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Activation of Insulin-like Growth Factor I Receptor Signaling Pathway Is Critical for Mouse Plasma Cell Tumor Growth

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ABSTRACT

Plasma cell neoplasia in humans generally occurs as multiple myeloma, an incurable form of cancer. Tumors with marked similarity can be induced in mice by a variety of agents, including chemicals, silicone, and oncogene-containing retroviruses, suggesting the use of murine tumors as an informative model to study plasma cell disease. Herein, we have focused on the role of insulin-like growth factor I receptor (IGF-IR) signaling in the development of plasma cell disease. The insulin receptor substrate 2/phosphatidylinositol 3′-kinase/p70S6K pathway was found to be either constitutively or IGF-I-dependently activated in all plasma cell tumors. Biological relevance was demonstrated in that plasma cell lines with up-regulated IGF-IR expression levels exhibited mitogenic responses to IGF-I. More importantly, expression of a dominant-negative mutant of IGF-IR in these lines strongly suppressed tumorigenesis *in vivo*. Taken together, these results demonstrate that up-regulation and activation of IGF-IR and the downstream signaling pathway involving insulin receptor substrate 2, phosphatidylinositol 3′-kinase, and p70S6K may play an important role in the development of a broad spectrum of plasma cell tumors.

INTRODUCTION

Plasma cells represent the end stage in B cell development and tumors of this cell type are found in both humans and mice. In humans, plasma cell disease can occur as isolated plasmacytoma or, more commonly, multiple myeloma, an incurable form of cancer. Similar tumors in mice either arise spontaneously (1) or can be induced by a variety of agents, including oils (2, 3), silicone (4), and retroviruses containing oncogenes such as *raf/myc* (5–7) and *abl/myc* (8). Recent studies have demonstrated a number of important similarities between the murine disease and human myeloma, suggesting that the murine system may serve as a useful model for this form of neoplasia. These similarities include: (*a*) a role for T cells in disease progression (9); (*b*) a critical role for IL-6 in tumor development (10); (*c*) bone marrow localization with associated bone destruction (11, 12); (*d*) constitutive activation of signal transducers and activators of transcription 3 (13, 14).

Although biochemical lesions such as *ras* gene activation or p53 mutation have been associated with a number of forms of cancer, very little is known about such alterations in B lineage tumors. An activated c-*myc* gene appears essential for development of most murine plasma cell tumor (15) but is less common in human myeloma. Activation of *myc* results most frequently from chromosomal translocation but can also be achieved by inclusion of the *myc* gene in a retroviral construct or other undefined mechanisms (16). Although *myc* rearrangements are rare in multiple myeloma, a variety of other translocations are routinely observed, most involving one of the immunoglobulin loci and a second locus, such as fibroblast growth factor receptor 3 (17) or cyclin D1 (18). In addition, *myc* activating translocations are frequently observed in Burkitt's lymphoma (19, 20), suggesting that gene activation by translocation may be a common mechanism for deregulation in B lineage neoplasia in both species. Biochemical pathways affected by such translocations have not been defined in terms of association with tumor development. The identification of deregulated pathways is of obvious importance to both further an understanding of the biology of these diseases and to identify potential targets in specific pathways for therapeutic intervention.

One of the emerging themes in loss of growth arrest involves either overexpression or aberrant activation of growth factor receptors, leading to deregulation of signaling pathways affecting cell growth, differentiation, and/or apoptosis. Among such receptors is the type II tyrosine kinase receptor family, which includes the insulin receptor, IGF-IR,[2] and *ros* oncogene (21, 22). Insulin and IGF-I are the physiological ligands for the corresponding receptors that share a number of properties. Both contain two α subunits responsible for ligand binding and two β subunits containing intracellular tyrosine kinase domains. Ligand binding induces receptor clusterization, autophosphorylation, and internalization. The activated receptor phosphorylates many important intracellular molecules on tyrosine, including SHC and IRS, leading to activation of MAPK and PI 3′K pathways, respectively (23). Whereas activation of the insulin receptor is generally implicated in glucose metabolism, stimulation of the IGF-IR pathway is strongly associated with cell proliferation, malignant transformation, and antiapoptotic effects (24).

Up-regulation of IGF-IR has been noted in several human cancers, and a role for this receptor in neoplastic development has been strongly suggested by the modulation of tumor growth after introduction of antisense oligonucleotides, antireceptor antibody, or dominant-negative mutants (23, 25). Furthermore, embryonic fibroblasts from IGF-IR knockout mice are resistant to transformation by a variety of highly transforming proteins, including oncogenes, activated tyrosine kinase receptors, and viral proteins (23). Taken together, these findings suggest that IGF-IR may play an important role in a number of neoplastic conditions. It was, therefore, of interest to determine whether this receptor might contribute to lymphoid malignancies and, if so, to define associated signaling pathways. In the present study, we describe a critical role for IGF-IR in plasma cell tumor development and identify such a downstream signaling pathway involving IRS-2, PI 3′K, and p70S6K.

MATERIALS AND METHODS

Cell Lines and Culture. Cell lines used in the present studies include the following: B cell lines WEHI231, A20, and NFS-1; plasma cell lines S107 and

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[2] The abbreviations used are: IGF-IR, insulin-like growth factor I receptor; IGF-I, insulin-like growth factor I; IRS, insulin receptor substrate; PI 3′K, phosphatidylinositol 3′-kinase; MAPK, mitogen-activated protein kinase; SHC, Src homology and collagen.

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X24 (oil-induced), 7.2 and 12.2 (*raf/myc*-induced), and 121.1 and 128.3 (*abl/myc*-induced). All lines were grown in RPMI 1640 containing 10% FCS and 2-mercaptoethanol.

Cell Lysates, Immunoprecipitation, and Immunoblot Analysis. Prior to lysis, cells were serum starved for 2 h in Dulbecco's modified essential medium containing 25 μM Na_3VO_4. They were either not treated or were stimulated with murine IL-4 (100 ng/ml) or human IGF-I (100 ng/ml; Intergen) for 10 min at 37°C. Cell pellets were lysed in a Triton X-100-containing buffer as reported (26). Insoluble proteins were removed by centrifugation, and protein concentration was determined using a Bio-Rad protein assay kit. Equivalent amounts of cell lysates (0.5–2 mg/sample) were immunoprecipitated with anti-IGF-IR serum (Santa Cruz Biotechnology; 5 μl/sample), anti-IRS-2 (Ref. 27; 5 μl/sample), anti-SHC (Transduction Laboratories, Inc.; 5 μl/sample), or anti-p70S6K (Upstate Biotechnology, Inc., 5 μl/sample). Washed immunoprecipitates were electrophoresed on 8% SDS-PAGE gels, transferred to Immobilon membrane (Millipore), and immunoblotted with antiphosphotyrosine (anti-pTyr, Upstate Biotechnology, Inc., 2 μg/ml), anti-PI 3′K (Upstate Biotechnology, Inc., 1:1000 dilution), or anti-p70S6K (1:1000). For direct Western analysis, anti-phospho-MAPK (1:1000, New England Biolabs) was used. Proteins were detected using an ECL system from Amersham Pharmacia Biotech.

***In Vitro* IGF-IR Immune Complex Assay.** Cell lines were treated as described above, and equivalent amounts of cell lysates were immunoprecipitated with anti-IGF-IR serum. An immune complex assay to determine receptor kinase activity was performed as described previously (26).

The p70S6K Activity Assay. Various cell lines were similarly treated as stated above and lysed. Equivalent amounts of cell lysates were immunoprecipitated with anti-p70S6K (Santa Cruz Biotechnology; SC230; 2 μg/sample) or anti-Grb2 (Santa Cruz Biotechnology; SC255; negative control). Washed immunoprecipitates were subjected to a S6K activity assay using a kit from Upstate Biotechnology, Inc., according to the manufacturer's instructions. The peptide AKRRRLSSLRA was used as a substrate in the activity assay. The results were the mean value from two independent experiments.

Mitogenic Assay. Cultured cells were washed twice with PBS and seeded at 2×10^5 cells/well in 24-well plates in RPMI 1640 containing 0.5% BSA (Sigma) with or without IGF-I (100 ng/ml) for 44 h. Cells were pulsed with [^{3}H]thymidine (Amersham Pharmacia Biotech; 1 μCi/well) for an additional 4 h prior to harvest. [^{3}H]Thymidine incorporation was measured in triplicate samples, and the mean value was calculated. Fold increase of [^{3}H]thymidine incorporation was calculated by dividing the mean value of untreated wells by that of corresponding IGF-I-stimulated wells.

Transfection and *in Vivo* Tumorigenesis. Electroporation for gene transfer into suspension cells was performed as reported (28). Cell lines were transfected with 10 μg of pBPV vector containing a lysine to arginine mutation at the ATP binding site (pBPV-IGF-IRKR, kindly provided by Dr. Renato Baserga) together with 1 μg of pZipneo as carrier DNA. Neomycin-resistant cells were selected by growth in the presence of 750 μg/ml of G418 (Life Technologies, Inc.). Parental or IGF-IRKR transfectants were injected i.p. (2×10^6 cells/mouse) into female BALB/c mice 24 h after pristane priming. Tumor development was monitored twice per week.

RESULTS

IGF-IR Is Up-regulated in *raf/myc* and Chemical (Oil)-induced Plasma Cell Tumors and Autophosphorylated in Response to IGF-I Stimulation. Because up-regulation of IGF-IR levels and increased IGF-IR activity has been implicated in several types of cancer (25), we were interested in determining whether IGF-IR up-regulation and activation would contribute to the pathogenesis of lymphoid neoplasias, particularly plasma cell disease. To initially assess receptor levels, equivalent amounts of cell lysates from plasma cell tumor and B cell lymphoma lines were immunoblotted with specific antibody against IGF-IR β chain (Fig. 1*A*). Whereas *abl/myc* plasma cell tumors (121.1 and 128.3) expressed only the basal levels of IGF-IR, the levels increased by 2- and 5-fold in chemical-induced (S107 and X24) and *raf/myc*-induced (12.2 and 7.2) plasma cell tumors, respectively. B cell lymphomas (WEHI231 and NFS-1) only expressed basal levels of IGF-IR when compared to *raf/myc* and chemical plasma cell tumors. IGF-IR up-regulation was reproducibly detected in five *raf/myc* lines (data not shown), indicating that increase in IGF-IR is a common phenomenon in plasma cell tumors induced by *raf/myc* oncogenes.

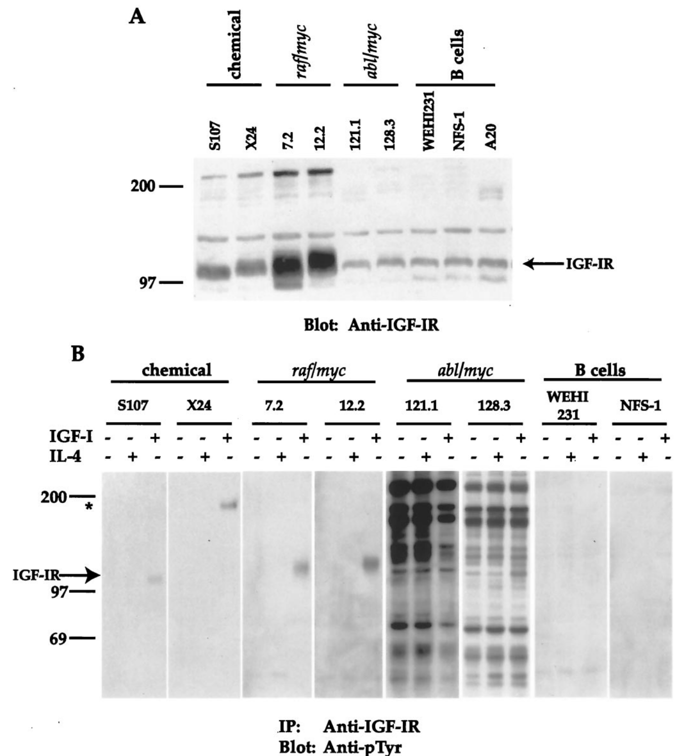

Fig. 1. IGF-IR is up-regulated in *raf/myc*- and chemical-induced plasma cell tumors and activated in response to IGF-I stimulation. *A,* equivalent amounts of cell lysates from the indicated plasma and B cell lines were subjected to SDS-PAGE. The separated proteins were transferred to Immobilon membrane and immunoblotted with anti-IGF-IR antibody. *B,* cells were serum starved for 2 h, either not stimulated or stimulated with IL-4 or IGF-I for 10 min, and lysed. Equivalent amounts of cell lysates were immunoprecipitated (*IP*) with anti-IGF-IR serum. Immunoprecipitates were electrophoresed and blotted as above using anti-pTyr. *, the 180-kDa phosphoprotein subsequently identified as IRS-2. *Arrows,* positions of IGF-IR. Marker sizes are indicated in kDa.

To demonstrate that up-regulated IGF-IR detected in both *raf/myc*- and chemical-induced plasma cell tumors was functional, we next analyzed receptor autophosphorylation. Up-regulated IGF-IR was phosphorylated in response to IGF-I in both *raf/myc*-induced (12.2 and 7.2) and chemical-induced S107 lines (Fig. 1*B*). The lower level of autophosphorylation in S107 correlates with lower protein expression (Fig. 1*A*). Although IGF-IR autophosphorylation was not observed in the chemical-induced X24 line, which may be attributable to the lower expression of IGF-IR, a 180-kDa tyrosine-phosphorylated protein was detected in IGF-IR immunoprecipitates in response to IGF-I stimulation. This protein was subsequently identified as IRS-2, a substrate of IGF-IR (see Fig. 2). Because only phosphorylated IGF-IR is able to associate with IRS molecules *in vivo*, detection of IRS-2 in the IGF-IR immunoprecipitates indicates that IGF-IR in X24 line is also functionally active. In *abl/myc* plasma cell lines, many phosphorylated proteins spanning a large size range were co-immunoprecipitated with anti-IGF-IR indicating that constitutively activated *abl* tyrosine kinase phosphorylates numerous proteins *in vivo,* some of which associate with IGF-IR. In striking contrast to the

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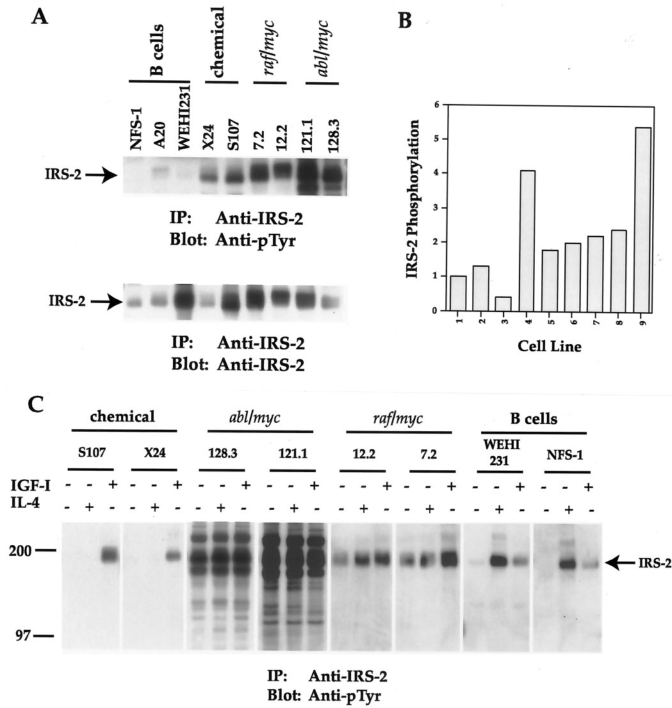

Fig. 2. IRS-2 is activated in all plasma cell tumors. *A,* cells were grown in medium containing 10% FCS and directly lysed. Lysates were immunoprecipitated (*IP*) with anti-IRS-2 serum, electrophoresed, and blotted with anti-pTyr (*top panel*). The same Immobilon membrane was reblotted with anti-IRS-2 (*bottom panel*). *B,* phosphorylation of IRS-2 was normalized based on the protein expression levels. *Columns 1–9,* lines NFS-1, A20, WEHI231, X24, S107, 7.2, 12.2, 121.1, and 128.3, respectively. *C,* cells were serum starved for 2 h, either not stimulated or stimulated with IL-4 or IGF-I for 10 min, and lysed. Immunoprecipitates were analyzed as in *A, top panel. Arrows,* positions of IRS-2. Marker sizes are indicated in kDa.

plasma cells, no constitutive or IGF-I-dependent IGF-IR autophosphorylation was detected in the two B cell lymphoma lines, WEHI231 and NFS-1. Although IL-4 is known to be a growth and differentiation factor for B lymphocytes, stimulation of all lines tested with IL-4 did not induce IGF-IR activation. Taken together, these results demonstrate that IGF-IR is up-regulated in both *raf/myc*- and chemical-induced plasma cell tumors and that the receptor is functionally active in response to IGF-I.

IRS-2 Is Constitutively Phosphorylated in all Plasma Cell Tumors but not in B Cell Lymphomas. To determine whether IGF-IR activation leads to downstream signaling, we next searched for substrates that might be phosphorylated in response to IGF-I. IRS molecules have been demonstrated to be major substrates of the insulin receptor and IGF-IR *in vivo* (27, 29). Analysis of IRS-1 revealed only sporadic phosphorylation in some lines (not shown). In contrast, IRS-2 was constitutively phosphorylated on tyrosine in six of six plasma cell lines (Fig. 2*A, top panel*), irrespective of the tumor-inducing agent. Weak phosphorylation was observed in one B cell line, A20, with two others remaining largely negative. The marked difference in IRS-2 phosphorylation between plasma and B cells could not be attributed to IRS-2 protein levels, because the highest expression was observed in the WEHI231 B cell line, and the X24 plasma cell tumor was one of the lowest (Fig. 2*A, bottom panel*). After normalizing for IRS-2 expression levels, constitutive phosphorylation of IRS-2 was shown to be 2–5-fold higher in plasma cell tumors than in B cell lymphomas (Fig. 2*B*).

Potential ligands known to be upstream activators of the IRS-2 pathway include IGF-I and IL-4 (27, 29). We therefore assessed the effects of these two factors on plasma and B cell lines after serum starvation. Constitutive tyrosine phosphorylation of IRS-2 was still observed in the two *raf/myc* (12.2 and 7.2) and the two *abl/myc* (121.1 and 128.3) lines (Fig. 2*C*) but not in chemical-induced tumors (S107 and X24) or B cell lines (WEHI231 and NFS-1). IRS-2 phosphorylation was readily induced in the chemical tumors by IGF-I, but not IL-4. IGF-I also enhanced IRS-2 phosphorylation in the *raf/myc* lines. In contrast, this phosphorylation was constitutive in *abl/myc* tumors. The IRS-2 response to IGF-I thus correlates with IGF-IR levels and autophosphorylation described in Fig. 1. B cell lines also responded to IGF-I, although maximal stimulation was observed with IL-4. These results indicate that normal serum levels of IGF-I in conjunction with up-regulated IGF-IR may be sufficient to constitutively activate this pathway in both chemical- and *raf/myc*-induced tumors. Phosphorylation of IRS-2 in the *abl/myc* lines may be attributable to the kinase activity of v-*abl,* consistent with the results described above demonstrating numerous phosphorylated proteins associated with the IGF-IR in these lines (Fig. 1*B*). We therefore conclude that IRS-2 is a major downstream substrate of IGF-IR in tumors induced by chemicals and *raf/myc* retrovirus. It may also be directly phosphorylated by v-*abl* in *abl/myc* lines. Constitutive phosphorylation of IRS-2 occurs under

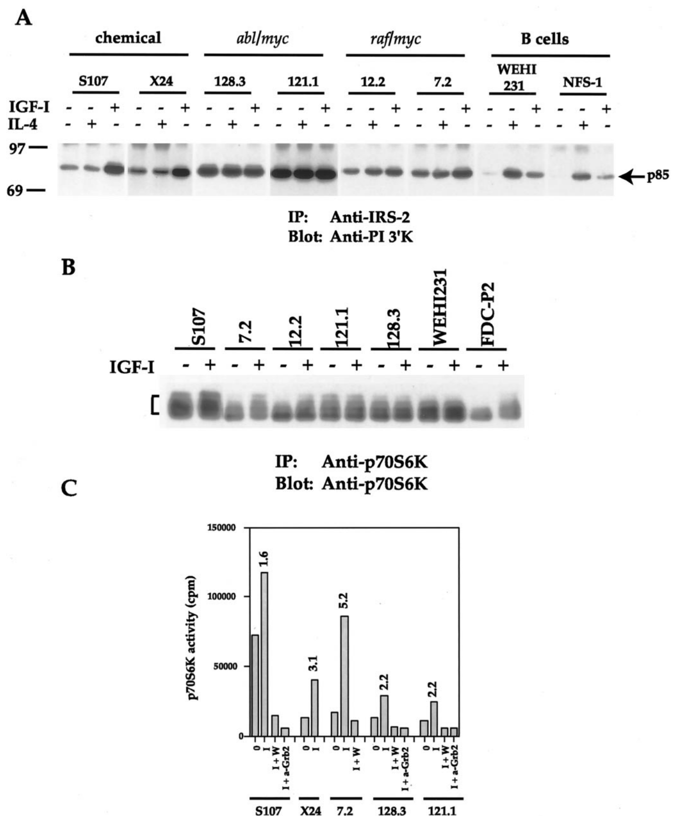

Fig. 3. Activation of PI 3′K is demonstrated by IRS-2/PI 3′K association *in vivo* and by p70S6K activation in plasma cell tumors. *A,* cells were serum starved for 2 h, either not stimulated or stimulated with IL-4 or IGF-I for 10 min, and lysed. Equivalent amounts of cell lysates were immunoprecipitated (*IP*) with anti-IRS-2 serum and blotted with anti-PI 3′K. *Arrow,* p85 subunit of PI 3′K. Marker sizes are indicated in kDa. *B,* cells were serum starved for 2 h, either not stimulated or stimulated with IGF-I for 10 min, and lysed. Equivalent amounts of cell lysates were immunoprecipitated with anti-p70S6K serum and blotted with the same antibody. *Bracket,* p70S6K migrating at different speeds. *C,* various cell lines were serum starved and either untreated or stimulated with IGF-I for 10 min. When wortmannin (*w*; 100 nM) was included, it was added 20 min before IGF-I stimulation. Equivalent amounts of cell lysates were immunoprecipitated with anti-p70S6K or anti-Grb2 (negative control). Washed immunoprecipitates were subjected to an *in vitro* p70S6K activity assay according to the protocol from the company. Results are the mean value of two individual experiments.

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normal growth conditions in all plasma cell tumors but not B cell lymphomas.

PI 3′K Is Associated with Tyrosine-phosphorylated IRS-2 in all Plasma Cell Lines. Tyrosine-phosphorylated IRS molecules have been shown to associate with PI 3′K by binding to the *src* homology 2 domain of the p85 subunit resulting in subsequent PI 3′K activation (30). To assess signal transduction from IRS-2 to PI 3′K in plasma cell tumors, we analyzed the association of IRS-2 with the p85 subunit of PI 3′K in co-immunoprecipitation experiments. As seen in Fig. 3*A,* constitutive association of p85 with IRS-2 was detected in all plasma cell lines, irrespective of inducing agent, and this association could be further enhanced in the chemical and *raf/myc* lines upon IGF-I, but not IL-4, stimulation. The highest level of association was observed in the two *abl/myc* lines and could not be further enhanced with ligands, likely indicating that IRS-2 is already maximally phosphorylated after *abl* transformation as suggested above (Fig. 2). In contrast, none of the B cell lines evidenced this constitutive activation. WEHI231 and NFS-1 demonstrated maximal association in response to IL-4, consistent with the preferential phosphorylation of IRS-2 after IL-4 stimulation (Fig. 2). Activation of PI 3′K was also detected by measuring phosphatidylinositol phosphate production in all plasma cell tumors either IGF-I dependently (chemical-induced and *raf/myc*-induced) or constitutively (*abl/myc*-induced; data not shown). Thus, the constitutive association of IRS-2 with PI 3′K in plasma but not B cell tumors strongly suggests that activation of the PI 3′K pathway through IRS-2 may be critical for plasma cell tumor development.

p70S6K Is Activated in Response to IGF-I Stimulation in Plasma Cell Tumors. p70S6K has been defined previously to be a signaling molecule downstream of PI 3′K (31). Thus, we measured p70S6K activation by both mobility shifting assay (Fig. 3*B*) and kinase activity assay (Fig. 3*C*). IGF-I stimulation of chemical (S107) and *raf/myc* (7.2 and 12.2) lines resulted in the slower migrated bands as detected by a p70S6K specific antibody. On the other hand, the constitutive shifting bands were detected from both *abl/myc* lines (121.1 and 128.3), consistent with constitutive activation of PI 3′K (see Fig. 3*A*). In WEHI231 B cell lymphoma line, only the two faster migrating bands were detected in the presence of IGF-I. Induction of p70S6K activation by IGF-I in FDC-P2 myeloid progenitor line was reported previously (32) and confirmed by the mobility shifting assay (Fig. 3*B*).

To directly prove that p70S6K is activated in plasma cell tumor lines, its activity was measured by immunoprecipitating equivalent amounts of cell lysates with anti-p70S6K followed by an *in vitro* phosphorylation assay using peptide AKRRRLSSLRA as substrate. As shown in Fig. 3*C,* all of the plasma cell tumor lines responded to IGF-I for p70S6K induction, ranging from 1.6- to 5.2-fold. The specific detection of p70S6K activity was demonstrated by using an isotype-matched anti-Grb2 antibody for immunoprecipitation as a negative control. Dependency on PI 3′K for p70S6K activity was established by including wortmannin, a known inhibitor of PI 3′K, which completely reversed IGF-I-induced p70S6K activities in several lines analyzed. We reproducibly observed that IGF-I was able to weakly induce p70S6K activity in the two *abl/myc* lines, suggesting some other pathways other than IRS-2 may be induced by IGF-I to stimulate extra PI 3′K activity in *abl*-transformed lines. Together, the p70S6K results substantiate the observation of activation of IRS-2/PI 3′K among the all plasma cell tumors.

The SHC/MAPK Pathway Is Constitutively Activated in *abl/myc* Plasma Cell Tumors. SHC can be tyrosine phosphorylated by the activated IGF-IR through the interaction of phosphotyrosine

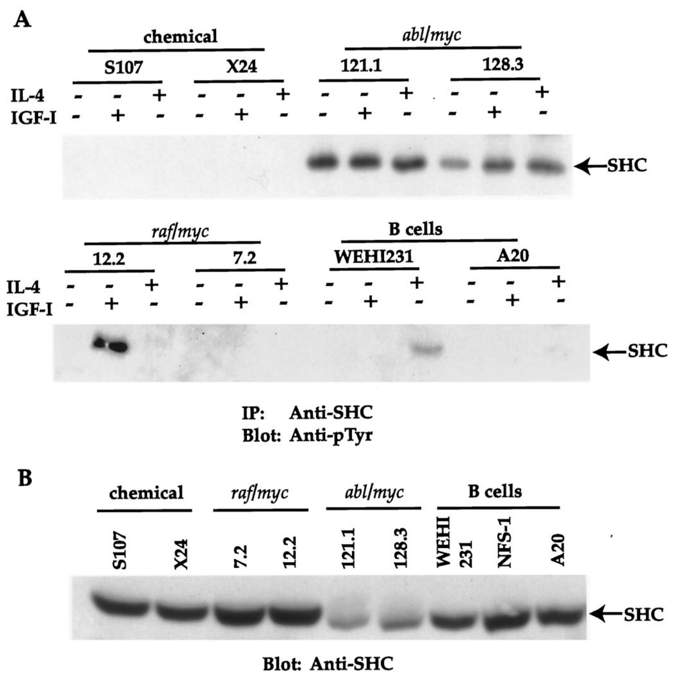

Fig. 4. SHC phosphorylation is detected mainly in *abl/myc* plasma cell tumors. *A,* cells were serum starved for 2 h, either not stimulated or stimulated with IL-4 or IGF-I for 10 min, and lysed. Equivalent amounts of cell lysates were immunoprecipitated (*IP*) with anti-SHC serum and blotted with anti-pTyr. *B,* equivalent amounts of cell lysates (100 μg/lane) were subjected SDS-PAGE, and transferred proteins were immunoblotted with anti-SHC. *Arrows,* p52 SHC protein.

binding domain from SHC with the NP*X*pY motif located at the juxtamembrane domain of IGF-IR (33). The phosphorylated SHC interacts with the *src* homology 2 domain of Grb2, thus activating SOS/Ras/Raf/MAPK cascade. To understand whether this cascade is also involved in the tumor development of mouse plasma cells, we tested SHC phosphorylation. As shown in Fig. 4*A,* although the p52 SHC phosphorylation was only detected in 12.2 line in response to IGF-I among the all *raf/myc* and chemical tumors, constitutive phosphorylation of SHC on tyrosine was detected in both 121.1 and 128.3 *abl/myc* lines. Phosphorylation of SHC was also observed in WEHI231 B lymphoma in response to IL-4. Similar expression levels of p52 SHC were detected from all lines (Fig. 4*B*).

MAPK activation was subsequently tested using anti-phospho-MAPK antibody. Again, the constitutive phosphorylation of p42 MAPK was found in *abl/myc* lines (Fig. 5*A*; the lower level of phosphorylation presented in the 128.3 line in resting condition was not reproducible). In contrast, we were not able to detect any MAPK activation in both *raf/myc* and chemical plasma cell tumors. Constitutive activation of MAPK was also found in the two B cell lymphomas (Fig. 5*B*). Again, weak induction of MAPK phosphorylation was observed in the WEHI231 B cell line in response to IL-4, consistent with SHC phosphorylation (see Fig. 4*A*). Similar levels of p42 Erk2 expression were detected among the all lines (Fig. 5, *bottom panels*). Together, these results indicate that SHC/MAPK pathway is mainly active in *abl/myc*-induced plasma cell tumors.

IGF-I Is Mitogenic for *raf/myc*- and Chemical-induced Cell Lines *in Vitro*. Having demonstrated IGF-IR up-regulation and activation of a functional signaling pathway through IRS-2, PI 3′K, and p70S6K, we proceeded to evaluate the biological relevance of IGF-I stimulation in terms of cell proliferation. As seen in Fig. 6, addition of IGF-I in the absence of serum led to a 4.3- and 8-fold increase in

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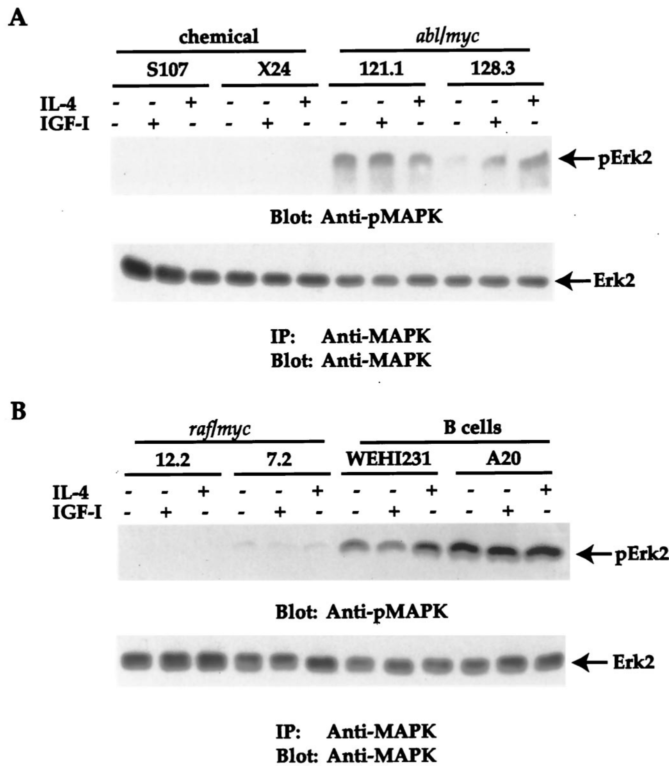

Fig. 5. The MAPK is constitutively active in *abl/myc* plasma cell tumors. Cells were serum starved for 2 h, either not stimulated or stimulated with IL-4 or IGF-I for 10 min, and lysed. Equivalent amounts of cell lysates (100 μg/lane) were subjected SDS-PAGE, and transferred proteins were immunoblotted with anti-phospho-MAPK (*A* and *B, top panels*). Equivalent amounts of cell lysates were immunoprecipitated (*IP*) with anti-MAPK, and transferred proteins were blotted with the same antibody (*A* and *B, bottom panels*).

proliferation of the 7.2 and 12.2 *raf/myc* lines, respectively, compared to non-IGF-I-treated controls. IGF-I was also able to induce a 2-fold increase in mitogenesis in the S107 chemical-induced line. In contrast, neither *abl/myc* lines (121.1 and 128.3) nor B cell lines (WEHI231 and A20) responded to IGF-I *in vitro,* correlating with the absence of IGF-IR up-regulation or IGF-I-dependent activation of the IGF-IR/IRS-2/PI 3′K cascade in these cell lines. These results demonstrate that IGF-I can be an important mitogen-inducing *in vitro* cell proliferation of *raf/myc* and chemical-induced plasma cell tumors but not earlier stage B lymphomas. The lack of proliferation to IGF-I in *abl/myc* tumors suggests that *abl* may bypass any requirement for IGF-IR, possibly by direct activation of the IRS-2/PI 3′K/p70S6K and SHC/MAPK pathways.

Expression of an ATP Binding Site Mutant of IGF-IR in *raf/myc* Lines Suppresses Tumorigenicity *in Vivo*. Demonstration of an *in vitro* biological role for IGF-I in plasma cell tumor proliferation raises the possibility that this pathway may be directly involved in *in vivo* tumorigenesis. To test this hypothesis, we expressed an ATP binding site mutant of IGF-IR (IGF-IRKR; Ref. 34) in the two lines expressing IGF-IR at the highest levels, 7.2 and 12.2. As shown in Fig. 7*A,* an increase in receptor protein levels of more than 2-fold was observed in both 7.2/IGF-IRKR and 12.2/IGF-IRKR-1 transfectants. Expression of IGF-IRKR mutant in these lines was also confirmed by flow cytometric analysis (not shown). IGF-IR tyrosine phosphorylation was largely eliminated in the IGF-IRKR mutant transfectants in comparison to that in the parental lines (Fig. 7*B*). Correspondingly, the kinase activity of IGF-IR was similarly reduced in both transfectants (Fig. 7*C*). Thus, the mutant receptor acts in a dominant negative fashion to *trans*-inhibit phosphorylation of endogenous receptor and provides us a tool to analyze the role of IGF-IR *in vivo*.

The BALB/c syngeneic mice were subsequently injected with the cell lines expressing dominant negative mutants as well as parental controls and observed for tumor development. As can be seen in Fig. 8, mice injected with parental lines began developing tumors by approximately day 10, with nearly 100% incidence by day 30. In striking contrast, expression of IGF-IRKR in line 7.2 suppressed tumorigenesis in all animals for more than 30 days postinjection. Two independently derived 12.2 transfectants were similarly assayed. No tumors were observed in 60% of mice receiving the 12.2/IGF-IRKR-1 line. Tumors that did develop were markedly delayed. The second line, 12.2/IGF-IRKR-2, was selected for high IGF-IR expression by fluorescence activated cell sorting. Inoculation of this line resulted in 100% of animals remaining tumor free for the duration of the experiment. These results strongly suggest that IGF-I is not only a proliferating factor for plasma cell tumor growth *in vitro*, but expression and activation of IGF-IR are also likely to be obligatory events for the development of or the maintenance of these tumors *in vivo*.

DISCUSSION

In the present study, we have attempted to identify signal transduction pathways that may be deregulated during development of plasma cell disease. For this analysis, we have used tumors induced by chemicals (oils), a retrovirus expressing a serine/threonine kinase (*raf/myc*), and a retrovirus expressing a tyrosine kinase (*abl/myc*). Initial focus has been directed toward the IGF-IR, because this pathway has recently been suggested to play a role in neoplasias involving several cell types (25). Herein, we provide evidence that IGF-IR is up-regulated and activated in both *raf/myc*- and chemical-induced, but

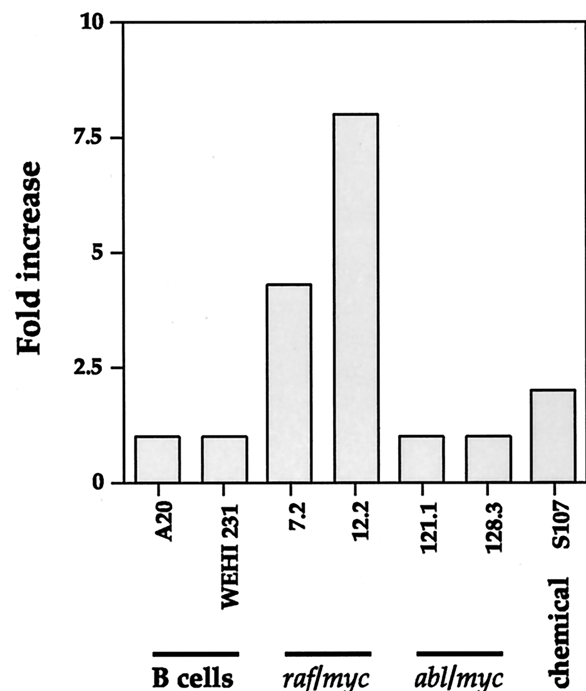

Fig. 6. IGF-I is a mitogen for *raf/myc*- and chemical-induced plasma cell lines *in vitro*. The indicated cell lines were seeded at 1×10^5 cells/well in 24-well plates and either not treated or incubated with 100 ng/ml of IGF-I for 44 h followed by a 4-h pulse with 1 μCi of [^{3}H]thymidine. Cells were harvested, and [^{3}H]thymidine content was determined. The fold increase of mitogenesis was calculated for triplicate samples by dividing the cpm of nonstimulated samples by the cpm of IGF-I stimulated counterparts.

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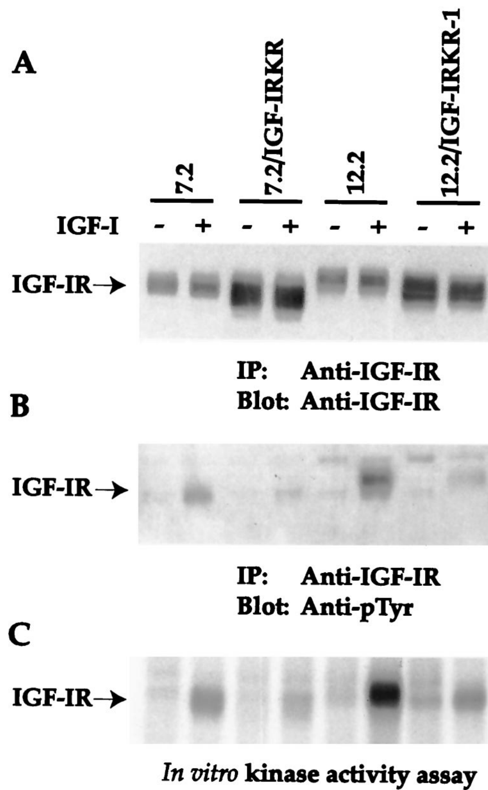

Fig. 7. Endogenous IGF-IR activity is inhibited by expression of the IGF-IRKR mutant in *raf/myc* tumors. 7.2, 12.2, and their IGF-IRKR-transfected counterparts were serum starved for 2 h, either not stimulated or stimulated with IGF-I for 10 min, and lysed. Equivalent amounts of cell lysates were immunoprecipitated (*Ip*) with anti-IGF-IR serum and blotted with anti-IGF-IR (*A*) or anti-pTyr (*B*). In *C*, anti-IGF-IR immunoprecipitates from the same lines were subjected to an *in vitro* kinase activity assay using [γ-^{32}P]ATP.

not *abl/myc*-induced, plasma cell tumors (Fig. 1). As a consequence, an IGF-I-responsive downstream cascade including IRS-2, PI 3′K and p70S6K (Figs. 2 and 3) is activated in the former two tumor types. This cascade is also activated in the *abl/myc* lines in the absence of IGF-IR up-regulation, most likely by direct phosphorylation of IRS-2 by v-*abl,* because v-*abl* is co-precipitated with IRS-2 *in vivo*.[3] When *raf/myc* and chemical lines are grown under normal conditions (10% FCS), IRS-2 is constitutively phosphorylated (Fig. 2*A*) and associates with PI 3′K (Fig. 3*A*). Thus, the IRS-2/PI 3′K portion of the cascade is constitutively activated in all plasma cell tumors, irrespective of the inducing agent, representing identification of a common deregulated biochemical pathway in plasma cell malignancy.

The signaling pathways used by three kinds of plasma cell tumors and B cell lymphomas differ greatly. Whereas *raf/myc* and chemical tumors respond to IGF-I for the activation of IRS-2/PI 3′K/p70S6K cascade, *abl/myc* plasma cell tumors additionally possess an activated SHC/MAPK pathway. Activation the SHC/MAPK pathway was reported previously in *abl*-transformed pre-B cell lymphomas (35). Although no explanation could be provided to address how *raf*-transformed tumors did not activate MAPK pathway, its sole stimulation by *abl/myc* tumors may represent the differences of IGF-IR *versus abl* tyrosine kinase in activating this pathway. Furthermore, whether IGF-I is the only ligand existing in the serum responsible for IRS-2 phosphorylation in both chemical and *raf/myc* tumors remains to be determined, because no constitutive activation of the up-regulated IGF-IR was observed in these two plasma cell tumors (see Figs. 1*A* and 2).

The IRS-2/PI 3′K/p70S6K cascade is not constitutively activated in a series of B cell lymphomas even in the presence of serum and can only be activated by exogenous ligand, preferentially IL-4 (see Figs. 2–4). Furthermore, constitutive activation of MAPK in the two B cell lymphomas may indicate that the cooperation between PI 3′K and MAPK may be obligatory for the B cell lymphoma development. The definitive role played by IRS-2/PI 3′K/p70S6K cascade in the pathogenesis of B cell and plasma cell tumor development awaits further determination. We are currently attempting to express several mutant molecules potentially blocking either IRS-2/PI 3′K/p70S6K or SHC/MAPK cascade in these two sets of B lymphoid malignancies and to test tumorigenicity *in vivo*.

The biological significance of deregulation of the IGF-IR pathway was demonstrated both *in vitro* and *in vivo*. Lines with IGF-IR up-regulation responded mitogenically to exogenous IGF-I. Response was proportional to the level of receptor expression (Fig. 6), indicating that IGF-I is a functional growth factor for these cells. Of greater significance, in terms of lymphoid tumor biology, are the results of *in vivo* experiments with lines transfected with a kinase inactive form of the IGF-IR. Expression of IGF-IRKR in the two highest receptor-expressing lines inhibited endogenous IGF-IR autophosphorylation and kinase activity (Fig. 7). Results of *in vivo* inoculation of these lines revealed that for the highest IGF-IR expresser (12.2), 60% of animals were protected from tumor development, and tumors that did develop were markedly delayed. Using a second 12.2 transfectant selected for higher mutant receptor levels, tumor-free incidence was increased to 100% (Fig. 8). For the 7.2 line, tumor-free survival was similarly striking in that 100% of animals remained negative for tumor development for the course of the experiment. These studies strongly suggest that activation of the IGF-IR pathway is obligatory for either the development or survival of plasma cell tumors *in vivo.* The pathological importance of up-regulation and activation of this receptor is readily noted in that its ligand, IGF-I, is one of the major growth factors present in plasma and other body fluids. Thus, a potential paracrine loop between IGF-I and up-regulated receptor may promote deregulated growth. We speculate that expression of KR mutant will greatly inhibit growth of chemical-induced tumors, because the level of endogenous IGF-IR up-regulation was lower than that of *raf/myc* lines. Another prediction from these studies is that *abl/myc* tumors, in which IGF-IR is not up-regulated, will be unaffected in terms of *in vivo* growth

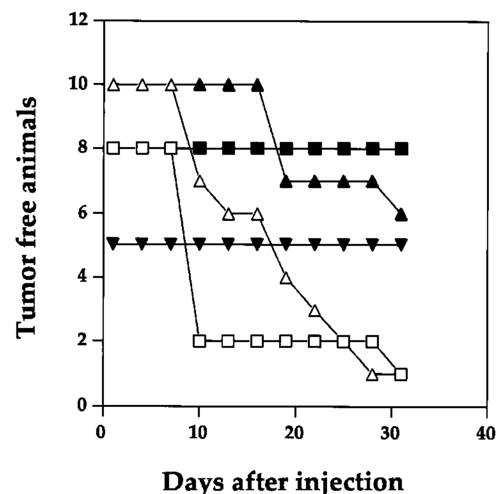

Fig. 8. Tumorigenicity of two *raf/myc* plasma cell tumors is suppressed after expression of the IGF-IRKR mutant. The parental 7.2 and 12.2 plasma cell lines and their IGF-IRKR-expressing transfectants were injected i.p. into syngeneic BALB/c mice at 2×10^6 cells/mouse. Tumor development was monitored twice weekly. The numbers of tumor-free animals are plotted *versus* days after injection. Cell lines: □, 7.2; ■, 7.2/IGF-IRKR; △, 12.2; ▲, 12.2/IGF-IRKR-1; ▼, 12.2/IGF-IRKR-2.

[3] M. Heller and S. Rudikoff, unpublished observations.

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by the dominant negative mutant of IGF-IR. To date, we have been unable to express the mutant in these lines, so that an experimental test of this hypothesis awaits further studies.

Although IGF-IR up-regulation in both retrovirally and chemically induced tumors further supports the suggestion of a key role in tumorigenesis, mechanisms underlying receptor induction are currently unknown. The highest levels of receptor protein are associated with *raf*-mediated tumor development, suggesting that *raf* overexpression and/or cooperation with *myc* leads to up-regulation. On the other hand, several other proteins, including IRS-2, PI 3′K, and members of the Bcl-2 family, are not up-regulated.[4] It will be interesting to test whether *raf/myc* expression would change the status of p53 and WT1 tumor suppressor genes, because mutations of them leading to inactivation of their tumor suppressing functions greatly enhanced the IGF-IR promoter activity (36, 37). In addition, N-*myc* overexpression also led to increased IGF-IR expression and promoter activity (38). Currently, we are testing whether IGF-IR up-regulation relies on the transcriptional activation and, if so, to dissect the promoter region of IGF-IR.

Although molecular mechanisms associated with human myeloma are largely unknown, the current studies using the murine model raise the possibility that the IGF-I/IGF-IR pathway may also be important for the development of human disease. In fact, it has been demonstrated previously that some myeloma lines undergo a mitogenic response to IGF-I (39), although subsequent steps in a signaling cascade have not been defined. Preliminary results[5] indicate significant IGF-IR expression in myeloma cell lines, and studies are in progress to determine whether the IGF-IR pathway plays a role in the development of these tumors. Pursuit of this line of investigation may eventually lead to a better understanding of molecular mechanisms involved in myeloma and may help identify possible points in biochemical pathways amenable to therapeutic intervention.

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[5] N. Ge and S. Rudikoff, unpublished observations.

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