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Janus Kinase-dependent Activation of Insulin Receptor Substrate 1 in Response to Interleukin-4, Oncostatin M, and the Interferons*

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In addition to a role in response to insulin and insulin-like growth factors, insulin receptor substrate 1 (IRS-1) is phosphorylated in response to IL-4, the interferons (IFNs) and oncostatin M (OSM). Here mutant cell lines lacking JAK1, JAK2, or Tyk2 were used to determine the role(s) of the Janus kinase (JAK) family of protein-tyrosine kinases in IRS-1 phophorylation. 32D cells, which do not express IRS proteins, were analyzed for any requirement for these proteins in response to the IFNs. For the mutant human fibrosarcoma cell lines, phosphorylation of IRS-1 through the insulin-like growth factor receptor is independent of JAK1, JAK2, or Tyk2. In contrast, phosphorylation of IRS-1 mediated by the Type I IFNs, IL-4, and OSM is JAK-dependent. For the αβ-IFNs, activation of IRS-1 is dependent on JAK1 and Tyk2, consistent with the interdependence of these kinases in the IFN-αβ response. Neither IRS-1 nor IRS-2 was detectably activated by IFN-γ. Consistent with this, activation of neither IRS proteins appears to be an absolute requirement for an antiproliferative or an antiviral response to the IFNs. For IL-4 and OSM phosphorylation of IRS-1 in the human fibrosarcoma cells is largely dependent on JAK1 but can also be mediated through Tyk2 or JAK2. Activation of phosphatidylinositol 3′-kinase in response to IL-4 and OSM, at least, was also JAK-dependent. The JAKs are, therefore, required not only for STAT activation but also for the activation, through a variety of different types of cytokine receptor, of an additional signaling pathway(s) through IRS-1 and phosphatidylinositol 3′-kinase.

Receptors that have an intrinsic tyrosine kinase domain recruit and activate a variety of signal transducers. Insulin receptor substrate 1 (IRS-1)[1] is a cytosolic protein of ~180 kDa in mass that is tyrosyl-phosphorylated at multiple sites through activated insulin and insulin-like growth factor 1 (IGF-1) receptors (1, 2). An $L(X)_4NPXY(p)XSXP$ motif has been identified in the insulin receptor as being important for the recruitment of the IRS proteins. IRS-1 contains multiple YM*X*M and Y*XX*M tyrosine motifs, which when phosphorylated can recruit Src homology 2 domain-containing proteins, including the p85α regulatory subunit of PI 3′-kinase, SHP-2, Grb-2, Crk, and Nck (3, 4). Phosphorylated IRS-1, therefore, can mediate a variety of responses including a mitogenic response and activation of PI 3′-kinase.

Common themes involving the role of tyrosine kinases and proteins with Src homology 2 domains have emerged to describe broadly the mechanism of signal transduction by many growth factors and cytokines. Signal transduction pathways utilizing the JAK (Janus kinase) family of protein-tyrosine kinases and the STATs (signal transducers and activators of transcription) are activated in response to polypeptide ligands including many cytokines, some growth factors and the interferons (IFNs). STAT activation mediated through the JAKs occurs in receptor complexes at the cell membrane. There are four known mammalian JAKs: JAK1, JAK2, JAK3, and Tyk2 (5–9). Each is ~130 kDa in mass and has a C-terminal tyrosine kinase domain, an adjacent kinase-related domain, and five additional domains extending toward the N-terminus with similarity between the family members. JAK1, JAK2, and Tyk2 appear to be widely expressed, whereas the expression of JAK3 occurs predominantly, if not exclusively, in cells of the hematopoietic system. There are seven mammalian STAT genes (1–6, including 5A and 5B) (10–16). Different cytokines activate different combinations of JAKs and STATs. JAK autophosphorylation/activation occurs on receptor dimerization/oligomerization in response to ligand with consequent phosphorylation of the receptor. Specific STATs, associated with or recruited to the JAK/receptor complex, are phosphorylated, released, migrate to the nucleus, and, with or without additional factors, activate transcription (reviewed in Refs. 17 and 18).

The activation of IRS-1 is not restricted to receptors having intrinsic kinase activity. IRS-1, or the highly related IRS-2 (19), can be phosphorylated through receptor-associated kinases in response to a number of ligands including IL-4, the IFNs (20–22), and oncostatin M (OSM) (this paper). IL-4 is produced by T cells (23). It can induce IgE production by activated B cells, class II HLA expression by B cells and macrophages, act as a co-stimulant for B and T cell proliferation (24), and stimulate differentiation into Th2 cells from uncommitted T cell precursors (25). The best characterized IL-4, receptor, Type I, consists of a high affinity α subunit and a common γ subunit (γ_c), which is also utilized by the IL-2, IL-7, IL-9, and IL-15 receptors (21). There is increasing evidence for an additional Type II IL-4 receptor(s), including one that utilizes the high affinity α subunit and a β subunit shared with the IL-13 receptor (26–28). JAK3 is activated through the γ_c subunit of the Type I receptor and JAK1, JAK2, and Tyk2 through the

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[1] The abbreviations used are: IRS-1, insulin receptor substrate 1; IGF, insulin-like growth factor; PI, phosphatidylinositol; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; JAK, Janus kinase; IFN, interferon; IGFR, IGF receptor; STAT, signal transducer and activators of transcription; OSM, oncostatin M; TBST, Tris-buffered saline/Tween 20.

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Type II receptor (29, 30). The IL-4 receptor α subunit, like the insulin and IGF receptors, contains an $L(X)_4NPXY(p)XSXP$ motif, which mediates IRS recruitment (20).

OSM, LIF, CNTF, IL-6, IL-11, and cardiotrophin belong to a family of pleiotropic cytokines, which share a common gp130 receptor subunit and have overlapping biological activities mediated in part at least through JAKs and STATs (31). In the human fibrosarcoma cell lines used here, OSM utilizes gp130 and an OSM-specific β subunit.[2] Neither gp130 nor the OSM-β receptor subunit contains an $L(X)_4NPXY(p)XSXP$ motif.

The interferons (IFNs), although first identified as antiviral agents, can also affect cell growth, differentiation, and function, and they play a major role in the immune response. There are three major antigenic types of human IFN: α, β, and γ. The Type I IFNs ($\alpha\beta$) utilize the same receptor(s) (32–35); Type II IFN (γ) acts through a separate receptor (36–39). For both types of IFN, two receptor subunits have been cloned. The IFN receptors do not contain the $L(X)_4NPXY(p)XSXP$ motif identified in the insulin and IL-4 receptors (20).

An essential role for JAK/STAT pathways is accepted for a number of cytokines. A role for the JAKs in the recruitment/activation of additional signaling molecules is, however, less well established. Mutant human fibrosarcoma cell lines lacking individual JAKs, originally isolated as being defective in signal transduction in response to the IFNs (40–42), have been used widely in the analysis of JAK/STAT pathways in response to a variety of ligands (17, 18, 43). Here they have been used, together with murine 32D cells that lack IRS-1 and IRS-2, to determine the JAK dependence of IRS protein phosphorylation in response to IL-4, OSM, and the IFNs and to analyze the IRS protein dependence of the IFN response.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit polyclonal antisera were raised against the JH2 domain of JAK1 (44), the JH1 and JH6 domains of JAK2,[3] amino acids 289–450 of TYK2 (45), and the last 12 residues in the C terminus of IRS-1 (46). The STAT 2C antiserum was a gift from C. Schindler (11, 47). The monoclonal antibodies to STAT1α were purchased from Santa Cruz Biotechnology, and the monoclonal antibodies to TYK2 and p85α were gifts from S. Pellegrini and D. Cantrell (Imperial Cancer Research Fund, London, United Kingdom). A mixture of 4G10 (UBI) and PY20 (ICN) anti-phosphotyrosine monoclonal antibodies was used throughout. Peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies were from Amersham Life Science.

Interferons and Cytokines—IFN-α was a highly purified mixture of human subspecies (Wellferon, 1.5×10^8 IU/mg of protein; Ref. 48) provided by Wellcome Research Laboratories, Beckenham, Kent, United Kingdom. Recombinant human and murine IFN-γ (4×10^7 and 1×10^7 IU/mg of protein, respectively) was from Dr. G. Adolf, Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria. Recombinant murine IL-3 (10 IU/ml) was used in the growth of the 32D cells. Recombinant Human IFN-α A/D (*Bgl*) hybrid (2×10^8 IU/mg of protein) was from Drs. S. Pestka and M. Brunda (Hoffman La Roche). IFN treatment of cells was at 10^3 IU/ml unless otherwise stated. OSM was supplied by G. Ciliberto (IRBM, Pomezia (Roma), Italy) and used at a concentration of 100 ng/ml. rhIL-4 was purchased from Serotec and used at a concentration of 20 ng/ml.

Cell Culture—Parental 2C4 cells and mutant U4A, γ2A, and U1D cells were derived as described previously (40, 41, 49). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. 2C4 and γ2A were maintained in 400 μg/ml G418, and U1D and U4A were maintained in Hygromycin (250 μg/ml). U4A cells stably transfected with JAK1 (U4/J1) or JAK2 (U4/J2) were maintained in both G418 and Hygromycin at the above concentrations (50). U4A cells stably transfected with TYK2 (U4/T2) were maintained in Hygromycin (as above) and Puromycin (0.5 μg/ml).

32D (myeloid progenitor) cell lines were maintained in RPMI containing 10% (v/v) heat-inactivated fetal calf serum and IL-3 (10 IU/ml). 32D cells expressing transfected IRS proteins were as described (51, 52) and maintained in medium containing 5–10 mM histidinol (Sigma).

Immunoprecipitation, SDS-PAGE, and Western Blotting—Immunoprecipitations were carried out as described previously (22). Briefly, cells were lysed in ice-cold lysis buffer (1% Triton X-100 (v/v), 10% glycerol (v/v), 50 mM Hepes, 150 mM NaCl, 1 mM EDTA, pH 8, 200 μM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 100 mM sodium fluoride 1.5 mM magnesium chloride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Nuclei were removed by centrifugation. Appropriate antibodies and a mixture of protein A- and protein G-Sepharose (Pharmacia Biotech Inc.) were added and incubated for 18 h at 4 °C. The immunoprecipitates were washed in ice-cold lysis buffer (with 0.1% Triton X-100 (v/v)) electrophoresed on 6.5% polyacrylamide-SDS gels (62) and blotted to Immobilon™ polyvinylidene difluoride (Millipore) membranes. Membranes were blocked with 5% bovine serum albumin (fraction V) (w/v) in TBST (10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 1 mM EDTA, 0.1% Tween 20 (v/v)) containing 1 mM sodium orthovanadate for 1 h, incubated with the relevant primary antibody for 1 h, washed in TBST, incubated for 30 min with peroxidase-conjugated secondary antibody, rewashed, and exposed to enhanced chemiluminescence (ECL, Amersham Life Science), followed by fluorography (Kodak AR). When reprobed, membranes were stripped in 0.1 M glycine, pH 2.5, for 1 h, washed in phosphate-buffered saline and TBST, and incubated with the appropriate primary and secondary antibodies as above.

In Vitro PI 3′-Kinase Assay—Cells were lysed in ice-cold lysis buffer (1% Nonidet P-40 (v/v), 50 mM Hepes, 150 mM NaCl, 200 μM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 10 mM iodoacetamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Nuclei were removed by centrifugation. IRS-1 antibodies and a mixture of protein A- and protein G-Sepharose (Pharmacia) were added and incubated for 18 h at 4 °C. The immunoprecipitates were washed in ice-cold lysis buffer, twice in 100 mM Tris, pH 7.4, 0.5 M LiCl, 100 μM sodium vanadate and twice in 50 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, pH 8, containing 100 μM sodium vanadate, resuspended in 10 μl of lipid mixture (1 mg/ml each of L-α-phosphatidylinositol and L-α-phosphatidyl-L-serine, dispersed by sonication in 25 mM Hepes buffer, pH 7.4, 1 mM EDTA). The reaction was initiated by the addition of 50 nM [γ-^{32}P]ATP (5 μCi) and 125 μM ATP in 40 μl of 12.5 mM $MgCl_2$, 25 mM Hepes, pH 7.4, 125 mM NaCl. After 15 min at 25 °C, the reaction was terminated by the addition of 500 μl of chloroform:methanol (1:2) in 0.12 M HCl. 125 μl of chloroform and 125 μl of 10 mM HCl was then added. The mixture was vigorously vortexed and centrifuged to separate the phases. 200 μl of the organic phase was removed and washed with 400 μl of methanol, 100 mM HCl, 2 mM EDTA (1:1). The organic phase was removed and dried *in vacuo* and resuspended in chloroform. The phospholipids were separated by thin layer chromatography (TLC) in propan-1-ol:acetic acid (2 N) (65:35) (v:v), and visualized by autoradiography.

Proliferation Assay with 32D Cells—32D parental cells and IRS-1/2 transfectants were plated at 4×10^3 cells/well in 96-well plates in RPMI, 10% FCS plus 10 IU/ml rmIL-3 in the presence or absence of interferons (1×10^3 IU/ml) as indicated. At days 3 and 5, cell numbers were measured by Coulter counter (Coulter Electronics, United Kingdom) and corrected for coincidence.

Densitometry and Phosphoimaging—Densitometry was carried out on autoradiographs of an appropriate exposure using a LKB Ultroscan XL laser densitometer. Three regions of any given band were analyzed and an average obtained, which was corrected against a signal representing the loading of the analyzed protein. For the PI 3′-kinase assay, quantitation of the signals was with a Molecular Dynamics Storm 860 PhosphorImager, directly from the TLC plate.

Statistical Analysis—The dependence upon JAK1 of IRS-1 phosphorylation in response to IL-4, OSM, or IFN-α in the appropriate cell lines was assessed by Fisher's exact test. The association of PI 3′-kinase activity with immunoprecipitation of IRS-1 was assessed by Friedman's nonparametric analysis of variance (61), and the effect of IFN on growth of murine 32D cells was assessed by standard analysis of variance.

RESULTS

Phosphorylation of IRS-1 through the IGF-1 Receptor—IRS-1 is constitutively phosphorylated on tyrosine in the wild-type and mutant human fibrosarcoma cell lines. The phosphorylation is sustained in the absence of serum and is variable in cells passaged using trypsin, but is ablated in the presence of antibody to the IGF-1 receptor (IGFR). It is independent of the JAKs and is thought to reflect activation of the intrinsic IGFR kinase through an autocrine loop. Examples of the data are included with those for IL-4 (Fig. 1*A*). Treatment with antibody to the IGFR was,

[2] J. M. Smith and I. M. Kerr, manuscript in preparation.

[3] A. Ziemiecki, unpublished results.

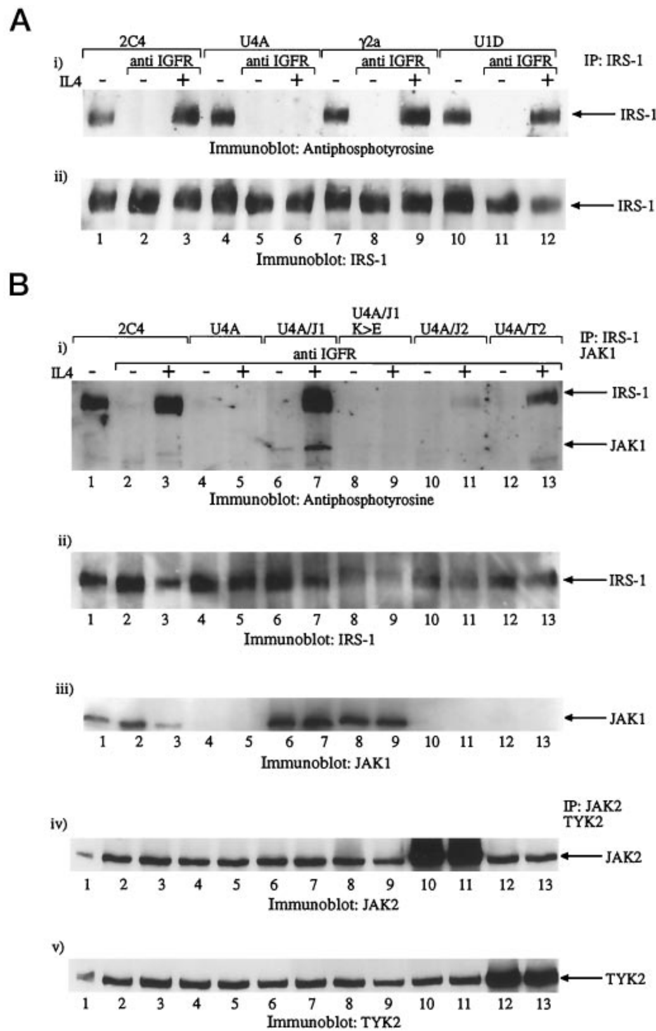

FIG. 1. **Abrogation of the constitutive phosphorylation of IRS-1 through the IGFR reveals IRS-1 phosphorylation in response to IL-4 in wild-type and JAK-negative cell lines.** *A*, Wild-type (2C4) and JAK1 (U4A)-, JAK2 (γ2A)-, and Tyk2 (U1D)-negative cell lines were incubated in the absence (*lanes 1*, *4*, *7*, and *10*) or presence (*lanes 2*, *3*, *5*, *6*, *8*, *9*, *11*, and *12*) of antibody to the IGFR for 24 h, treated where indicated (+) with IL-4 for 15 min, lysed, and IRS-1 immunoprecipitates analyzed by SDS-PAGE and Western blotting sequentially, with antibody to phosphotyrosine (*i*) or IRS-1 (*ii*). *B*, IRS-1 phosphorylation in response to IL-4 in JAK1-negative U4A cells expressing wild-type JAK1 (U4A/J1), kinase-negative JAK1 (U4A/J1.K→E), JAK2 (U4A/J2), or Tyk2 (U4A/T2). Cells were incubated in the absence (*lane 1*) or presence of antibody to the IGFR and treated with IL-4 as in *A*. IRS-1 and JAK1 (*i–iii*) and JAK2 and Tyk2 (*iv–v*) were immunoprecipitated and analyzed by SDS-PAGE and Western blotting sequentially with antibodies to phosphotyrosine (*i*), IRS-1 (*ii*), JAK1 (*iii*), JAK2 (*iv*), and Tyk2 (*v*).

therefore, routinely carried out to lower the constitutive levels of phosphorylation of IRS-1 throughout all of the experiments in which cells of this type were used. Such treatment in the presence or absence of IFNs was without effect on cell growth or the response to the IFNs over several days.

IRS-1 Phosphorylation in Response to IL-4—Tyrosyl phosphorylation of IRS-1 was observed in response to IL-4 in wild-type cells (2C4) and in cells lacking JAK2 (γ2A) and Tyk2 (U1D), but not in cells lacking JAK1 (U4A) (Fig. 1*A*, *lanes 3*, *9*, *12*, and *6*, respectively). In contrast, the constitutive phosphorylation of IRS-1 through the IGFR was not affected by the absence of JAK1, JAK2 or Tyk2 (Fig. 1, *lanes 1*, *4*, *7*, and *10*). For IL-4, phosphorylation of IRS-1 was restored in the U4A cells lacking JAK1 on stable transfection with wild-type (U4A/JAK1) but not a kinase-negative mutant of JAK1 (U4A/

TABLE I
Tyrosine phosphorylation of IRS1

Comparing the data for wild-type (2C4) and JAK1-negative (U4A) cells and U4A cells complemented with JAK1 (U4/J1) by Fisher's exact test, the *p* values for the requirement of JAK1 for the phosphorylation of IRS-1 in response to IL-4, OSM, and IFNα are 0.001, 0.05, and 0.002, respectively.

	Tyrosine phosphorylation of IRS1 (%)[a]							
	2C4	U4A	U1D	γ2A	U4/J1	U4/J1K > E	U4/J2	U4/T2
IL-4								
Fig. 1*A* (*i*)								
No αIGFR[b]	48	77	67	52				
No IL-4[c]	1	1	1	1				
IL-4[c]	100	1	94	96				
Fig. 1*B* (*i*)								
No IL-4[c]	2	1			3	4	3	3
IL-4[c]	100	1			115	2	10	37
OSM								
Fig. 2*B* (*i*)								
No OSM[c]	16	4	3	2				
OSM[c]	100	9	40	32				
Fig. 2*C* (*i*)								
No OSM[c]		7			8		5	6
OSM[c]		8			100		15	44
Fig. 2*D* (*i*)								
No OSM[c]	2	1			6	6		
OSM[c]	100	16			72	4		
IFN								
Fig. 4*A* (*i*)								
No IFN[c]	3	3	9	8	8			
IFN[c]	100	3	3	80	105			
IL-4 + OSM + IFN								
Fig. 5 (*A–C*) (*i*)								
No IL-4, OSM, IFN[c]	<10	<10			<10			
IL-4[c]	100	1			79			
OSM[c]	100	19			100			
IFN[c]	100	4			96			

[a] Relative values for tyrosyl phosphorylation of IRS-1 in the experiments shown in Figs. 1, 2, 4, and 5, monitored by densitometry (see "Experimental Procedures") are presented as percent of the signal for wild-type, 2C4 (or U4A/J1 in Fig. 2*C* (*i*)) cells in the presence of ligand, corrected for protein loading. Similar results were obtained in three to seven independent experiments for the comparison of 2C4, U4A, and U4/J1 cells with all three ligands and in duplicate for the remaining cell lines.

[b] Where αIGFR is neutralizing antibody to IGFR-1 receptor.

[c] In presence of αIGFR antibody.

JAK1.K→E; Ref. 50) (Fig. 1*B*, *lanes 7* and *9*). Interestingly, JAK1, JAK2, and Tyk2 are all activated in response to IL-4 in these cells and phosphorylation in response to IL-4 was restored by overexpression of Tyk2 (Fig. 1*B*, *lane 13*) and, to a lesser extent, JAK2 (Fig. 1*B*, *lane 11*), as well as by JAK1 (*lane 7*). Activation of IRS-1 by IL-4, but not through the IGFR, is, therefore, dependent on JAK activation in these cells. The data on IRS-1 phosphorylation in response to IL-4 are summarized in Table I, together with data for IRS-1 phosphorylation in response to OSM and IFNα.

IRS-1 Phosphorylation in Response to OSM—IRS-1 is also rapidly phosphorylated in response to OSM in the human fibrosarcoma cell lines (Fig. 2*A*). Such phosphorylation is substantially reduced in the absence of JAK1 (compare Fig. 2*B* (*i*, *lanes 3* and *9*), Fig. 2*D* (*i*, *lanes 3* and *5*), and Fig. 5*B* (*i*, *lanes 2* and *4*), but less so in the absence of Tyk2 or JAK2 (Fig. 2*B*, *i*, *lanes 6* and *12*)). The response is restored in the U4A cells lacking JAK1 on stable transfection with wild-type JAK1 (Figs. 2*C* (*i*, *lane 6*), 2*D* (*i*, *lane 7*), and 5*B* (*i*, *lane 6*)) and to a lesser extent on transfection with Tyk2 or JAK2 (Fig. 2*C*, *i*, *lanes 4* and *8*) but not on transfection with a kinase-negative mutant of JAK1 (JAK1.K→E, Fig. 2*D*, *i*, *lane 9*). The ablation of the residual tyrosyl phosphorylation of IRS-1 observed in U4A cells by the kinase-negative JAK1.K→E (Fig. 2*D*, *i*, *lanes 9*, *5* and *2*)

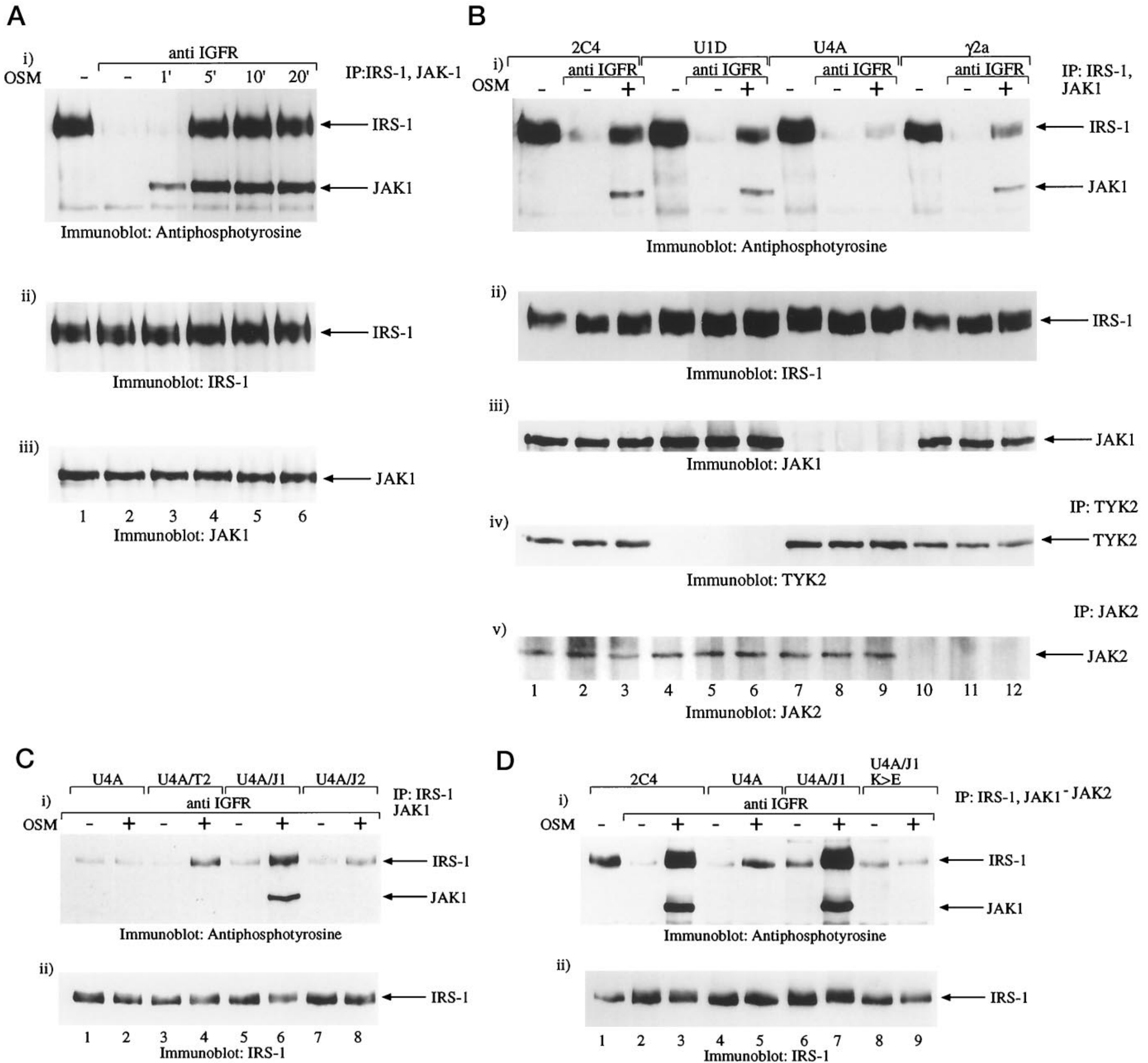

FIG. 2. **Kinetics (*A*) and JAK dependence (*B–D*) of IRS-1 phosphorylation in response to OSM.** Wild-type and JAK-negative cells were incubated in the absence or presence of antibody to the IGFR (where indicated) as in Fig. 1. *A*, wild-type cells incubated for the indicated times with OSM were lysed and IRS-1 and JAK1 immunoprecipitates analyzed by SDS-PAGE, Western transfer, and blotting sequentially with antibody to phosphotyrosine (*i*), IRS-1 (*ii*), and JAK1 (*iii*). *B*, wild-type and Tyk2 (U1D)-, JAK1 (U4A)-, and JAK2 (γ2A)-negative cells incubated for 15 min at 37 °C with (+) or without (−) OSM were lysed, and IRS-1 and JAK1 (*i–iii*) and Tyk2 or JAK2 (*iv* and *v*) immunoprecipitates analyzed by SDS-PAGE, Western transfer, and blotting with antibody to phosphotyrosine (*i*), IRS-1 (*ii*), JAK1 (*iii*), Tyk2 (*iv*), and JAK2 (*v*). *C* and *D*, IRS-1 phosphorylation in response to OSM in JAK1-negative U4A cells expressing wild-type JAK1 (U4A/J1), kinase-negative JAK1 (U4A/J1.K→E), JAK2 (U4A/J2), or Tyk2 (U4A/T2). Cells incubated for 15 min at 37 °C with (+) or without (−) OSM were lysed and IRS-1 and JAK1 immunoprecipitated and analyzed by SDS-PAGE, Western transfer, and blotting with antibody to phosphotyrosine (*i*) and IRS-1 (*ii*).

is consistent with a role for TYK2 and/or JAK2 in this phosphorylation. The data are summarized in Table I.

JAK Dependence of the Phosphorylation of IRS-1 in Response to the Type I Interferons—IRS-1 is rapidly phosphorylated on tyrosine in response to IFNs α and β in the human fibrosarcoma cell lines. It is detectable within 5 min (data not presented) and is clearly observable in parallel with STAT1 activation at 300 IU/ml, reaching a maximum at 1000 IU/ml (Fig. 3*A*). It was observed with a highly purified mixture of natural α-IFNs (Wellferon), recombinant human A/D (*Bgl*) hybrid, and β-IFNs (Fig. 3*A*). In contrast, phosphorylation in response to IFN-γ was barely detectable at concentrations of 1000, 20,000, and 100,000 IU/ml (Fig. 3*B*, *lanes 7*, *2–5*, and *8*) despite good JAK activation in response to IFN-γ at ≤1000 IU/ml (Fig. 3*B*, *lane 7*). Phosphorylation of IRS-1 in response to IFNs-α β was not observed in cells lacking JAK1 (U4A) or Tyk2 (U1D) but was restored in U4A cells stably expressing transfected wild-type JAK1 (U4A/J1) (Fig. 4, *i*, *lane 11*). It was not restored by a kinase-negative JAK1.[4] This is in accord with the interdependence of JAK1 and Tyk2 activation in response to the Type I IFNs (Ref. 42 and Fig. 4, *iii* and *v*, *lanes 5* and *9*) and establishes the JAK dependence of IRS-1 activation in response to Type I IFNs in these cells (summarized in Table I).

[4] N. C. Rogers, unpublished data.

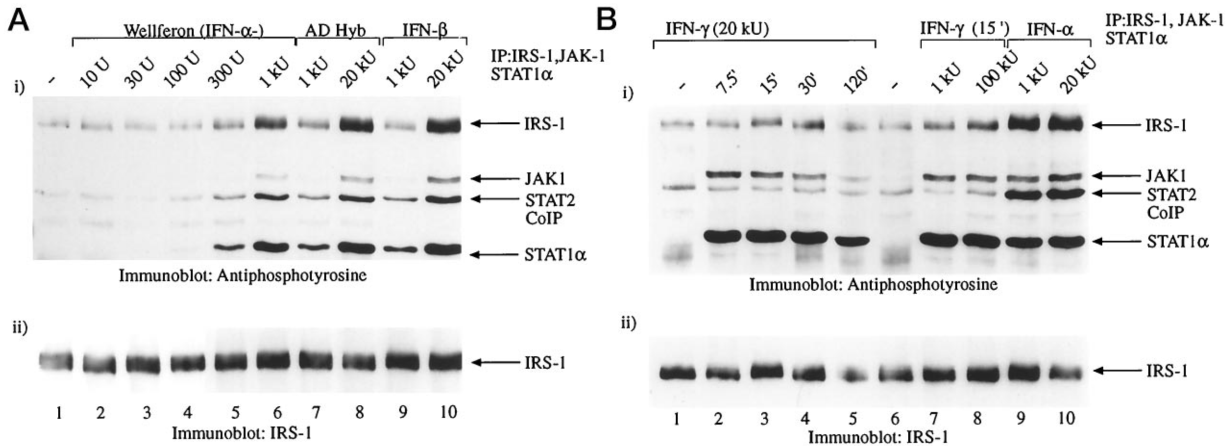

FIG. 3. **Kinetics and dose response of IRS-1 phosphorylation in response to the IFNs.** Wild-type (2C4) cells were incubated in the presence of antibody to the IGFR for 24 h and treated with Wellferon, rIFN-α A/D (*Bgl*) hybrid or IFN-β for 15 min at the concentrations indicated (*A*). The cells were lysed and IRS-1, JAK1 and STAT1α immunoprecipitated and analyzed by SDS-PAGE, Western transfer, and blotting sequentially with antibody to phosphotyrosine (*i*) and IRS-1 (*ii*). *B*, wild-type cells were treated with 20,000 IU IFN-γ for the times indicated (*lanes 2–5*) and for 15 min with IFN-γ (*lanes 7* and *8*) or IFN-α (Wellferon) (*lanes 9* and *10*) at the concentrations indicated. The cells were lysed and IRS-1, JAK1, and STAT1α immunoprecipitated and analyzed by SDS-PAGE, Western transfer, and blotting sequentially with antibody to phosphotyrosine (*i*) and IRS-1 (*ii*).

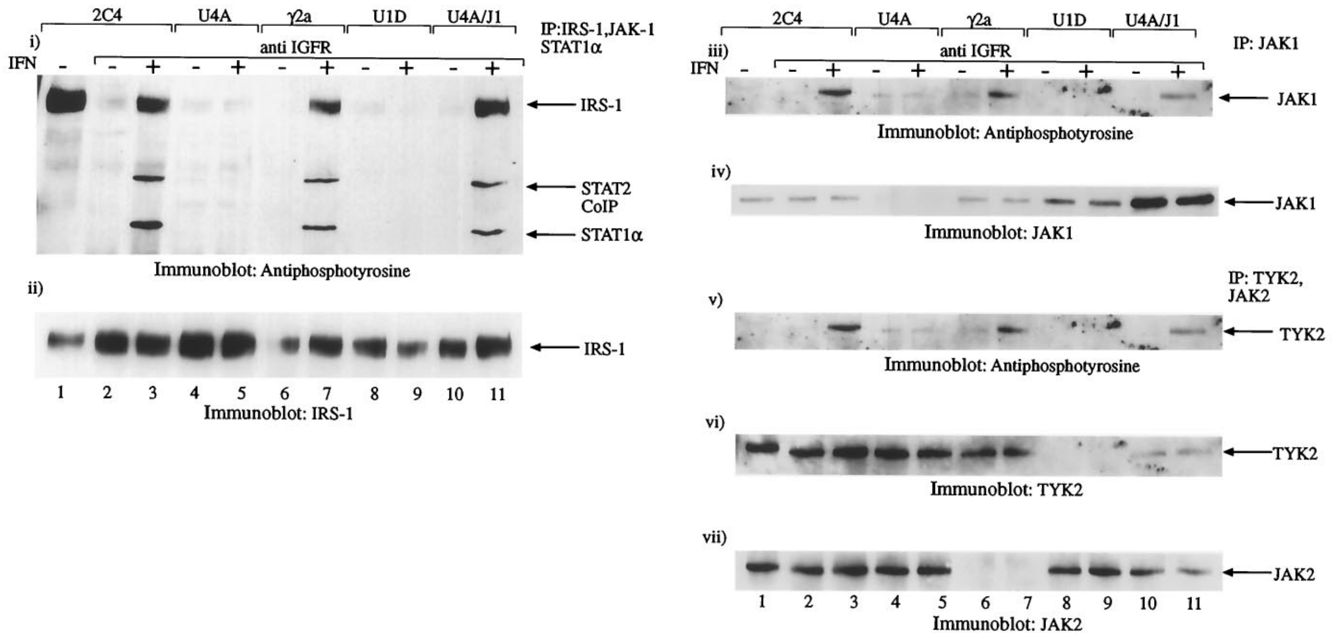

FIG. 4. **JAK dependence of IRS-1 phosphorylation in response to IFN-α.** Wild-type and JAK-negative cells were incubated in the absence or presence of antibody to the IGFR (where indicated) as in Fig. 1. Wild-type and JAK1 (U4A)-, JAK2 (γ2A)-, and Tyk2 (U1D)-negative cells and JAK1-negative U4A cells expressing wild-type JAK1 (U4A/J1) were incubated at 37 °C for 15 min with (+) or without (−) IFN-α (Wellferon) and lysed and IRS-1 and STAT1α were immunoprecipitated and analyzed by SDS-PAGE and Western transfer and sequential blotting with antibodies to phosphotyrosine (*i*) and IRS-1 (*ii*). The cell lysates were re-immunoprecipitated for JAK1 and analyzed by SDS-PAGE and Western transfer and sequential blotting with antibodies to phosphotyrosine (*iii*) and JAK1 (*iv*). The lysates were further immunoprecipitated for Tyk2 and JAK2 and analyzed by SDS-PAGE and Western transfer and sequential blotting with antibodies to phosphotyrosine (*v*), Tyk2 (*vi*), and JAK2 (*vii*).

JAK Dependence of PI 3′-Kinase Activation in Response to IL-4, OSM, and Type I Interferons—The p85α subunit of PI 3′-kinase associates with IRS-1 in response to IL-4 (52, 55), OSM, and less substantially IFN-α (Wellferon) (Fig. 5, *A–C*, *lanes 2* and *6*). Such association is dependent on JAK1 in these cells. It is not observed in U4A cells but is restored in U4A cells expressing JAK1 (Fig. 5, *A–C*, *lanes 4* and *6*, respectively). The JAK dependence of PI 3′-kinase recruitment/activity was also evident when monitored by an *in vitro* kinase assay (Fig. 5*D*, *i* and *ii*). For IL-4 and IFN-α significant activity (*lanes 4* and *5*) over background (*lane 2*) was observed in wild-type 2C4 cells but not in U4A cells lacking JAK1 (*lanes 8* and *9*). For OSM there was a substantial reduction in activity in the U4A cells lacking JAK1 (compare *lanes 3* and *7*). The activity observed in response to all three ligands was substantially increased in U4A cells overexpressing wild-type JAK1 (Fig. 5*D*, *i* and *ii*, *lanes 10–13*).

IRS-1 and IRS-2 Are Not Essential for an Inhibitory Effect of the IFNs on Cell Growth—The growth of mouse 32D cells, which do not express IRS-1 or IRS-2 (52), was consistently inhibited by IFN-α (recombinant human A/D (*Bgl*) hybrid highly active on mouse cells) or recombinant murine IFN-γ, at 1000 IU/ml over 3–5 days (Fig. 6). On comparison of 32D cells with 32D cells stably expressing IRS-1 or IRS-2, only minor

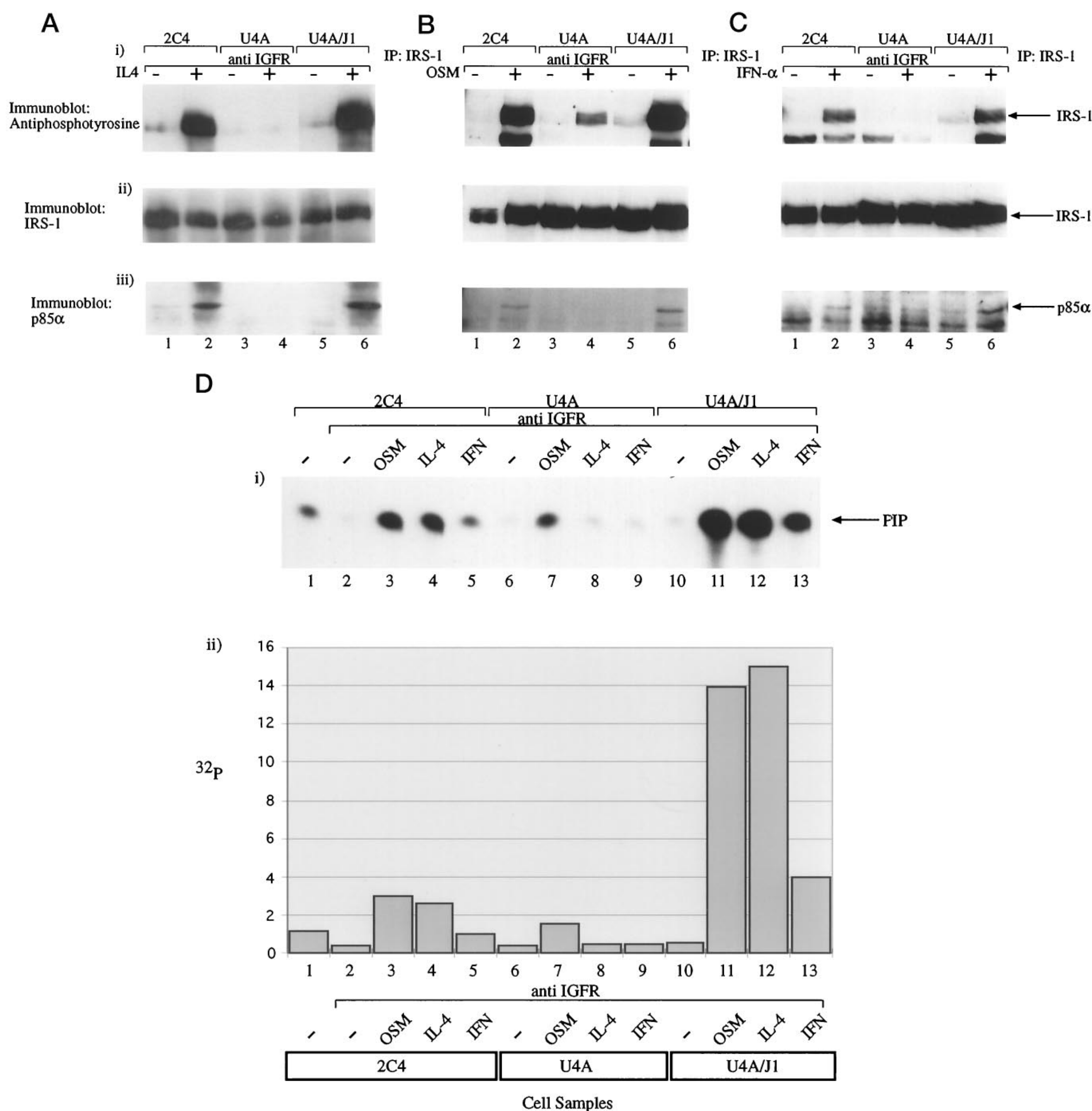

FIG. 5. **PI 3′-kinase activation.** JAK dependence of the association of the p85α subunit of PI 3′-kinase with IRS-1 (*A–C*) and PI 3′-kinase activity (*D*) in response to IL-4, OSM, and IFN-α. Wild-type and JAK1-negative (U4A) cells and U4A cells stably transfected with wild-type JAK1 (U4A/J1) were incubated in the presence of antibody to the IGFR for 24 h and treated where indicated (+) with IL-4 (*A*), OSM (*B*), and IFN-α (*C*, Wellferon) at 37 °C for 15 min. The cells were lysed and immunoprecipitated with antibody to IRS-1 and analyzed by SDS-PAGE, Western transfer, and sequential blotting (*A–C*) with antibody to phosphotyrosine (*i*), IRS-1 (*ii*), and p85α (*iii*), or the immunoprecipitates analyzed by an *in vitro* PI 3′-kinase assay (*D*) in the presence of [γ^{32}P] ATP and the PI 4′-kinase inhibitor, adenosine (see "Experimental Procedures") and the products separated by TLC and analyzed by autoradiography (*i*) or quantification using a PhosphorImager (*ii*). In *i*, *PIP* indicates the position of the radioactive product and the co-migrating phosphatidylinositol phosphate standard. The *abscissa* in *ii* indicates the level of incorporated ^{32}P determined by PhosphorImager analysis. Essentially identical results were obtained on three to four independent occasions for the individual cytokines. The statistical analysis of the association of PI 3′-kinase activity with IRS-1 immunoprecipitation was performed by Friedman's nonparametric analysis of variance (61), giving p values of <0.01, 0.08, and <0.001 for IL-4, OSM, and IFN-α (Wellferon), respectively.

differences were observed in the inhibitions of cell growth in response to the IFNs (data not shown). IRS-1 and IRS-2 are not, therefore, essential for the inhibition of cell growth in response to IFNs α or γ in these cells.

DISCUSSION

The phosphorylation of IRS-1 through a constitutive IGFR-mediated pathway and in response to the IFNs, IL-4, and OSM has been investigated. Phosphorylation through the IGFR shows no dependence on JAK1, JAK2, or Tyk2 (Fig. 1), as has previously been assumed to be the case. In contrast, phosphorylation in response to the IFNs, IL-4, and OSM is JAK-dependent (Figs. 1 and 2). Despite easily detectable levels of expression, neither constitutive nor induced tyrosine phosphorylation of IRS-2 was observed in multiple experiments in these cells. This suggests an important possible difference(s) in receptor/JAK/IRS interaction for the two IRS protein isoforms.

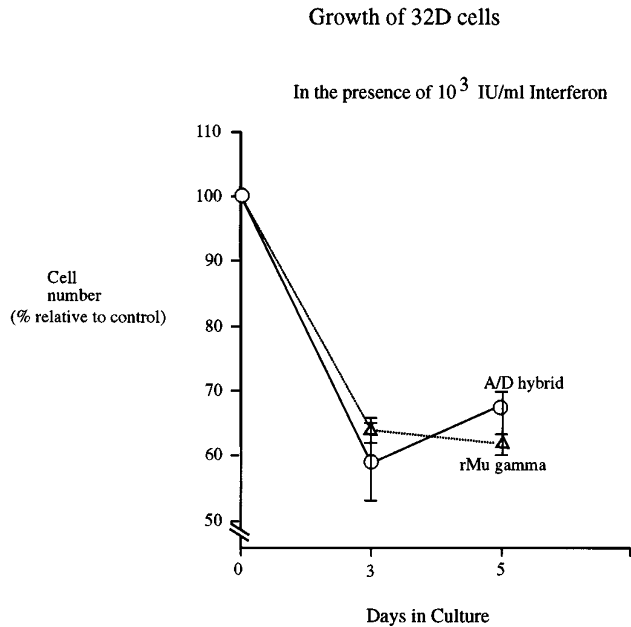

FIG. 6. **Inhibition of cell growth by the IFNs in the absence of IRS-1 and IRS-2.** Murine 32D cells, which do not express endogenous IRS-1 or IRS-2, were incubated with or without 1000 IU/ml of (○) Human A/D hybrid IFN (highly active on mouse cells) or (△) recombinant murine IFN-γ. Cell numbers at 3 or 5 days are presented as a percentage of controls. The experiments have been repeated independently three times. An analysis of variance for the effect of human A/D hybrid IFN and recombinant murine IFN-γ at 3 days, 5 days, and over the combined period gives p values of <0.001 in all cases.

The basis for the differential is under investigation.

IRS-1 and the IFNs—Phosphorylation of IRS-1 was observed in response to a natural mixture of α IFNs (Wellferon) and to individual recombinant IFNs. Of these IFN-β consistently appeared slightly less effective, but the difference, if any, was marginal (Fig. 3*A*). IFN-γ, on the other hand, was clearly less effective, if effective at all (Fig. 3*B*). The phosphorylation in response to the α IFNs is JAK-dependent (Fig. 4 and Table I). It was not observed in the absence of JAK1 or Tyk2, in accord with the interdependence of the activation of JAK1 and Tyk2 in response to the Type I IFNs. 32D cells do not express IRS-1 or IRS-2 but retain an IFN response, including activation of the JAK/STAT pathway and the inhibition of cell growth (Fig. 6).[5,6] Although not essential for the inhibition of cell growth, transfected IRS-1 and IRS-2 did marginally affect the inhibitory response to the IFNs, and it remains possible that signaling through these molecules may play a greater role in this aspect of the response in other cell types.

Of the viruses tested (EMC, SFV, coccal, and vaccinia) none grew sufficiently well in 32D cells or in 32D cells transfected with IRS-1 or 2 for an antiviral response to the IFNs to be assayed.[7] Although with IFN-γ a substantial antiviral response (using EMC) is routinely observed at concentrations of <30 IU/ml in the human fibrosarcoma cell lines (53),[8] phosphorylation of IRS-1 (or IRS-2) was not observed with this IFN at concentrations of less than 1000 IU/ml and was, at best, barely detectable at concentrations of up to 100,000 IU/ml (Fig. 3*B*). Taken together the results indicate that phosphorylation of IRS-1 is JAK-dependent, but, unlike the JAK/STAT pathway, the JAK/IRS pathway does not appear essential to those aspects of the IFN response tested in the cells utilized here. This suggests a likely ancillary role in more specialized aspects of the IFN response. A more central role in the response in particular cell types cannot, however, be excluded.

IRS-1, IL-4, and OSM—In the human fibrosarcoma cell lines, IL-4 and OSM each activate JAK1, JAK2, Tyk2, and IRS-1 (Figs. 1 and 2). IL-4 activates STAT6 and STAT3[9] and OSM, STATs 1, 3, and 5.[10] Expression of JAK3 was not detected in these non hematopoietic human fibrosarcoma cell lines. In the case of IL-4, it is assumed that the activation of the JAKs observed here is either through the Type II IL-4 receptor, which utilized the common IL-4 α subunit together with the β subunit of the IL-13 receptor, or a novel receptor. For both cytokines, the phosphorylation of IRS-1 was substantially reduced in the absence of JAK1. For IL-4 the dependence on JAK1 appeared complete (Fig. 1*A*, *lane 6*), and the absence of JAK2 or Tyk2 was without marked effect (Fig. 1*A*, *lanes 9* and *12*). With OSM a residual response was frequently observed in the absence of JAK1 (*e.g.* Fig. 2*B* (*i*), 2*D* (*i*), and Fig. 5*B* (*i*), *lanes 9*, *5*, and *4*, respectively). This parallels the data for STAT activation by IL-6 and OSM in these cells for which a major but incomplete dependence on JAK1 is observed, the residual activation being through JAK2 and Tyk2 (Ref. 50).[2] Consistent with this here, as for STAT activation (50), the residual response was ablated on over expression of a dominant negative JAK1 (JAK1.K→E) in the U4A cells (Fig. 2*D*, *i*, *lanes 9*, *5*, and *2*) and a reduced level of IRS-1 phosphorylation was observed in response to OSM in cells lacking Tyk2 or JAK2 (Fig. 2*B*, *i*, *lanes 6* and *12*). For both cytokines the phosphorylation of IRS-1 in the U4A cells lacking JAK1 was restored on stable transfection with JAK1 (Fig. 1*B* (*i*, *lane 7*); Fig. 2, *C* (*i*, *lane 6*) and *D* (*i*, *lane 7*); and Fig. 5*B* (*i*, *lane 6*)) and substantially or partially restored on transfection with Tyk2 or JAK2 (Fig. 1*B* (*i*, *lanes 11* and *13*) and Fig. 2*C* (*i*, *lanes 4* and *8*)). Taken together the results establish the JAK dependence of IRS-1 phosphorylation in response to IL-4 and OSM in these cells. The dependence for IL-4 in particular is predominantly on JAK1, but the partial restoration of the response by Tyk2 and JAK2 for both cytokines indicates that IRS-1 phosphorylation can be mediated directly or indirectly through JAK1, JAK2, or Tyk2. Phosphorylation through JAK2 or Tyk2 (or JAK3) may, therefore, play a more important role in other cell types and/or with alternative receptors. It would be of interest to know if the pattern of phosphorylation of IRS-1-mediated through JAK1, JAK2, and Tyk2 are the same, but this will not be trivial to establish. For OSM the phosphorylation of IRS-1 in the different cell lines parallels that of the gp130 receptor subunit and may therefore simply reflect that of the receptor. It must, however, be emphasized that there is no apparent $L(X)_4NPXY(p)XSXP$ motif in the gp130 subunit of the OSM receptor, analogous to that utilized as an IRS-1 docking site in the IL-4 receptor and recruitment through the JAKs remains possible. Co-immune precipitation of IRS-1 with the JAKs has been reported (21, 54). Substantial additional work will therefore be required to determine the precise roles of the JAKs and receptor subunits in the recruitment and phosphorylation of IRS-1 in response to IL-4, IFNs, and OSM.

Activation of PI 3′-kinase through association of the p85α subunit with IRS-1 is well established (22, 55–58). Consistent with this ligand and JAK-dependent association of the p85α subunit of PI 3′-kinase with IRS-1 and PI 3′-kinase “activation” were observed here for IL-4, OSM, and less obviously IFN-α (Wellferon) (Fig. 5). It is recognized that the PI 3′-kinase complex may be preactivated independent of agonist stimulation

[5] D. Watling and N. C. Rogers, unpublished data.
[6] L. Platanias, personal communication.
[7] M. S. Burfoot and D. Watling, unpublished data.
[8] D. Watling, unpublished data.
[9] M. S. Burfoot, unpublished data.
[10] J. M. Smith, M. S. Burfoot, and N. Broughton, unpublished data.

when recruited to phosphorylated IRS-1. It will be of interest to determine whether PI 3′-kinase activation in response to these ligands is exclusively through IRS-1 or whether additional JAK-dependent PI 3′-kinase recruitment/activation pathways operate in these cells.

Signaling through the JAKs—Experiments with JAK and STAT-negative mutant cell lines have established the essential role of JAK/STAT pathway in IFN-inducible gene expression and the antiviral response (53). Such pathways are not, however, sufficient for all aspects of the response. Indeed, simple comparison with the other cytokines, all of which activate a number of response pathways, suggests that this is unlikely to be the case and the activation of a number of second message pathways by the IFNs has frequently been reported. The role of the JAKs in such pathways is less well established. An additional JAK1-dependent signal appears essential for the antiviral response to IFN-γ (53) and activation of phospholipase A_2 in response to IFN-α is dependent on JAK1 (59). The JAK1 dependence of IRS-1 phosphorylation in response to IL-4 has recently been reported (60). Here the dependence of such phosphorylation upon JAK activation has been clearly established for three different ligands operating through distinct types of receptor. In the case of the $\alpha\beta$ IFNs, the dependence on JAK1 and Tyk2 reflects the interdependence of JAK activation in response to the IFNs (41, 42). For OSM and IL-4, there is a major dependence on JAK1 in these cells, but phosphorylation of IRS-1 can also be mediated through JAK2 or Tyk2. For IL-4, OSM, and, less obviously, IFN-α, a similar and likely consequential (in part at least) JAK dependence of PI 3′-kinase activation has also been observed. It will be of considerable interest to determine the molecular mechanisms underlying the JAK dependence of these activations and to investigate further the roles of the JAKs in the activation of additional pathways in response to these and other ligands.

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