2 (Eastman Kodak, Rochester, NY), anti-myc 9E10 (Babco, Richmond, CA), and anti-hemagglutinin (HA) 12CA5 (Boehringer Mannheim, Mannheim, Germany) antibodies. IM-9 cells were cultured in RPMI 1640 medium containing 10% FCS. Ba/F3 cells were maintained in RPMI 1640 medium containing 10% FCS and 0.25 ng/ml mouse interleukin 3 (IL-3). Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium containing 10% FCS. Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FCS.

Plasmid Construction

To construct the C-1 deletion mutant of murine IRS-1, the *XmaI*-*Eco81I* fragment corresponding to amino acids 869-1201 was excised, and the remaining cDNA was blunted and religated. The deletion mutants of IRS-1, C-2 (which encodes aa 1-741), C-3 (aa 1-573), and C-4 (aa 1-421), were constructed by PCR and epitope tagged with the FLAG sequence at the carboxyl terminus. All constructs of the mutants were verified by direct sequencing and subcloned into an expression vector, pUC-CAGGS (Ueno *et al.*, 1995). The Δ BH4 (lacking aa 10-31), the Δ loop (lacking aa 35-79), the Δ BH3 (lacking aa 100-118), and the Δ BH1-BH2 (lacking aa 118-239) mutants of human Bcl-2 were constructed by PCR or the site-directed mutagenesis and subcloned into the pcDNA3myc vector (tagged with the myc sequence). All these deletion mutants of Bcl-2 lack the transmembrane domain (aa 219-239). Rat IRS-3 and human Bax and Bik genes were isolated by reverse transcription-PCR of total RNA isolated from rat liver and human peripheral lymphocyte and sequenced by an autosequencer (Applied Biosystems, Foster City, CA). Rat IRS-3 cDNA was tagged with the FLAG sequence (DYLDLDDDL) or the HA (YPYDVPDYA) sequence at the 3' terminus. Human Bcl-2, Bcl-X_L, Bax, and Bik genes were subcloned into the pcDNA3myc vector and tagged with the myc sequence (EQKLLSEEDLN). Retrovirus vector pSR α SVtkneo was used to transfect cDNAs into Ba/F3 cells, as reported previously (Ueno *et al.*, 1996). To coexpress two cDNAs in Ba/F3 cells, we used an internal ribosomal entry site (IRES) that was subcloned between two cDNAs in pSR α SVtkneo. To construct the loop fragment tagged with the FLAG peptide (LF), the cDNA of human Bcl-2 corresponding to the region aa 31-75 was amplified and tagged with the FLAG sequence

by PCR. The construct was subcloned into an expression vector, pMX-puro vector (Onishi *et al.*, 1996). All constructs generated by PCR were verified by sequencing.

Transfection, Immunoprecipitation, Immunoblotting, and Kinase Assay

Transfection into 293 cells was carried out by the calcium phosphate method as described previously (Ueno *et al.*, 1995). To establish CHO-IR/IRS-1/Bcl-2 cells, Bcl-2/pSSR α -bsr (carrying the blasticidin-resistant gene) was introduced into the CHO-IR/IRS-1 cells (Yonezawa *et al.*, 1994), and stable clones were selected in the presence of 5 μ g/ml blasticidin (Funakoshi, Tokyo, Japan) as reported (Ueno *et al.*, 1995). Immunoprecipitation and immunoblotting were performed as described (Ueno *et al.*, 1995). To detect serine/threonine phosphorylation of Bcl-2 by insulin stimulation, cells were labeled with [³²P]orthophosphoric acid as described previously (Tobe *et al.*, 1993). Then cells were stimulated with insulin (100 nM) at 37°C for 5 min, lysed, and immunoprecipitated with anti-Bcl-2 antibody. The kinase activity of PI3K was measured as described previously (Yamamoto-Honda *et al.*, 1996).

Immunostaining

Cells were fixed in 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.1% Nonidet P-40 in PBS for 10 min, and blocked in PBS containing 5% normal goat serum (Life Technologies, Gaithersburg, MD) and 1 mg of BSA fraction V (Sigma, St. Louis, MO)/ml for 1 h. Then the cells were incubated with indicated antibodies for 1 h, followed by incubation with FITC- or Texas Red-conjugated goat secondary antibodies for 1 h. The cells were visualized under a Bio-Rad (Hercules, CA) MRC 1024 confocal microscope.

Survival Assay

For flow cytometry, cells were fixed with 70% ethanol for 5 h at -20°C, treated with 100 μ g/ml RNase A at 37°C for 30 min, and stained with 50 μ g/ml propidium iodide for 30 min. Then cells were subjected to fluorescence-activated cell sorting (FACS) analysis by a FACSort (Becton Dickinson, Mountain View, CA). The survival ratio was calculated as the rate of a number of viable cells to the total number of viable and apoptotic cells, which were counted after trypan blue staining.

RESULTS

Association between IRS Proteins and the Bcl-2 Family Proteins

We first examined the tyrosine-phosphorylated proteins associated with Bcl-2 when RTKs are activated by ligand binding. We used IM-9 cells for these experiments, because IM-9 cells, derived from B lymphocytes, express relatively large amounts of insulin receptors (Van *et al.*, 1976) and Bcl-2 (our unpublished results). When cells were stimulated with insulin, an ~180-kDa tyrosine-phosphorylated protein was coprecipitated with Bcl-2 (Figure 1A). Because the ~180-kDa proteins tyrosine phosphorylated with insulin stimulation are known to be IRS-1 (Sun *et al.*, 1991) and IRS-2 (Sun *et al.*, 1995), and IM-9 cells express more IRS-1 than IRS-2 (our unpublished results), we assumed that this phosphoprotein could be IRS-1. IRS proteins are substrates for many growth factors such as insulin (Sun *et al.*, 1991), insulin-like growth factor-1 (IGF-1) (Chuang *et al.*, 1993; Myers *et al.*, 1993), IL-4 (Keegan *et al.*, 1994), IL-9 (Vin *et al.*, 1995), IL-13 (Welham *et al.*, 1995), and growth hormone (GH) (Souza *et al.*, 1994; Ridderstrale *et al.*, 1995). So far, four IRS proteins, namely

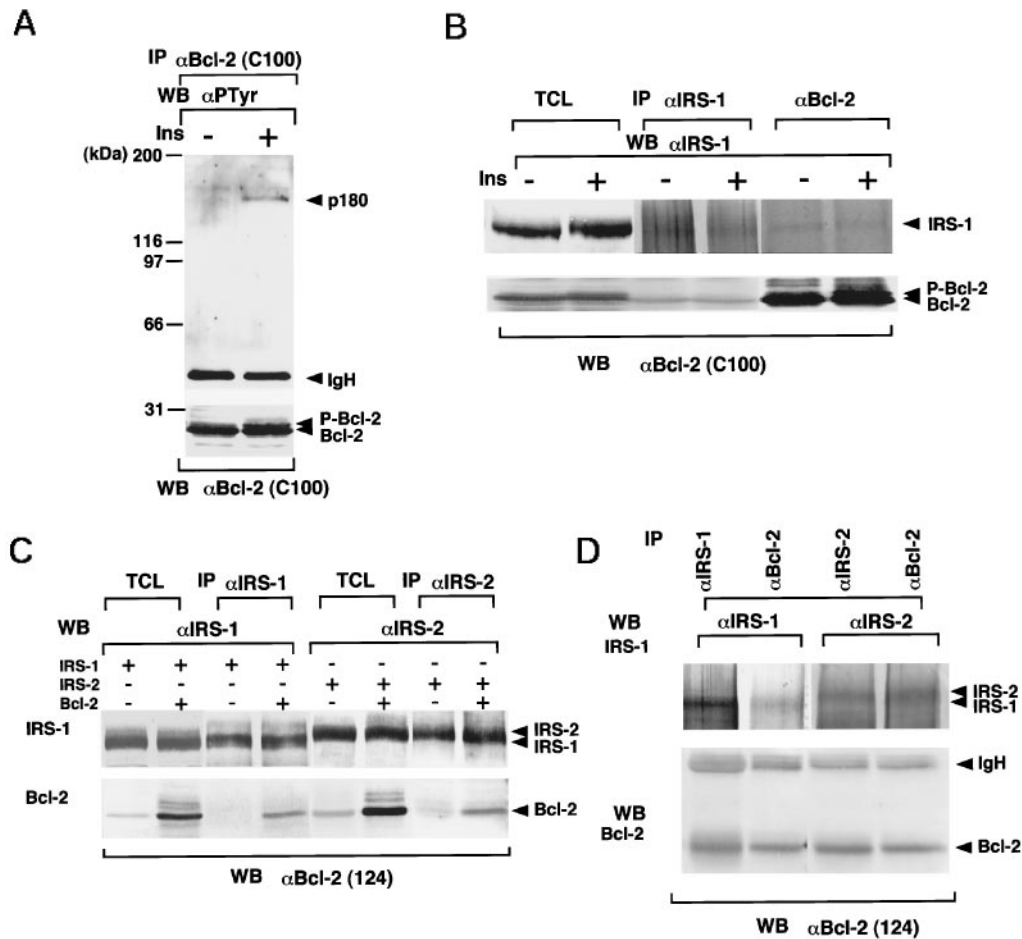


Figure 1. Association among IRS-1, IRS-2, and Bcl-2. (A) A 180-kDa tyrosine phosphoprotein associated with Bcl-2 in insulin-stimulated IM-9 cells. IM-9 cells stimulated with insulin (100 nM) at 37°C for 5 min were lysed, immunoprecipitated (IP) with C100 anti-Bcl-2 antibody, and immunoblotted (WB) with anti-phosphotyrosine antibody 4G10 (upper panel) or C100 (lower panel). P-Bcl-2, phosphorylated Bcl-2. (B) A 180-kDa tyrosine phosphoprotein associated with Bcl-2 in insulin-stimulated IM-9 cells is IRS-1. IM-9 cells were lysed, immunoprecipitated (IP) with J36 anti-IRS-1 antibody or C100 anti-Bcl-2 antibody, and immunoblotted (WB) with C-20 anti-IRS-1 antibody or C100 anti-Bcl-2 antibody. TCL, total cell lysates. (C) IRS-1 and IRS-2 associate with Bcl-2. Murine IRS-1 or IRS-2 in pRcCMV was cotransfected with human Bcl-2/pUC-CAGGS in 293 cells. Then lysates were immunoprecipitated and immunoblotted with indicated antibodies. +, transfected; -, not transfected. (D) IRS-1 and IRS-2 associate with Bcl-2 in murine hepatocytes. Two milligrams of lysates from murine hepatocytes (C57BL/6J Jcl mice) were immunoprecipitated and immunoblotted with the indicated antibodies.

IRS-1, IRS-2, IRS-3 (Lavan *et al.*, 1997b), and IRS-4 (Lavan *et al.*, 1997a), have been identified. Moreover, GAB1 (Holgado-Madruga *et al.*, 1996) and GAB2 (Gu *et al.*, 1998) genes have been cloned that are structurally related to IRS proteins. IRS-1, IRS-2, and IRS-4 are structurally closely related to each other (Lavan *et al.*, 1997a). However, IRS-3 lacks the region corresponding to the carboxyl half-domain of IRS-1 (Lavan *et al.*, 1997b). As shown in Figure 1B, IRS-1 and Bcl-2 are constitutively associated in IM-9 cells, and such association was not affected by insulin stimulation. As reported previously, phosphorylated Bcl-2 can be detected as a shifted band by using anti-Bcl-2 monoclonal antibody C100 (Figure 1, A and B) (Scatena *et al.*, 1998). In Figure 1B, we found that IRS-1 coprecipitated nonphosphorylated but not phosphorylated Bcl-2, indicating that IRS-1 does not bind to phosphorylated Bcl-2. The reason will be discussed later. By

cotransfection, IRS-2 also associated with Bcl-2 (Figure 1C). The association was also observed in the lysates from murine hepatocytes (Figure 1D), indicating that not only transfected but also endogenous IRS-1/IRS-2 and Bcl-2 associate with each other. IRS-1 also bound to Bcl-X_L but not to Bax or Bik (Figure 2A). Interestingly, Bcl-2 and Bcl-X_L are antiapoptotic members, and Bax and Bik are proapoptotic members of the Bcl-2 family proteins. To determine the domains of Bcl-2 and IRS-1 that bind with each other, we used deletion mutants of Bcl-2 and IRS-1 (Figure 2, B and C). As shown in Figure 2B, only the Δ loop mutant of Bcl-2 failed to bind to IRS-1, indicating that IRS-1 binds to the loop domain of Bcl-2. This result is reasonable because Bcl-2 and Bcl-X_L possess the loop domain, whereas Bax and Bik do not. On the other hand, using deletion mutants of IRS-1, we found that Bcl-2 binds to the carboxyl half-region of IRS-1 (amino acids

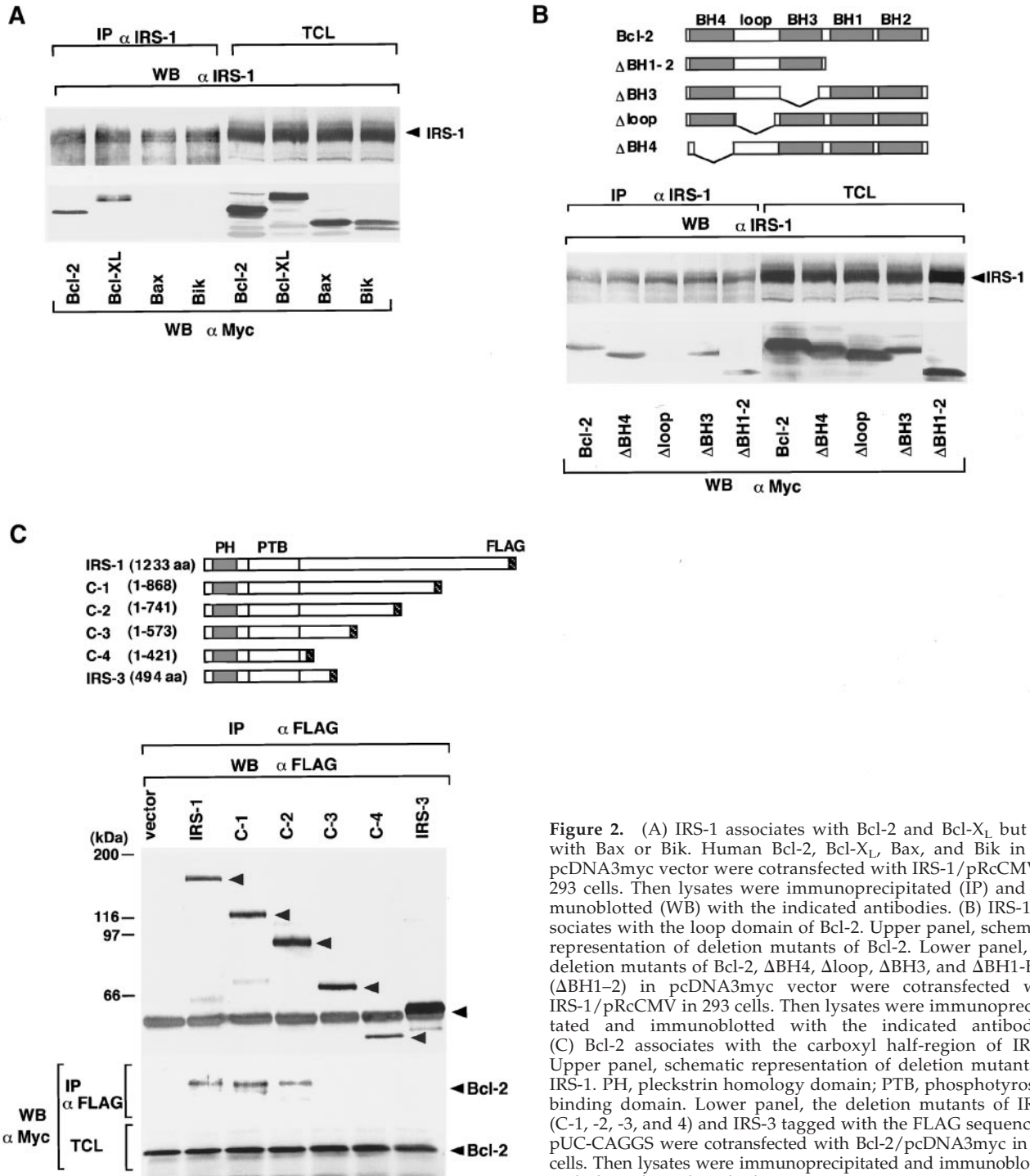


Figure 2. (A) IRS-1 associates with Bcl-2 and Bcl-X_L but not with Bax or Bik. Human Bcl-2, Bcl-X_L, Bax, and Bik in the pcDNA3myc vector were cotransfected with IRS-1/pRcCMV in 293 cells. Then lysates were immunoprecipitated (IP) and immunoblotted (WB) with the indicated antibodies. (B) IRS-1 associates with the loop domain of Bcl-2. Upper panel, schematic representation of deletion mutants of Bcl-2. Lower panel, the deletion mutants of Bcl-2, Δ BH4, Δ loop, Δ BH3, and Δ BH1-BH2 (Δ BH1-2) in pcDNA3myc vector were cotransfected with IRS-1/pRcCMV in 293 cells. Then lysates were immunoprecipitated and immunoblotted with the indicated antibodies. (C) Bcl-2 associates with the carboxyl half-region of IRS-1. Upper panel, schematic representation of deletion mutants of IRS-1. PH, pleckstrin homology domain; PTB, phosphotyrosine binding domain. Lower panel, the deletion mutants of IRS-1 (C-1, -2, -3, and 4) and IRS-3 tagged with the FLAG sequence in pUC-CAGGS were cotransfected with Bcl-2/pcDNA3myc in 293 cells. Then lysates were immunoprecipitated and immunoblotted with the indicated antibodies.

574-1233; Figure 2C). IRS-3 that lacks the corresponding domain of IRS-1 could not associate with Bcl-2 (Figure 2C).

Colocalization of IRS-1 and Bcl-2

It has been reported that IRS-1 localizes to the cytoplasm and that stimulation with insulin induces its association with the

insulin receptor through its phosphotyrosine binding domain (Craparo *et al.*, 1995; Eck *et al.*, 1996), whereas Bcl-2 localizes to the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane (Lithgow *et al.*, 1994). Because IRS-1 and Bcl-2 associated with each other (Figure 1B), we examined the subcellular localization of both

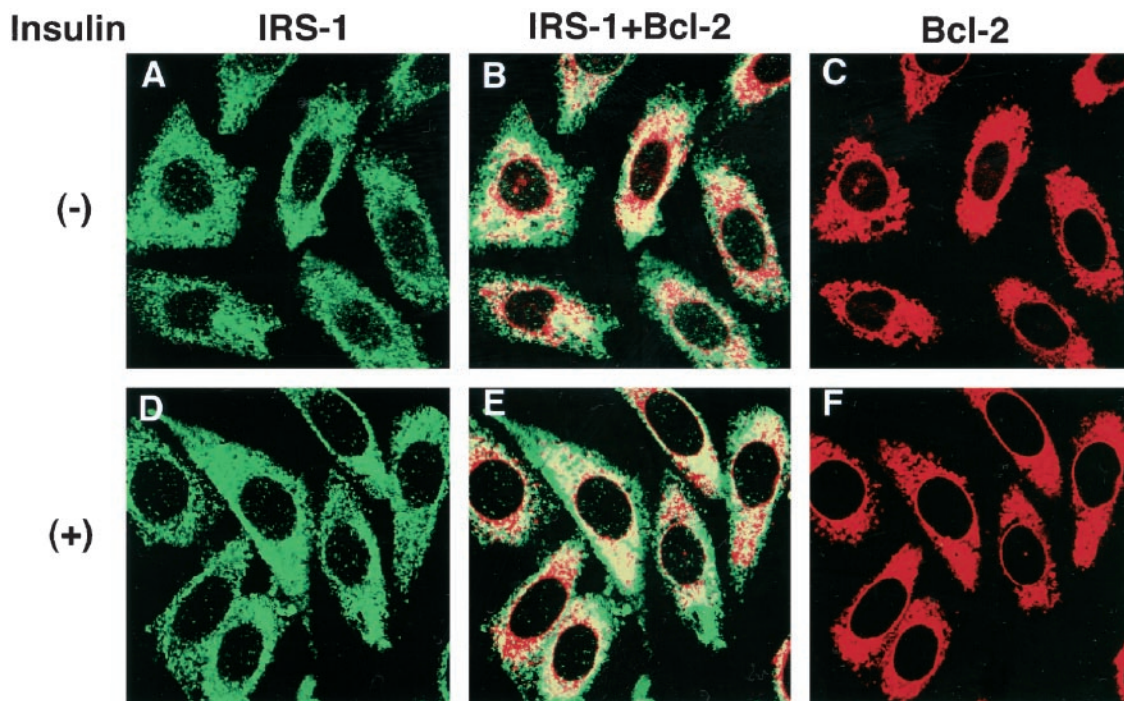


Figure 3. Colocalization of IRS-1 and Bcl-2. A part of IRS-1 colocalizes with Bcl-2, and insulin stimulation of cells does not affect localization of IRS-1 and Bcl-2. CHO-IR/IRS-1/Bcl-2 cells were treated with (D–F) or without (A–C) insulin (100 nM) at 37°C for 10 min. Then cells were fixed and incubated with anti-IRS-1 antibody (C-20; A, B, D, and E) and anti-Bcl-2 antibody (clone 124; B, C, E, and F), followed by FITC-conjugated antirabbit (for C-20) or Texas Red-conjugated anti-mouse (for anti-Bcl-2 antibody) secondary antibodies.

molecules and the effects of growth factor stimulation. To this end, we established a stable transfectant of CHO cells expressing the insulin receptor, IRS-1, and Bcl-2 (CHO-IR/IRS-1/Bcl-2 cells). By immunostaining with anti-IRS-1 and anti-Bcl-2 antibodies, IRS-1 and Bcl-2 were revealed to colocalize at the perinuclear region (Figure 3, A–F). Similar results were obtained when IRS-1 and Bcl-2 were cotransfected into NIH3T3 and COS7 cells (our unpublished results). When these cell lines were stimulated with insulin, however, there was no alteration in the subcellular localization of either molecule. The similar results were obtained when these cells were stimulated with insulin for a shorter or longer period (from 1 min up to 12 h) at a lower temperature (up to 4°C). Consistent with our data, a previous report regarding the subcellular localization of IRS-1 indicated that, after insulin stimulation, IRS-1 did not translocate to the plasma membrane and was tyrosine phosphorylated at the cytoplasm by internalization of activated insulin receptors (Kublaoui *et al.*, 1995).

IRS-1 Enhances Antiapoptotic Activity of Bcl-2

From the observations described above, we presumed that IRS proteins regulate the apoptosis of cells by modulating the Bcl-2 function. It has been shown that overexpression of Bcl-2 suppresses apoptosis of Ba/F3 cells when deprived of IL-3. To analyze the survival effects of IRS proteins and their regulatory mechanisms for Bcl-2, we examined whether they could suppress apoptosis of cells induced by IL-3 withdrawal. Ba/F3 cells express endogenous IRS-2 and Bcl-2;

however, the expression levels of them are relatively low. Therefore, we established stable transfectants of IRS-1, IRS-2, IRS-3, and Bcl-2, in which IRS-1 and IRS-2 but not IRS-3 associate with Bcl-2 (Figure 4A). We confirmed that the Ba/F3-derived cell lines used in these experiments express comparable levels of insulin receptors, and when treated with insulin, the levels of autophosphorylation of the insulin receptor were also approximately equal (Figure 4B). Interestingly, IRS-1 and IRS-2 could suppress apoptosis, but IRS-3 could not (Figures 4C and 5A). Because the results of the survival rate, calculated as described in MATERIALS AND METHODS, correlated well with those of the PI staining and the FACS analysis of cells (Figures 4C and 5A), we used the survival rate as an antiapoptotic level in the subsequent experiments. In the presence of a tyrosine kinase inhibitor, genistein (50 $\mu\text{g}/\text{ml}$), or in the absence of FCS, IRS-1 and IRS-2 could not suppress the apoptosis of cells (our unpublished results). We thus deduced that the IRS-1-mediated signals, which were activated by growth factors such as insulin and IGF-1 included in FCS, could be required to exert survival effects. In fact, the FCS we used in this study contained 15 nM insulin quantified by the immunoradiometric assay (IRMA). Moreover, even if the FCS concentration was reduced to 0.5%, we obtained similar results when insulin was added to the medium (10 nM; our unpublished results). Because IRS-1 and IRS-2 can interact with the PI3K-Akt pathway, it is reasonable to assume that they suppress apoptosis by activating the PI3K-Akt pathway. However, IRS-3 also has binding sites for the p85 subunit of

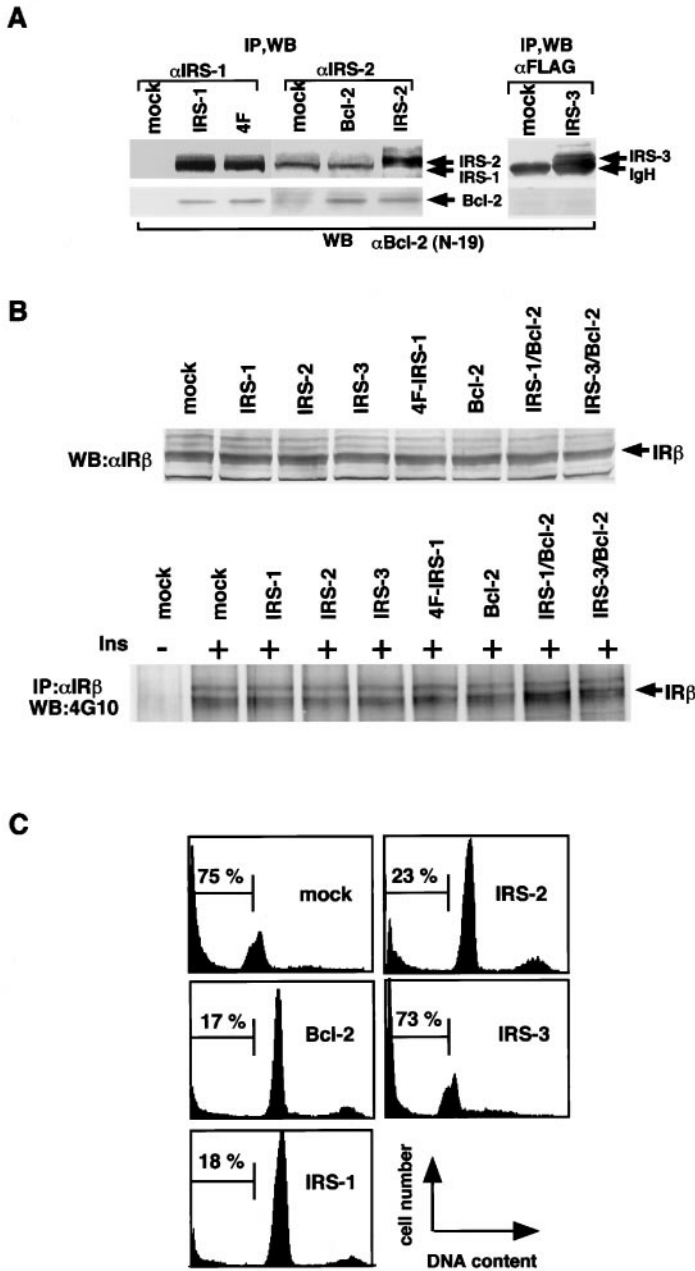


Figure 4. IRS-1 and IRS-2 but not IRS-3 suppress apoptotic cell death. (A) IRS-1, 4F-IRS-1 (4F), and IRS-2 but not IRS-3 associate with Bcl-2 in Ba/F3 cells. Lysates from the indicated cell lines were immunoprecipitated and immunoblotted with the indicated antibodies. P-Bcl-2, phosphorylated Bcl-2. (B) Upper panel, lysates from the indicated cell lines were immunoblotted with anti-insulin receptor β chain (α IR β). Lower panel, lysates from the indicated cell lines were immunoprecipitated and immunoblotted with indicated antibodies. (C) IRS-1 and IRS-2 but not IRS-3 suppress apoptosis of Ba/F3 cells deprived of IL-3. Stable transfectants of IRS-1, IRS-2, IRS-3, 4F-IRS-1, and Bcl-2 were deprived of IL-3 and cultured in RPMI 1640 medium containing 5% FCS. FCS used in this study contained 15 nM insulin quantified by IRMA. The FACS analysis was performed 48 h after IL-3 depletion.

PI3K (Lavan *et al.*, 1997b) and can activate PI3K (Figure 5B) but cannot suppress apoptosis (Figures 4C and 5A). On the other hand, a quadruple tyrosine-phenylalanine mutant of IRS-1, 4F-IRS-1, which lacks all the major binding sites for p85 (Tyr-460, Tyr-608, Tyr-939, and Tyr-987) and significantly loses the ability to activate PI3K (Yamamoto-Honda *et al.*, 1996; Figure 5B), can bind to Bcl-2 (Figure 4A) and suppressed apoptosis at a level similar to that of IRS-1 (Figure 5A). We next examined whether the series of truncated mutants of IRS-1 could suppress apoptosis and found that C-1 and C-2 could suppress apoptosis but C-3 and C-4 mutants could not (Figure 5C). Because C-1 and C-2 can bind

to Bcl-2, whereas C-3 and C-4 cannot (Figure 2C), these findings suggest that, to exert the survival effect of IRS-1, association with Bcl-2 is more important than association with the PI3K pathway. The next issue to be addressed is whether IRS-3 is merely unable to suppress apoptosis or whether it interferes with the antiapoptotic effect of the IRS-1/2-Bcl-2 pathway. To test this, we established three Ba/F3 lines coexpressing IRS-1/Bcl-2, IRS-3/Bcl-2, and IRS-1/IRS-3. Although the cells coexpressing IRS-1/Bcl-2 showed an enhanced survival rate compared with the cells expressing either one of them alone, the cells coexpressing IRS-3 and Bcl-2 underwent apoptosis as rapidly as the mock

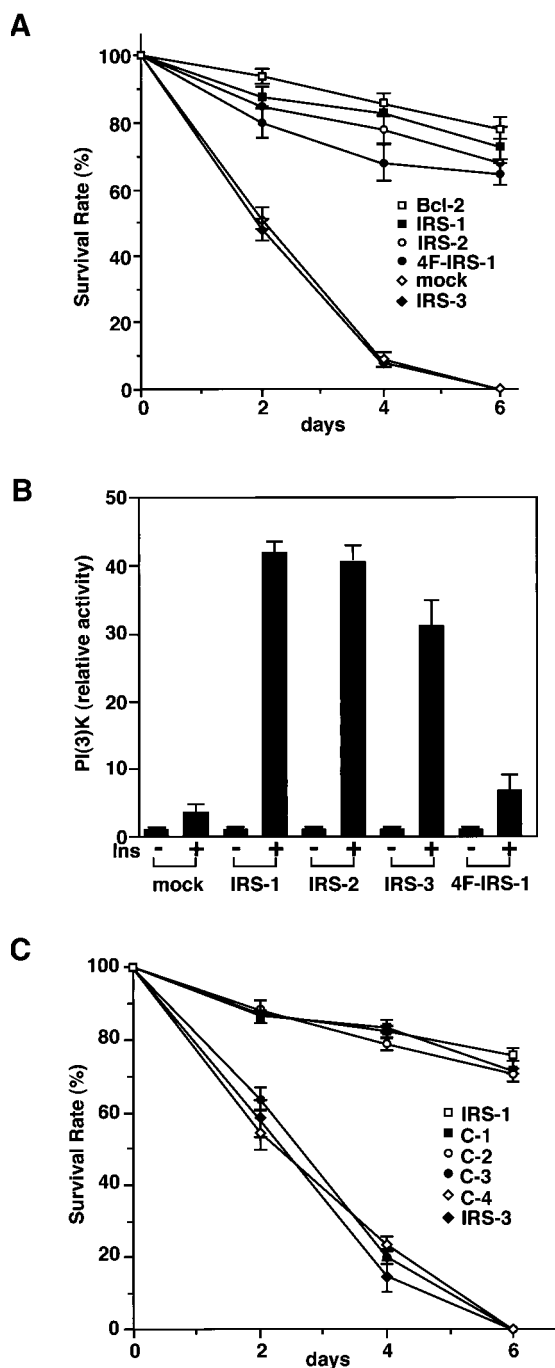


Figure 5. (A) IRS-1 and IRS-2 but not IRS-3 suppress apoptosis of Ba/F3 cells deprived of IL-3. Stable transfectants of IRS-1, IRS-2, IRS-3, 4F-IRS-1, Bcl-2, and mock cells were deprived of IL-3 and cultured in RPMI 1640 medium containing 5% FCS. FCS used in this study contained 15 nM insulin quantified by IRMA. (B) PI3K activity of insulin-stimulated cell lines. Stable transfectants of IRS-1, IRS-2, IRS-3, 4F-IRS-1, and mock cells treated with or without insulin were lysed and immunoprecipitated with 4G10. The immunoprecipitates were subjected to the PI3K assay. (C) Survival rate of stable transfectants expressing deletion mutants of IRS-1, C-1, C-2, C-3, and C-4.

cells, and the cells coexpressing IRS-1 and IRS-3 showed a significantly reduced survival rate compared with the cells expressing IRS-1 alone (Figure 6A), indicating that IRS-3 abrogates the survival effects of IRS-1 and Bcl-2. Consistent with this, tyrosine phosphorylation of endogenous IRS-2 was severely impaired in the cells expressing IRS-3 when stimulated with insulin (Figure 6B). This result was confirmed in the cells coexpressing IRS-1 and IRS-3 by the finding that phosphorylation of IRS-1 was significantly suppressed compared with that in the cells expressing IRS-1 alone (our unpublished results).

To ascertain the importance of association between IRS-1 and Bcl-2 in survival of cells, we constructed a deletion mutant of Bcl-2, which consists of the loop domain of human Bcl-2 (aa 31-75) epitope tagged with the FLAG peptide (LF). We expect that the LF peptide dominant-negatively blocks signals from IRS-1 to Bcl-2 and thus abolishes the antiapoptotic effects of IRS-1 and Bcl-2. We cotransfected the construct into mock, Bcl-2, and IRS-1 cells and examined the effect on the survival of cells. As expected, we found in the experiment that the cells transfected with the LF peptide become sensitive to apoptosis induced by IL-3 depletion under the same conditions of Figure 6A (Figure 6C).

IRS-1 Suppresses Phosphorylation of Bcl-2 Induced by Insulin

To investigate the mechanisms involved in the regulation of the antiapoptotic function of Bcl-2 by IRS proteins, we examined their effects on phosphorylation of Bcl-2, because serine/threonine phosphorylation sites are present within the loop domain of Bcl-2 (Chang *et al.*, 1997; Maundrell *et al.*, 1997). To confirm that the insulin treatment induces phosphorylation of Bcl-2, and the phosphorylation site is located within the loop domain, we labeled Ba/F3 cells expressing Bcl-2 and Δ loop-Bcl-2 with [³²P]orthophosphoric acid and stimulated with insulin. The deletion mutant of Bcl-2 that lacks the loop domain (Δ loop-Bcl-2) was not phosphorylated by insulin treatment (Figure 7A), indicating that the phosphorylation sites of Bcl-2 by insulin were located within the loop domain. Because the expression level of endogenous Bcl-2 in Ba/F3 cells is relatively low, we could not clearly compare the phosphorylation levels induced by insulin treatment (our unpublished results). Therefore, we used double transfectants of Ba/F3, IRS-1/Bcl-2, IRS-3/Bcl-2, and Bcl-2 cells that express approximately the same amount of Bcl-2 (Figure 7B). Insulin treatment of these cells induced phosphorylation of Bcl-2, but coexpression of IRS-1 significantly suppressed insulin-induced phosphorylation, whereas phosphorylation of Bcl-2 in IRS-3/Bcl-2 cells was rather enhanced compared with that in Bcl-2 cells (Figure 7B). Because the phosphorylation levels of Bcl-2 correlate well with the results of Figure 6A, they support our hypothesis that IRS-3 blocks activation of the IRS-1/2-Bcl-2 pathway in a dominant-negative manner and thus abrogates the survival effects of Bcl-2. To confirm the importance of the phosphorylation level of Bcl-2 in the regulation of apoptosis by IRS proteins, we next generated stable transfectants that coexpress the Δ loop mutant with mock, IRS-1, and IRS-3 cells. The Δ loop mutant lacks the phosphorylation site(s) by insulin stimulation (Figure 7A), and possesses an enhanced ability to inhibit apoptosis compared with the full-length protein (Chang *et al.*, 1997; Maundrell *et al.*, 1997). As ex-

IRS proteins regulate the phosphorylation of Bcl-2. As expected, we found that overexpression of IRS-1 suppresses

pected, IRS-3 cells coexpressing the Δ loop mutant became resistant to apoptosis. This result supports our hypothesis that the dominant-negative effect of IRS-3 is exerted through up-regulating phosphorylation of Bcl-2. In the Figure 8, we present our hypothesis on the mechanism of how IRS proteins regulate survival of cells through controlling phosphorylation of Bcl-2. We could not obtain data as to whether the association between IRS-1 and Bcl-2 is direct; therefore, it is possible that internal molecule(s) exist between them.

DISCUSSION

In this study, we searched for regulatory molecules of Bcl-2 in the signaling pathway of the insulin receptor and found that IRS-1 binds to the loop domain of Bcl-2. The association between IRS-1 and Bcl-2 was observed regardless of insulin stimulation (Figure 1B). However, IRS-1 could not coprecipitate phosphorylated Bcl-2, as shown in Figure 1B. We can raise two possibilities to explain these data. First, when phosphorylated, Bcl-2 dissociates from IRS-1. Second, a part of Bcl-2 not bound to IRS-1 is mainly phosphorylated with insulin treatment, whereas the rest of Bcl-2 constitutively associated with IRS-1 is not phosphorylated. Because nonphosphorylated Bcl-2 coprecipitated with IRS-1 and IRS-1 coprecipitated with Bcl-2 did not decrease with insulin stimulation (Figure 1B), we concluded that the latter possibility is more probable. Constitutive association between IRS-1 and Bcl-2 does not conflict with previous data as described in RESULTS. Kublaoui *et al.* (1995) reported that the distribution of IRS-1 is 80% cytosolic, 20% internal membrane associated, and essentially undetectable in the plasma membrane. After insulin stimulation, the phosphorylation state of IRS-1 in the internal membrane parallels that of the insulin receptor, but cytosolic IRS-1 phosphorylation remains constant. They hypothesized that insulin action is mediated by receptor internalization (Kublaoui *et al.*, 1995).

As shown in Figures 4 and 5, IRS-1 suppresses apoptotic cell death induced by growth factor withdrawal. The anti-apoptotic effects of IRS-1 and Bcl-2 were synergistic (Figure 6A). Our results that IRS-3 and the deletion mutants of IRS-1 that fail to bind to Bcl-2 (Figure 2C, C-3 and C-4) cannot suppress apoptosis (Figure 5C) indicate that the association between IRS-1 and Bcl-2 is important to suppress apoptosis. The data of Figure 6C support our assumption. Because IRS-1 binds to the loop domain of Bcl-2 that is the region of serine/threonine phosphorylation, it was most likely that

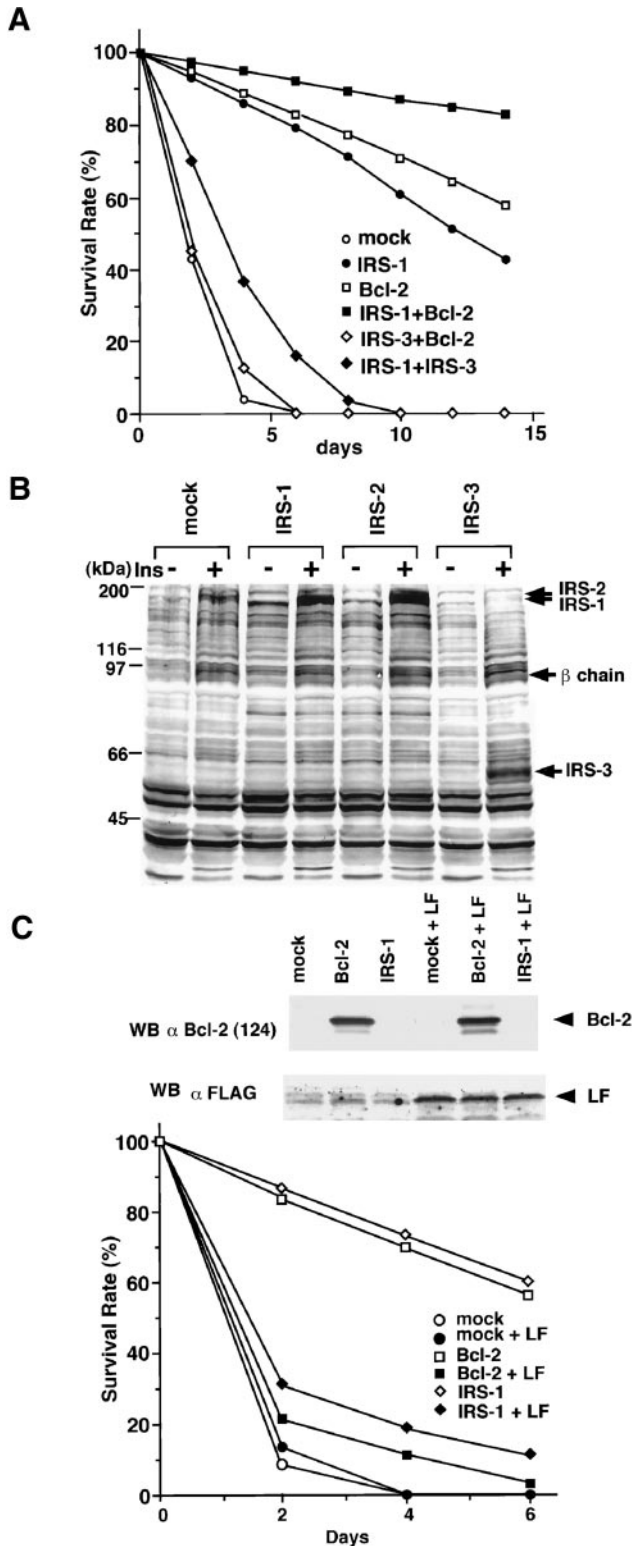


Figure 6. (A) Survival rate of double stable transfectants of Ba/F3 cells established by using retrovirus vectors carrying IRS-1-IRES-Bcl-2, IRS-3-HA-IRES-Bcl-2, or IRS-1-IRES-IRS-3, compared with those of Bcl-2, IRS-1, and Ba/F3 cells. (B) Stable transfectants expressing IRS-1, IRS-2, IRS-3, and mock cells were serum starved and stimulated with insulin (100 nM). Lysates from these cells were subjected to immunoblotting with 4G10. β chain, β subunit of the insulin receptor. (C) Coexpression of the loop fragment of Bcl-2 in Bcl-2 and IRS-1 cells severely impairs the survival of these cells. Upper panel, the lysates from indicated cell lines were subjected to the immunoblotting with anti-Bcl-2 (clone 124) or anti-FLAG M2 antibody. Lower panel, survival rate of indicated cell lines. LF, loop fragment tagged with the FLAG peptide.

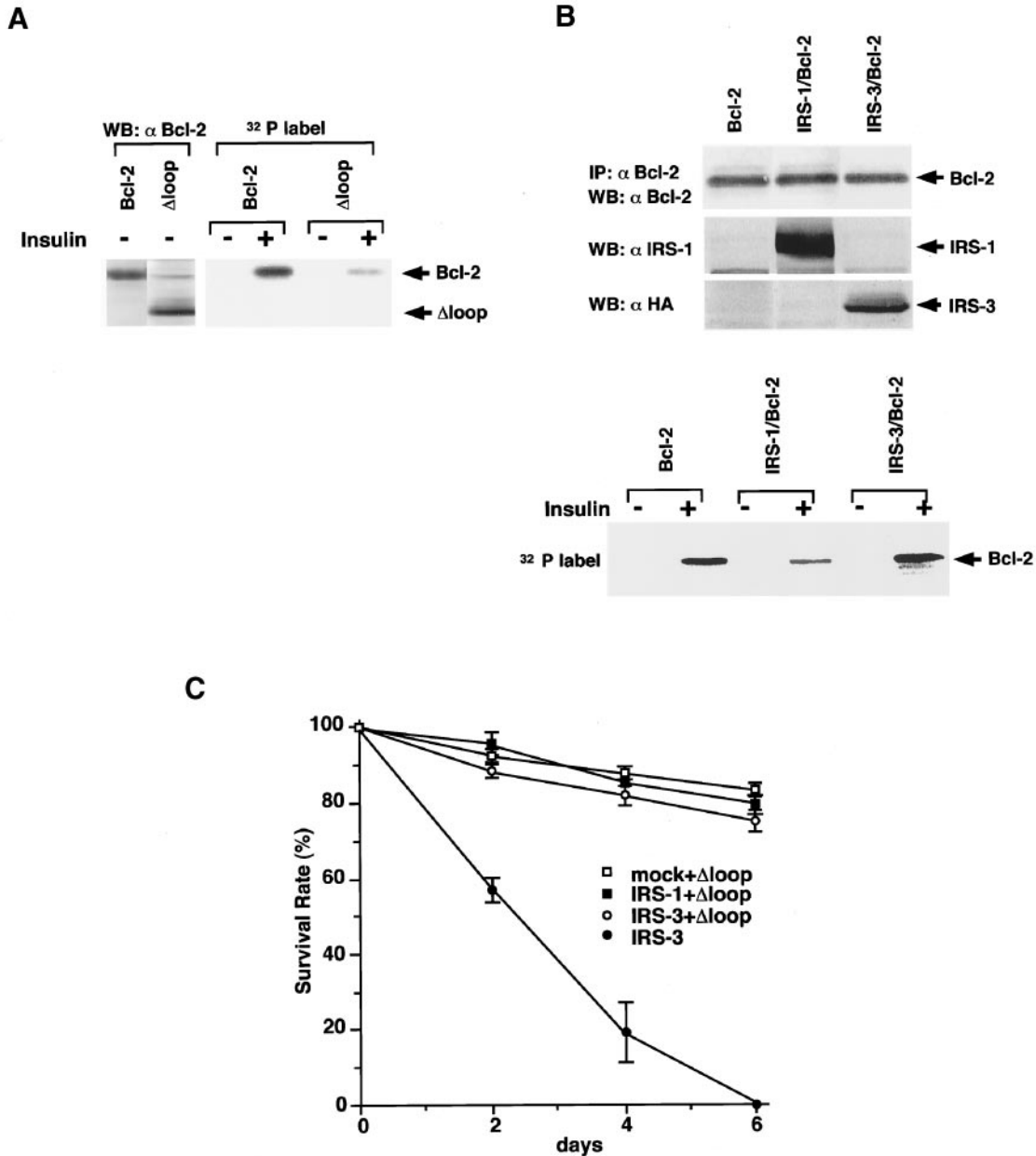


Figure 7. Effects of IRS proteins on phosphorylation of Bcl-2. (A) Insulin induces phosphorylation of Bcl-2 but not the Δloop mutant of Bcl-2. Left panel, total cell lysates from Bcl-2 and Δloop cells were subjected to immunoblotting with anti-Bcl-2 antibody (N-19). Right panel, Bcl-2 and Δloop cells were serum starved and labeled with [32P]orthophosphoric acid. Then cells were stimulated with or without insulin (100 nM), lysed, immunoprecipitated with anti-Bcl-2 antibody, and subjected to SDS-PAGE. The phosphorylated proteins were visualized by autoradiography. Δloop, deletion mutant of Bcl-2 lacking the loop domain. (B) IRS-1 but not IRS-3 suppresses insulin-induced phosphorylation of Bcl-2. Bcl-2, IRS-1/Bcl-2, and IRS-3-HA/Bcl-2 cells were serum starved and labeled with [32P]orthophosphoric acid. Phosphorylation of Bcl-2 was detected as in A. The expression levels of IRS-1, IRS-3, and Bcl-2 were indicated by immunoblotting with the indicated antibodies. (C) Survival rate of double stable transfectants of Ba/F3 cells established by using retrovirus vectors carrying IRS-1-HA-IRES-Δloop and IRS-3-HA-IRES-Δloop, compared with those of Δloop and IRS-3 cells. Δloop, deletion mutant of Bcl-2 lacking the loop domain.

IRS proteins regulate the phosphorylation of Bcl-2. As expected, we found that overexpression of IRS-1 suppresses phosphorylation of Bcl-2 and that IRS-3 has an opposite effect (Figure 7B).

The roles of phosphorylation of Bcl-2 are controversial. Many papers argue that dephosphorylation of Bcl-2 enhances the antiapoptotic function of Bcl-2 and phosphorylation inactivates its effects (Haldar *et al.*, 1995; Chang *et al.*,

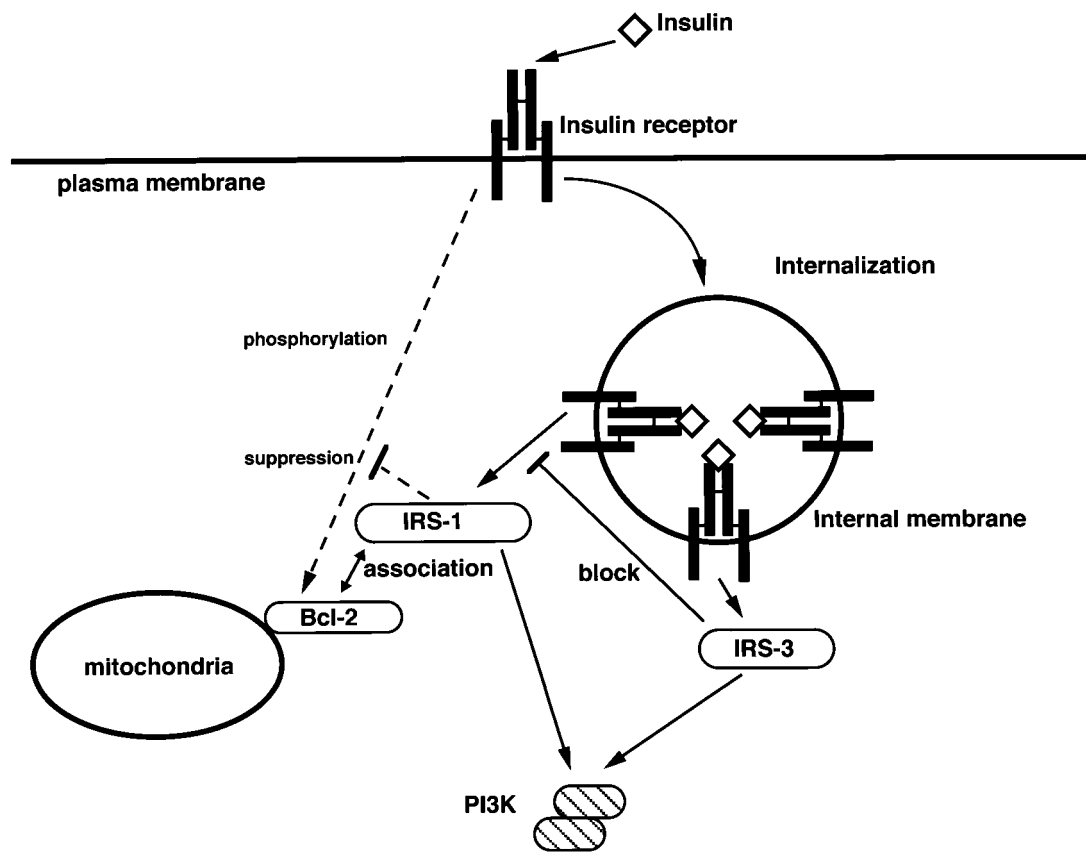


Figure 8. Schematic representation of our proposed model for the role of association between IRS-1 and Bcl-2 (see DISCUSSION). The pathways activated by unknown mechanisms are indicated by dotted lines.

1997; Maundrell *et al.*, 1997), although some reports contradict them (Ito *et al.*, 1997; Ruvolo *et al.*, 1998). Moreover, several recent studies insist that phosphorylation of Bcl-2 is a marker of M phase events (Ling *et al.*, 1998; Scatena *et al.*, 1998). However, the well-described phenomena that the loop deletion mutants of Bcl-2 possess enhanced antiapoptotic activity than wild-type Bcl-2 (Chang *et al.*, 1997; Maundrell *et al.*, 1997; Srivastava *et al.*, 1999; Wang *et al.*, 1999) cannot be explained only by their hypothesis. From the standpoint of the first group, it can be interpreted as follows; the loop-deleted mutant is not phosphorylated; therefore, its antiapoptotic activity is up-regulated. Because phosphorylation of Bcl-2 and cell survival inversely correlate well (Figures 4C, 5A, and 7B) and, by coexpressing the Δ loop mutant, IRS-3 cells became resistant to apoptosis (Figure 7C), our results support the first hypothesis. However, it is possible that phosphorylation of Bcl-2 can be independently regulated by the signal transduction pathway downstream of growth factor receptors and by cell cycle.

We could not present data indicating the mechanism for how IRS-1 suppresses phosphorylation of Bcl-2. From Figure 7, A and B, we can conclude that 1) insulin stimulation result in serine/threonine phosphorylation of Bcl-2 at the loop domain; 2) the expression level of IRS-1 is in inverse proportion to phosphorylation of Bcl-2; and 3) IRS-3 opposes the effect of IRS-1. Because, without insulin and FCS, the

survival effects of IRS-1 were not exerted, and the tyrosine kinase inhibitor genistein abolished its effects, we expect that the signals mediated through IRS-1 enhance the activity of serine/threonine phosphatases that dephosphorylate Bcl-2. Indeed, we have preliminary data that serine/threonine phosphatase inhibitors such as okadaic acid and cypermethline induce apoptosis of IRS-1/Bcl-2 cells at the same condition as Figure 6A (our unpublished results).

Several reports have already indicated that IRS-1 possesses an antiapoptotic activity. Growth factors such as insulin (Sun *et al.*, 1991), IGF (Chuang *et al.*, 1993; Myers *et al.*, 1993), and IL-4 (Keegan *et al.*, 1994) that use IRS-1 as a substrate for intracellular signaling are known to suppress apoptotic cell death (Barres *et al.*, 1992, 1993; Parry *et al.*, 1994). Hepatocellular carcinoma cell lines overexpressing IRS-1 are resistant to apoptosis (Tanaka and Wands, 1996b). However, when an IRS-1 mutant lacking the carboxyl-terminal domain is introduced into such cell lines, the cell becomes sensitive to apoptosis (Tanaka and Wands, 1996a). These reports suggest that IRS-1 has a critical role in tumorigenesis, and the carboxyl-terminal domain is essential for these activities. From our data (Figures 5C and 6A), we can deduce that the carboxyl-terminal-truncated mutant of IRS-1 dominant-negatively blocks the IRS-1/Bcl-2 cascade to induce apoptosis of hepatocellular carcinoma cells.

Recently, CHICO, a *Drosophila* homologue of vertebrate IRS-1–4, was reported to play an essential role in the control of cell size and growth of flies (Bohni *et al.*, 1999). Moreover, a recent study demonstrated that knockout mice lacking both IRS-1 and IRS-2 (*Irs-1^{-/-}Irs-2^{-/-}*) are lethal. The heterozygotes (*Irs-1^{-/-}Irs-2^{+/-}*) mice showed severe growth retardation. They also showed that apoptosis of the islet β cells of IRS-2-deficient mice were increased. These data indicate that, in mammals, IRS proteins are critical for development and regulation of apoptosis (Withers *et al.*, 1999). Taken together, our findings that IRS proteins associate with Bcl-2 and modulate its antiapoptotic function might provide a clue to understand the mechanism of how growth factors regulate cell survival, apoptosis, and organ development.

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REFERENCES

- Barres, B.A., Hart, I.K., Coles, H.S., Burne, J.F., Voyvodic, J.T., Richardson, W.D., and Raff, M.C. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70, 31–46.
- Barres, B.A., Schmid, R., Sendtner, M., and Raff, M.C. (1993). Multiple extracellular signals are required for long-term oligodendrocyte survival. *Development* 118, 283–295.
- Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1–4. *Cell* 97, 865–875.
- Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. (1993). *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74, 597–608.
- Boyd, J.M., *et al.* (1995). Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene* 11, 1921–1928.
- Chang, B.S., Minn, A.J., Muchmore, S.W., Fesik, S.W., and Thompson, C.B. (1997). Identification of a novel regulatory domain in Bcl-X(L) and Bcl-2. *EMBO J.* 16, 968–977.
- Chuang, L.M., Myers, M.J., Seidner, G.A., Birnbaum, M.J., White, M.F., and Kahn, C.R. (1993). Insulin receptor substrate 1 mediates insulin and insulin-like growth factor I-stimulated maturation of *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* 90, 5172–5175.
- Cleary, M.L., Smith, S.D., and Sklar, J. (1986). Cloning and structural analysis of cDNAs for *bcl-2* and a hybrid *bcl-2*/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* 47, 19–28.
- Craparo, A., O'Neill, T.J., and Gustafson, T.A. (1995). NonSH2 domains within insulin receptor substrate-1 and SHC mediate their phosphotyrosine-dependent interaction with the NPEY motif of the insulin-like growth factor I receptor. *J. Biol. Chem.* 270, 15639–15643.
- Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91, 231–241.
- del Peso, L., Gonzalez, G.M., Page, C., Herrera, R., and Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278, 687–689.
- Eck, M.J., Dhe, P.S., Trub, T., Nolte, R.T., and Shoelson, S.E. (1996). Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. *Cell* 85, 695–705.
- Gu, H., Pratt, J.C., Burakoff, S.J., and Neel, B.G. (1998). Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. *Mol. Cell* 2, 729–740.
- Haldar, S., Jena, N., and Croce, C.M. (1995). Inactivation of Bcl-2 by phosphorylation. *Proc. Natl. Acad. Sci. USA* 92, 4507–4511.
- Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L.J., Taylor, S.S., Scott, J.D., and Korsmeyer, S.J. (1999). Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. *Mol. Cell* 3, 413–422.
- Holgado-Madruga, M., Emlet, D.R., Moscatello, D.K., Godwin, A.K., and Wong, A.J. (1996). A Grb2-associated docking protein in EGF- and insulin-receptor signaling. *Nature* 379, 560–564.
- Ito, T., Deng, X., Carr, B., and May, W.S. (1997). Bcl-2 phosphorylation required for antiapoptosis function. *J. Biol. Chem.* 272, 11671–11673.
- Jacobson, M.D., Weil, M., and Raff, M.C. (1997). Programmed cell death in animal development. *Cell* 88, 347–354.
- Keegan, A.D., Nelms, K., White, M., Wang, L.M., Pierce, J.H., and Paul, W.E. (1994). An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1 phosphorylation and cell growth. *Cell* 76, 811–820.
- Klesse, L.J., and Parada, L.F. (1998). p21 ras and phosphatidylinositol-3 kinase are required for survival of wild-type and NF1 mutant sensory neurons. *J. Neurosci.* 18, 10420–10428.
- Kublaoui, B., Lee, J., and Pilch, P.F. (1995). Dynamics of signaling during insulin-stimulated endocytosis of its receptor in adipocytes. *J. Biol. Chem.* 270, 59–65.
- Kurada, P., and White, K. (1998). Ras promotes cell survival in *Drosophila* by downregulating hid expression. *Cell* 95, 319–329.
- Lavan, B.E., Fantin, V.R., Chang, E.T., Lane, W.S., Keller, S.R., and Lienhard, G.E. (1997a). A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. *J. Biol. Chem.* 272, 21403–21407.
- Lavan, B.E., Lane, W.S., and Lienhard, G.E. (1997b). The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J. Biol. Chem.* 272, 11439–11443.
- Ling, Y.H., Tornos, C., and Perez-Soler, R. (1998). Phosphorylation of Bcl-2 is a marker of M phase events and not a determinant of apoptosis. *J. Biol. Chem.* 273, 18984–18991.
- Lithgow, T., Van, D.R., Bertram, J.F., and Strasser, A. (1994). The protein product of the oncogene *bcl-2* is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. *Cell Growth & Differ.* 5, 411–417.
- Maudrell, K., *et al.* (1997). Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. *J. Biol. Chem.* 272, 25238–25242.
- Myers, M.J., Sun, X.J., Cheatham, B., Jachna, B.R., Glasheen, E.M., Backer, J.M., and White, M.F. (1993). IRS-1 is a common element in insulin and insulin-like growth factor-I signaling to the phosphatidylinositol 3'-kinase. *Endocrinology* 132, 1421–1430.

- Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74, 609–619.
- Onishi, M., *et al.* (1996). Applications of retrovirus-mediated expression cloning. *Exp. Hematol.* 24, 324–329.
- Parry, S.L., Hasbold, J., Holman, M., and Klaus, G.G. (1994). Hypercross-linking surface IgM or IgD receptors on mature B cells induces apoptosis that is reversed by costimulation with IL-4 and anti-CD40. *J. Immunol.* 152, 2821–2829.
- Ridderstrale, M., Degerman, E., and Tornqvist, H. (1995). Growth hormone stimulates the tyrosine phosphorylation of the insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase in primary adipocytes. *J. Biol. Chem.* 270, 3471–3474.
- Ruvolo, P.P., Deng, X., Carr, B.K., and May, W.S. (1998). A functional role for mitochondrial protein kinase Calpha in Bcl2 phosphorylation and suppression of apoptosis. *J. Biol. Chem.* 273, 25436–25442.
- Scatena, C.D., Stewart, Z.A., Mays, D., Tang, L.J., Keefer, C.J., Leach, S.D., and Pietenpol, J.A. (1998). Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and Taxol-induced growth arrest. *J. Biol. Chem.* 273, 30777–30784.
- Souza, S.C., Frick, G.P., Yip, R., Lobo, R.B., Tai, L.R., and Goodman, H.M. (1994). Growth hormone stimulates tyrosine phosphorylation of insulin receptor substrate-1. *J. Biol. Chem.* 269, 30085–30088.
- Srivastava, R.K., Mi, Q.S., Hardwick, J.M., and Longo, D.L. (1999). Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. *Proc. Natl. Acad. Sci. USA* 96, 3775–3780.
- 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* 352, 73–77.
- Sun, X.J., Wang, L.M., Zhang, Y., Yenush, L., Myers, M.J., Glasheen, E., Lane, W.S., Pierce, J.H., and White, M.F. (1995). Role of IRS-2 in insulin and cytokine signaling. *Nature* 377, 173–177.
- Tanaka, S., and Wands, J.R. (1996a). A carboxy-terminal truncated insulin receptor substrate-1 dominant negative protein reverses the human hepatocellular carcinoma malignant phenotype. *J. Clin. Invest.* 98, 2100–2108.
- Tanaka, S., and Wands, J.R. (1996b). Insulin receptor substrate 1 overexpression in human hepatocellular carcinoma cells prevents transforming growth factor beta1-induced apoptosis. *Cancer Res.* 56, 3391–3394.
- Tobe, K., *et al.* (1993). Insulin stimulates association of insulin receptor substrate-1 with the protein abundant Src homology/growth factor receptor-bound protein 2. *J. Biol. Chem.* 268, 11167–11171.
- Ueno, H., Hirano, N., Kozutsumi, H., Sasaki, K., Tanaka, T., Yazaki, Y., and Hirai, H. (1995). An epidermal growth factor receptor-leukocyte tyrosine kinase chimeric receptor generates ligand-dependent growth signals through the Ras signaling pathway. *J. Biol. Chem.* 270, 20135–20142.
- Ueno, H., Sasaki, K., Kozutsumi, H., Miyagawa, K., Mitani, K., Yazaki, Y., and Hirai, H. (1996). Growth and survival signals transmitted via two distinct NPXY motifs within leukocyte tyrosine kinase, an insulin receptor-related tyrosine kinase. *J. Biol. Chem.* 271, 27707–27714.
- Van, O.E., De, M.P., and Roth, J. (1976). Cell surface receptors for insulin and human growth hormone. Effect of microtubule and microfilament modifiers. *J. Biol. Chem.* 251, 6844–6851.
- Vin, T., Keller, S.R., Quelle, F.W., Witthuhn, B.A., Tsang, M.L., Lienhard, G.E., Ihle, J.N., and Yang, Y.C. (1995). Interleukin-9 induces tyrosine phosphorylation of insulin receptor substrate-1 via JAK tyrosine. *J. Biol. Chem.* 270, 20497–20502.
- Walker, F., Kato, A., Gonez, L.J., Hibbs, M.L., Pouliot, N., Levitzki, A., and Burgess, A.W. (1998). Activation of the Ras/mitogen-activated protein kinase pathway by kinase-defective epidermal growth factor receptors results in cell survival but not proliferation. *Mol. Cell. Biol.* 18, 7192–7204.
- Wang, S., Wang, Z., Boise, L., Dent, P., and Grant, S. (1999). Loss of the bcl-2 phosphorylation loop domain increases resistance of human leukemia cells (U937) to paclitaxel-mediated mitochondrial dysfunction and apoptosis. *Biochem. Biophys. Res. Commun.* 259, 67–72.
- Welham, M.J., Learmonth, L., Bone, H., and Schrader, J.W. (1995). Interleukin-13 signal transduction in lymphohemopoietic cells. Similarities and differences in signal transduction with interleukin-4 and insulin. *J. Biol. Chem.* 270, 12286–12296.
- Withers, D.J., Burks, D.J., Towery, H.H., Altamuro, S.L., Flint, C.L., and White, M.F. (1999). Irs-2 coordinates Igf-1 receptor-mediated beta-cell development and peripheral insulin signaling. *Nat. Genet.* 23, 32–40.
- Yamamoto-Honda, R., *et al.* (1996). Mutant of insulin receptor substrate-1 incapable of activating phosphatidylinositol 3-kinase did not mediate insulin-stimulated maturation of *Xenopus laevis* oocytes. *J. Biol. Chem.* 271, 28677–28681.
- Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 80, 285–291.
- Yonezawa, K., *et al.* (1994). Signal transduction pathways from insulin receptors to Ras. Analysis by mutant insulin receptors. *J. Biol. Chem.* 269, 4634–4640.