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Overexpression of Insulin Receptor Substrate-1, But Not Insulin Receptor Substrate-2, Protects a T Cell Hybridoma from Activation-Induced Cell Death1

Li Li, Xiulan Qi, Mark Williams, Yufang Shi, and Achsah D. Keegan2

The insulin receptor substrate (IRS) family of signaling molecules is expressed in lymphocytes, although their functions in these cells is largely unknown. To investigate the role of IRS in the protection of T cells from activation-induced cell death (AICD), we transfected the T cell hybridoma A1.1, which is IL-4 responsive but lacks expression of IRS family members with cDNA encoding IRS1 or IRS2. Stimulation of these clones with immobilized anti-CD3-induced expression of CD69 to the same level as the parental A1.1 cells. However, the A1.1 IRS1-expressing cells were markedly resistant to AICD, while the A1.1 IRS2-expressing cells were not. Inhibition of phosphatidylinositol 3'-kinase in the A1.1 IRS1-expressing cells did not abrogate their resistance to AICD. Fas mRNA was induced similarly by anti-CD3 in A1.1, A1.1 IRS1-expressing, and A1.1 IRS2-expressing cells. However, induction of Fas ligand (FasL) mRNA and functional FasL protein was delayed and decreased in IRS1-expressing cells, but not in IRS2-expressing cells. The induction of transcription from a 500-bp FasL promoter and a minimal 16-mer early growth response element linked to luciferase was also impaired in the IRS1-expressing cells. These results suggest that overexpression of IRS1, but not IRS2, protects A1.1 cells from AICD by diminishing FasL transcription through a pathway that is independent of the tyrosine phosphorylation of IRS1 and phosphatidylinositol 3'-kinase activity. The Journal of Immunology, 2002, 168: 6215–6223.

T lymphocyte homeostasis is critical for normal immune function. One means of regulating T cell homeostasis is through a process termed activation-induced cell death (AICD)1 (1). Activation of the TCR using specific Ag or Abs directed against TCR components induces the expression of a number of genes, including IL-2, CD69, and Fas ligand (FasL) (2). The interaction of the induced FasL with cell surface Fas results in cell death via apoptosis and the down-modulation of immune responses (3). Aberrant expression or function of Fas or FasL has been implicated in a number of diseases, including lymphoproliferative disease, autoimmunity, and cancer (3–8). The cytokine environment during T cell activation influences T cell differentiation and sensitivity to AICD (9). In the presence of IL-12, activated T cells differentiate into Th1, which secrete IL-2, TNF-α, and IFN-γ. Th1 cells have been reported to be sensitive to AICD (10). In the presence of IL-4, activated T cells differentiate into Th2 cells that produce IL-4, IL-5, IL-6, and IL-10. Th2 clones have been reported to express less FasL than Th1 clones after activation (10) and primary Th2 cultures have been shown to be intrinsically resistant to Fas signaling (10–13).

Since IL-4 is a critical factor in the differentiation of T cells to the Th2 type, it is possible that an IL-4-regulated cellular response participates in the development of the AICD-resistant phenotype. The binding of IL-4 to its cell surface receptor complex activates at least two independent signaling pathways (14, 15). One pathway, STAT6, is necessary for maximal Th2 cell differentiation (but not absolutely required) (16, 17). The second pathway, the insulin receptor substrate pathway (IRS), is very strongly up-regulated during the differentiation of Th2 cells, but not Th1 cells (16).

IRS family proteins play a central role in signal transduction by receptors for insulin, insulin-like growth factor I, and a growing number of cytokines, including IL-4 (17). The IRS proteins can become tyrosine phosphorylated on multiple tyrosine residues following ligand stimulation. These phosphorylated motifs then interact with proteins containing Src homology 2 domains such as the regulatory subunit of phosphatidylinositol 3'-kinase (PI-3K), growth factor receptor binding protein 2, Nck, c-Fyn, and the Src homology domain-containing protein tyrosine phosphatase-2 (SHP-2) (18). These signaling intermediates stimulate a variety of downstream biological effects, including mitogenesis, gene expression, glucose transport, and suppression of apoptosis. In addition to tyrosine phosphorylation sites, the IRS proteins contain numerous serine/threonine phosphorylation sites. Some of these sites are constitutively phosphorylated, while others appear to be regulated by extrinsic signals. It has been shown that Ser247 in IRS1 is phosphorylated by c-Jun NH2-terminal kinase (JNK) in response to TNF-α or anisomycin treatment and that this phosphorylation negatively modulates insulin signaling (19). While there have been many studies on the effect of tyrosine phosphorylation on downstream signals mediated by the IRS family of docking proteins, very little is known about the function of serine/threonine phosphorylation.

The IRS family is made up of four family members, IRS-1, -2, -3, and -4. Lymphocytes express IRS1 and/or IRS2 (18). These members share similar overall structure, including an NH2-terminal pleckstrin homology and protein tyrosine kinase binding domain as
well as a -COOH-terminal region containing numerous tyrosine phosphorylation sites. The common structure suggests some similarity of function among the IRS proteins. Indeed, all four IRS family members are able to associate with the p85 subunit of PI-3K via YXXM motifs, and both IRS1 and IRS2 can mediate anti-apoptotic functions through the regulation of PI-3K activity in some transfected cell lines (20). While many studies have focused on the similarities, newer analyses are beginning to reveal differences. Comparisons between IRS1 and IRS2 have found differences in their efficiency at recruiting SH2-domain containing molecules after insulin or IL-4 treatment (18). Moreover, the expression of IRS2 in fibroblasts lacking IRS1 does not reconstitute normal insulin or insulin-like growth factor I responses, suggesting that these two molecules can have important nonredundant function (21). Regions of the IRS molecules between the major modules are not highly homologous; however, very little is known about how they may contribute to signaling. Interestingly, IRS1 lacking all tyrosine residues retains the ability to mediate a mitogenic response to insulin in transfected cells, suggesting the presence of phosphotyrosine-independent mechanisms of signaling by IRS family members (22).

Normal T cells have been shown to express IRS family members to varying degrees. Human and murine thymocytes and human peripheral T cells express both IRS-1 and IRS-2 (23–25). Murine T cells express IRS2 and low levels of IRS-1 (16, 18, 26). Murine Th2 clones express large amounts of IRS2, and in vitro differentiation in the presence of IL-4, Th2 cells acquire elevated expression of IRS2, while in Th1 cells IRS2 expression remains low (16). Several murine thymic lymphoma cell lines demonstrate constitutive tyrosine phosphorylation of both IRS1 and IRS2 (A. D. Keegan, unpublished observation).

The role the IRS family members play in the regulation of T cell growth and survival is not clear. There is no major immunological change in mice lacking IRS1 or IRS3 (27, 28). In mice lacking IRS2 there is no gross change in immune function (29); however, there is a modest reduction in IL-5 production by Th2 cells, with no effect on T cell survival (26). Given the many redundant functions of the IRS family members, it is possible that the function of one member may be compensated for by that of another during development.

Therefore, to address the role of IRS family members in T cell function, we used a T cell hybridoma cell line, A1.1, that lacks expression of all IRS family members, but demonstrates IL-4-induced tyrosine phosphorylation of STAT6. These cells have been extensively used to analyze the signaling pathways activated by the TCR that lead to regulation of Fas and FasL expression and AICD (1, 30, 31). To investigate whether IRS proteins can influence AICD, we transfected IRS1 or IRS2 cDNA into A1.1 cells. We found that overexpression of IRS1, but not IRS2, protected A1.1 cells from AICD primarily through decreased induction of FasL expression.

## Materials and Methods

### Cells, reagents, and Abs

The murine T cell hybridoma (A1.1) (1) was maintained in RPMI 1640 complete medium (CM; Life Technologies, Gaithersburg, MD) that was supplemented with 2 mM L-glutamine, 50 mM 2-ME, 10% heat-inactivated FBS (Sigma, St. Louis, MO), and 1% penicillin/streptomycin (BioWhittaker, Walkersville, MD). The target cell lines L1210 and L1210 expressing Fas (obtained from Dr. P. Golstein, Centre National de la Recherche Scientifique-Institut National de la Santé et de la Recherche Médicale, Marseille, France) were maintained in RPMI 1640 CM. L cells and L cells expressing FasL were obtained from Dr. T. A. Ferguson (Washington University School of Medicine, St. Louis, MO) and were maintained in DMEM-CM. PMA and LY294002 were obtained from Calbiochem (San Diego, CA), FITC anti-mouse CD69 and anti-phaophotyrosine were obtained from BD Biosciences (San Diego, CA). Anti-IRS1, -IRS2, and -p85 of PI-3K were purchased from Upstate Biotech (Upstate Biotech, Uppsala, Sweden) according to the protocol recommended by the manufacturer. RNA samples were fractionated on 0.1% agarose/2.2 M formaldehyde denaturing gels and transferred onto Nytran membranes (Schleicher & Schuell, Keene, NH). The cDNA encoding mouse Fas and FasL were provided by Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan), and cDNA encoding early growth response gene (Egr)-1, -2, and -3 have been described previously (33, 34). The CDNA probes were labeled by random priming with [32P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Prehybridization and hybridization were conducted at 42°C in a solution containing 5× SSC (10× SSC is 1.5 M NaCl and 0.15 M sodium citrate), 2.5 mM EDTA, 0.1% SDS, 5× Denhardt’s solution, 2 mM sodium pyrophosphate, 50 mM sodium phosphate, and 50% formamide. After washing with 0.2× SSC and 0.1% SDS at 56°C for 1 h, hybridization signals were detected by autoradiography.

### FACS assay for CD69 and apoptosis

CD69 expression was examined by staining the cells with anti-CD69 Ab (PharMingen, San Diego, CA) before stimulation with anti-CD3 for 4 h after culturing the mixed cells. The radioactivity was measured with a gamma counter (Wallac, Turku, Finland). The spontaneous release of the target cells was estimated by mixing the target cells with effector cells at various ratios. The supernatant was harvested and 10 7 cells were mixed with 10 7 cells of 51Cr-labeled target cells. The samples were separated on 7.5% SDS-polyacrylamide gels before transfer to a polyvinylidene difluoride membrane. The membranes were then probed with a monoclonal antiphosphotyrosine Ab, RC20. The bound Abs were detected using enhanced chemiluminescence (Amersham, Aurlington Heights, IL). The blots were stripped and reprobed as necessary.

### Microcytotoxicity assay

The 51Cr release microcytotoxicity assay has been described previously (35). Briefly, target cells were labeled with 51Cr (NEN, Boston, MA) for 1 h at 37°C. After washing with PBS four times, labeled target cells were mixed with effector cells at various ratios. The supernatant was harvested 4 h after culturing the mixed cells. The radioactivity was measured with a gamma counter (Wallac, Turku, Finland). The spontaneous release of the target cells from the 51Cr-labeled target cells was usually <15%. The percent specific release was calculated using the following formula: experimental release – spontaneous release/total release – spontaneous release.
Luciferase assay

Luciferase activity was evaluated using a chemiluminescence assay kit (Tropix, Bedford, MA). One million cells were transiently transfected with 1 μg purified luciferase reporter plasmids containing the 5′ region of the murine FasL gene from −511 to +1 (36), a 16 mer encoding the critical CD3 response element from the FasL promoter containing an Egr binding site (33, 34) (termed FasL response element (FLRE)), three copies of a NF-κB site derived from the murine IL-2 promoter, six copies of a consensus AP-1 site, or two copies of a NF-κB site derived from mouse κ light chain enhancer cDNA (37) by electroporation. The cells were then cultured in CM overnight. After washing, harvested cells were stimulated by immobilized anti-CD3 for various times. Ten microliters of the extract from activated cells was mixed with 100 μl luciferase assay reagent, and luciferase activity was measured for 5 s in a ML2250 luminometer (Dynatech, Chantilly, VA). In each experiment 1.5 μg vector carrying β-galactosidase driven by the CMV promoter was also transfected as a control for transfection efficiency as previously described (36). Transfection without any plasmid was used to detect background luciferase activity, which was minimal.

Results

Characterization of A1.1 cells expressing IRS1 or IRS2

IRS family members are expressed in T lymphocytes (16, 18, 23–26); however, their function in T cell biology is unclear. Lack of IRS family member expression in mice does not cause a major effect on lymphocyte responses (26–29). Lack of IRS2 expression results in a decrease in T cell proliferation and a modest suppression of Th2 development when analyzed in vitro (26). The lack of both genes results in embryonic lethality (38). Therefore, to analyze the function of the IRS family members in T cell activation induced responses, we took advantage of a T cell hybridoma, A1.1, that lacks expression of all IRS family members, but can respond to IL-4 treatment with the tyrosine phosphorylation of STAT6. These cells were transfected with cDNA encoding IRS1 or IRS2 with a neomycin resistance gene or a neomycin resistance gene alone. Cells able to grow in the presence of G418 were selected and tested for IRS expression by Western blotting using the appropriate Ab. The ability of IL-4 to stimulate tyrosine phosphorylation of the transfected IRS1 or IRS2 in the selected clones was analyzed by immunoprecipitation, followed by immunoblotting (Fig. 1A). Clones expressing IRS1 demonstrated a basal level of phosphorylation that was substantially increased after IL-4 stimulation. Clones expressing IRS2 demonstrated minimal basal phosphorylation of IRS2 that was increased after IL-4 stimulation. In the parental A1.1 cells or A1.1 expressing the neomycin gene alone, we did not detect the expression or tyrosine phosphorylation of IRS1 or IRS2 as expected. To determine whether the expression of IRS1 or IRS2 in A1.1 cells influenced signaling through the TCR, we analyzed the induction of CD69 in A1.1, A1.1-IRS1, and A1.1-IRS2 before and after anti-CD3 treatment by FACS analysis (Fig. 1B). The A1.1 cells expressing IRS1 or IRS2 responded to anti-CD3 stimulation by the induction of cell surface CD69 to levels similar to the parental A1.1 cells and with similar kinetics. In addition, all showed similar levels of IL-2 production and stimulation of tyrosine phosphorylation of cellular substrates (data not shown), suggesting that global TCR signaling was not grossly affected by the transfected genes.

Expression of IRS1, but not IRS2, protects A1.1 cells from AICD

In previous studies we showed that overexpression of IRS1 in the IL-3-dependent myeloid cell line 32D protected from IL-3 withdrawal-induced death (32). Furthermore, it has been shown that elevated expression of IRS1 or IRS2 is able to protect a number of cell types from apoptosis and is often associated with the oncogenic phenotype (20, 39–42). Therefore, we tested the sensitivity of the IRS-expressing cell lines to AICD by propidium iodide staining of nuclear DNA content (Fig. 2). Interestingly, the cells overexpressing IRS1 were resistant to apoptosis induced by culture with plate-bound anti-CD3, while the cells expressing IRS2 were not. For example, control neomycin-resistant A1.1 cells responded to plate-bound anti-CD3 with an increase in the percentage of apoptotic cells from 5 to 47%, while the percentage of A1.1IRS1a cells rose from 8 to only 11%. A1.1IRS2a cells responded to anti-CD3 with an increase in the percentage of apoptotic cells from 5 to 47%. The addition of IL-4 during the stimulation did not further enhance the protection of AICD in either A1.1-IRS1 or A1.1-IRS2. These preliminary studies in A1.1 cells indicate that IRS1 specifically signals protection of T cells from AICD. This could be
through the regulation of molecules that activate the apoptotic signal (i.e., Fas or FasL) or by suppression of the apoptotic program itself.

Signal transduction for the protection of cell death by overexpressing IRS1 is through a PI-3K independent pathway

Previous studies in the 32D cell model demonstrated that the IRS1-dependent prevention of apoptosis was associated with the activation of PI-3K (20, 32). To examine whether the ability of IRS1 expression to protect A1.1 cells from AICD was dependent upon PI3K, we performed the anti-CD3 stimulation in the presence or the absence of the inhibitor of PI-3K, LY294002 (Fig. 3A). Inhibition of PI3K with this agent did not reverse the protection from AICD seen in the IRS1-expressing cells. In fact, at concentrations >3 μM, LY294002 suppressed AICD in all A1.1 cells, including parental cells. Similar results were obtained using wortmannin (data not shown). We further analyzed the association of IRS1 with the p85 subunit of PI-3K (Fig. 3B). The coprecipitation of p85 with IRS1 was increased significantly by IL-4 treatment, but not by anti-CD3; the ability of IRS1 and p85 to coprecipitate was correlated with the IL-4-induced tyrosine phosphorylation of IRS1 as shown previously (20), indicating that the IL-4 activated IRS1/p85 signaling pathway is present in these cells. However, stimulation through the TCR did not have an effect on tyrosine phosphorylation of IRS1 or on its association with p85. These results demonstrate that the signal transduction for the prevention of cell death by overexpressing IRS1 in A1.1 cells is not dependent upon tyrosine phosphorylation of IRS1 and the downstream activation of PI-3K.

Effect of IRS1 on expression of Fas and FasL after TCR stimulation

A1.1 cells commit to AICD in a Fas- and FasL-dependent manner (1, 30). To directly examine the effect of IRS1 expression on the levels of Fas and FasL induction, we performed Northern blot analysis (Fig. 4A). mRNA encoding Fas was detected in untreated cells and was increased after anti-CD3 treatment in A1.1 cells and in IRS1- and IRS2-expressing cells. The expression of Fas-L mRNA was undetectable in untreated cells, induced within 2 h, and induced to high levels within 4–5 h after anti-CD3 treatment in the parental A1.1 and the IRS2-expressing cells. Interestingly, the induction of Fas-L mRNA in the IRS1-expressing cells was not observed until 8 h of stimulation.

To assess the level of functional cell surface FasL, we performed 51Cr release assays using the L1210 T cell line expressing Fas as a result of transfection as a target cell (Fig. 4B). As a control, L1210 cells lacking Fas expression were also used. The parental A1.1, A1.1 IRS1-expressing (A1.1-IRS1), and A1.1 IRS2-expressing
(A1.1-IRS2) cells were treated with anti-CD3 for 0, 2.5, or 5 h before adding the labeled target cells. After stimulation, the A1.1 and A1.1-IRS2 cells showed cytotoxicity against L1210 Fas targets (20% specific lysis), but not against L1210. However, the A1.1-IRS1 showed no cytotoxicity against Fas-expressing target cells at this time point, which is consistent with the Northern blot results. After 5 h of anti-CD3 stimulation, the parental cells and the IRS2-expressing cells demonstrated 50–60% specific cytotoxicity, while the A1.1-IRS1 cells demonstrated ~20%. Inhibition of cytotoxic activity by IRS1 expression was also apparent after 8 h of activation (data not shown).

Since overexpression of IRS1 has been correlated with resistance to factor withdrawal-induced apoptosis in IL-3-dependent cells (32), we next tested whether IRS expression affected the sensitivity of A1.1 cells to be killed via cell surface Fas (Fig. 5). The parental A1.1, A1.1-IRS1, and A1.1-IRS2 cells were treated with PMA to induce Fas expression and render them sensitive to FasL-mediated killing without inducing FasL itself (30). Such treatment induced similar levels of Fas mRNA in these three cell types (data not shown). The ability of L cells expressing sense or antisense constructs for Fasl to induce apoptosis in these cells was analyzed. We found that all three cell types were able to be killed by FasL-expressing L cells, but not by FasL-negative cells. At a 1.5:1 E:T cell ratio the parental cells, A1.1-IRS1, and A1.1-IRS2 cells showed 29, 18, and 18% apoptosis, respectively, suggesting a modest effect of the IRS proteins on sensitivity to Fas-mediated apoptosis. This effect was not observed at a 4.5:1 E:T cell ratio; under these conditions all three targets demonstrated a high level of apoptosis (37–45%). Taken together, these results indicate that the protection from AICD by overexpressing IRS1, but not IRS2, is predominantly through delayed expression of Fasl, rather than through suppression of Fas signaling.

Effect of IRS expression on transcription factor activation by TCR

To determine whether the IRS1-specific effect on the induction of Fasl after TCR stimulation was acting at the level of transcription, we tested the inducibility of FasL promoter-luciferase constructs in transiently transfected A1.1 cells (Fig. 6). The FasL-luciferase construct consists of 511 bp of the murine FasL promoter (36). Stimulation of A1.1 and A1.1-IRS2 cells with anti-CD3 resulted in an increase in luciferase activity over time; there was an increase of 5- to 7-fold over background by 3 h of activation (data not shown).

We further tested the regulation of a minimal TCR-regulated response element derived from the FasL promoter that contains an Egr binding site, the FLRE (33, 34). Stimulation of the A1.1 and A1.1-IRS2 cells with anti-CD3 resulted in an increase in luciferase activity over time; there was an increase of ~5- to 7-fold over background by 3 h of treatment. By contrast, the A1.1-IRS1 cells showed only an ~2-fold increase over background with this promoter construct. These results are consistent with the Northern blotting results (Fig. 4A) and suggest that the mechanism of the IRS1 effect is at least in part via interference of the TCR-induced transcription from the minimal FLRE element of the FasL promoter.

There are a number of cis-acting elements in the FasL promoter that have been shown to interact with trans-acting transcription factors, including Egr1, Jun, and NF-κB.
The relative importance of NFAT, AP-1, NF-κB, and Egr genes in the regulation of the FasL promoter in T cell hybridomas and primary T cells has been investigated extensively (33, 34, 36, 43–49). Therefore, we tested the ability of TCR stimulation to activate these transcription factors in the A1.1 cells using multimerized cis-elements linked to luciferase (NFAT, AP-1, NF-κB) or by Northern blotting (Egr-1, Egr-2, Egr-3). All three cell types demonstrated similar patterns of induction of NFAT and NF-κB activity as measured by luciferase assay after anti-CD3 treatment (Fig. 7A). This result is consistent with our observation that these three cell types demonstrated similar levels of CD69 expression and IL-2 production after anti-CD3 stimulation. A1.1-IRS1 cells showed elevated activity of AP-1-luciferase compared with parental cells and A1.1-IRS2 cells at later time points, but not a deficiency. It is not clear whether this increase is functionally important, since pharmacologic activation of AP-1 by PMA did not mimic the suppression of FasL induction mediated by IRS1 expression (data not shown). All three cell lines demonstrated some level of induction of mRNA encoding Egr-1, -2, and -3. The increase in the level and kinetics of Egr by anti-CD3 varied somewhat for the three different types. However, we did not see a deficiency in Egr induction in these cells. These results indicate that the suppression of transcription of the FasL promoter by IRS1 expression is not caused by simple suppression of expression of important trans-acting factors such as NFAT, AP-1, NF-κB, and Egr.

Discussion

The regulation of T cell homeostasis is critical for the maintenance of self-tolerance and immune surveillance. Alterations in T cell homeostasis have been shown to lead to the development of autoimmunity, lymphoproliferative disease, and cancer (3–8). Elevation of IRS1 and IRS2 protein expression and tyrosine phosphorylation have been linked to the oncogenic potential of several cell types and are anti-apoptotic in transfected cell lines (32, 39, 42). However, we have found that IRS1 expression in the A1.1 T cell hybridoma renders them resistant to AICD by a mechanism that is probably independent of tyrosine phosphorylation.

While IRS family proteins are expressed in resting and activated thymocytes and peripheral T cells to varying degrees, their functions in T cell biology are unclear (16, 18, 23–26). There are no major immunological defects in the IRS1 or IRS2 single knockout mice (6). The A1.1 T cell hybridoma used in these experiments is resistant to AICD by FasL-mediated apoptosis (7). However, A1.1-IRS1 and A1.1-IRS2 cells are partially resistant to FasL-mediated killing (Fig. 5). IRS1 expression is not sufficient by itself to protect cells from apoptosis induced by FasL, since A1.1-IRS1 cells are less resistant than A1.1-IRS2 cells (7). The mechanism by which IRS1 protects cells from FasL-mediated apoptosis is not yet understood.

In summary, we have shown that IRS1 expression is sufficient to protect T cells from FasL-mediated apoptosis. This protective effect is likely due to the ability of IRS1 to activate transcription factors such as NFAT, AP-1, NF-κB, and Egr, which can act synergistically to enhance the expression of anti-apoptotic genes such as Bcl-2. Future studies will be needed to determine the specific mechanisms by which IRS1 protects cells from apoptosis and to identify other downstream targets of IRS1 that contribute to its anti-apoptotic effects.
animals (27, 29). Detailed immunological studies have been hampered by the severe diabetes and infertility of the IRS2 knockout mice (29, 50). Furthermore, double-deficient animals die in utero (38). Therefore, to address the function of the IRS family in T cells, we took advantage of the observation that the T cell hybridoma A1.1 lacks expression of IRS family members (Fig. 1). A1.1 cells have been used extensively as an in vitro model of AICD (1, 30, 31) and have provided valuable information on the regulation of Fas and FasL expression. This cell line was derived from the fusion of a CD4+/H11001 T cell and BW5147 and undergoes rapid apoptosis after anti-CD3 stimulation via a Fas-/FasL-dependent pathway (30). After anti-CD3 stimulation, expression of Fas mRNA is rapidly induced by a protein kinase C-dependent mechanism. FasL mRNA is dramatically induced and is dependent on both the protein kinase C pathway and the cyclosporin A-sensitive pathway.

Using this model system we found that IRS1 expression suppresses AICD, while IRS2 expression does not. Due to the ability of IRS family members to link to a PI-3K/PKB pathway (27), we expected the IRS-expressing cells to be intrinsically resistant to apoptosis. Using the A1.1 cells as targets for FasL-expressing L1210 cells, we found that both the IRS1- and IRS2-expressing cells were modestly protected from Fas-mediated apoptosis at low E:T cell ratios. However, this modest level of protection cannot explain the specific resistance of IRS1-expressing A1.1 cells to anti-CD3-induced apoptosis. Addition of IL-4 did not enhance the resistance to AICD, while it clearly induced the association of IRS1 with PI-3K (Fig. 3B) and induced PI-3K activity in the IRS1-expressing cells (data not shown). Furthermore, the PI-3K inhibitor, LY294002, did not reverse the protection from AICD observed in the IRS1-expressing cells.

A striking finding is that IRS1 mediates the protection from AICD, while IRS2 does not. In vivo and in vitro experiments have revealed important differences in the signaling capacities of IRS1 and IRS2. Tyrosine-phosphorylated IRS1 and IRS2 display differential abilities to associate with the various Src homology 2 domain-containing signaling molecules, including p85, growth factor receptor binding protein 2, SHP-2, Fyn, Crk, and phospholipase Cγ (18). However, the protective effect of IRS1 was observed in the absence of anti-CD3- or IL-4-induced tyrosine phosphorylation, suggesting that it may act via a phosphotyrosine-independent mechanism. Phosphotyrosine-independent effects of IRS1 have been reported in other systems (22). Both IRS1 and IRS2 contain >70 potential Ser/Thr phosphorylation sites that can be phosphorylated by a variety of kinases (JNK, ERK, casein kinase II, c-Akt, protein kinase C). In the mid-region of the IRS molecules (aa 555–898) there are a number of potential phosphorylation sites and two potential JNK binding motifs (19). However, three of five Ser sites in this region known to be phosphorylated in IRS1 are absent from IRS2 (17). One of the putative JNK binding sites is conserved, but the other is not well conserved. Strikingly, IRS2 does not have a Ser307 equivalent, the residue in IRS1 that is phosphorylated by JNK and whose phosphorylation regulates insulin receptor kinase activity (19). It is interesting to speculate that the IRS1-specific effect is mediated via phosphorylation of these serine residues after TCR stimulation.

FIGURE 7. Transcription factor activation. A, Parental A1.1 (prech), A1.1-IRS1 (prech), and A1.1-IRS2 (prech) cell lines were transfected with vectors containing multimerized elements for NFAT, AP-1, and NF-κB linked to luciferase by electroporation and were cultured overnight. Transfection with β-galactosidase was also performed to ensure equivalent levels of transfection. The luciferase activity in cell lysates was measured using the Tropix kit and a luminometer. The data represented show the relative light units (RLU) ± SEM. In some cases the SEM is too small to be observed in the graph. B, Parental A1.1 (prech), A1.1-IRS1 (prech), and A1.1-IRS2 (prech) cell lines were incubated in anti-CD3-coated plates for various times. Expression of mRNA for Egr-1, Egr-2, Egr-3, and GAPDH was examined by Northern blotting using specific probes. The relative intensities of the bands on the autoradiogram were determined using the public domain software National Institutes of Health Image. The relative ratio of Egr to GAPDH expression is shown.
The IRS1-specific suppression of AICD in this hybridoma T cell model system is most likely due to its effect on the induction of FasL itself. Strikingly, we found that significant levels of FasL mRNA were not detected until after 8 h of anti-CD3 stimulation in the IRS1-expressing cells. This delay in FasL mRNA was correlated with a delay in the expression of functional FasL on the cell surface. This substantial delay in functional FasL expression could be important in vivo where cellular interactions frequently occur transiently.

The effect of IRS1 on FasL expression appears to be at the level of the FasL promoter, since the induction of a FasL-luciferase reporter was delayed in IRS1-expressing cells. This delay in FasL mRNA was correlated with a delay in the expression of functional FasL on the cell surface. This substantial delay in functional FasL expression could be important in vivo where cellular interactions frequently occur transiently.

The studies reported herein have examined the mechanisms by which IRS family members regulate AICD in a T cell hybridoma model system. It is not yet clear how these findings relate to FasL expression in normal T cells. To examine their roles, we are currently developing retrovirus-based technology to overexpress/inhibit IRS1 or IRS2 expression in developing primary T cell cultures. Such studies could aid in the understanding of the regulation of T cell homeostasis and potentially provide a novel therapeutic target for the treatment of diseases such as cancer.

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References


