The Insulin-like Growth Factor-I Receptor Is Required for EWS/FLI-1 Transformation of Fibroblasts*

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Ewing's family of tumors is characterized by a well described reciprocal translocation, t(11;22)(q24;q12), which produces a fusion protein (EWS/FLI-1) that transforms mouse fibroblasts. The EWS/FLI-1 fusion protein has been shown to act as a potent chimeric transcription factor. Overexpression of insulin-like growth factor-I receptor (IGF-IR) has been implicated in many tumor models as playing a role in cell growth and tumorigenesis. In addition, blockade of the IGF-IR inhibits the growth of Ewing's family of tumors cells. Therefore, we first studied whether the presence of the IGF-IR is required for transformation by the EWS/FLI-1 fusion protein. To perform this study, we used two previously described fibroblast cell lines, R and W, derived from an IGF-IR knockout mouse and a wild-type littermate, respectively. Neither W nor R cells without the fusion protein formed soft agar colonies. However, W clones expressing the fusion message (WF cells) formed soft agar colonies, whereas R clones expressing the fusion message (R- cells) did not form soft agar colonies. Therefore, we chose to investigate whether altered signaling occurs from the IGF-IR when the EWS/FLI-1 fusion is present. WF cells demonstrated a greater degree of ligand-stimulated insulin receptor substrate-1 phosphorylation when compared with W cells, suggesting that expression of the EWS/FLI-1 fusion protein alters the IGF-IR signaling pathway.

Ewing's sarcoma family of tumors (ESFT) are characterized by a translocation that occurs in 95% of tumors (1). This translocation joins the EWS gene located on chromosome 22 to an ets family gene, either FLI-1 located on chromosome 11, t(11;22) (2) or ERG located on chromosome 21, t(21;22) (3, 4). The fusion protein is a result of expression in either of these translocations contains two primary domains. The EWS domain is a potent transcriptional activator, whereas the FLI-1 or ERG domain contains a highly conserved DNA binding domain (5–8). The EWS/ets translocation and the resulting fusion protein are thought to play a role in both the etiology and growth promotion of ESFT.

The EWS/FLI-1 fusion protein appears to play a role in maintaining cell growth. Growth inhibition has been demonstrated by three methods of inhibiting the EWS/FLI-1 fusion protein: 1) RNA antisense (9), 2) a dominant negative truncated protein with an intact DNA binding domain (10), and 3) antisense oligodeoxynucleotides (11, 12). Each of these models demonstrates that when the fusion protein or its transcript is decreased, tumor cell growth is reduced. The chimeric EWS/FLI-1 fusion protein generated as a result of the translocation transforms mouse fibroblasts, and this transformation requires both the EWS and FLI-1 functional domains to be intact (13). The mechanism whereby the EWS/FLI-1 fusion protein transforms cells remains unknown.

The insulin-like growth factor-I receptor (IGF-IR) is an a2-b2 heterodimeric receptor where the a-subunit contains the extra-cellular ligand binding domain and the b-subunit contains the transmembrane and intracellular tyrosine kinase domains. The IGF-IR binds IGF-I and IGF-II and to a lesser extent insulin. The receptor transduces its signal via autophosphorylation of tyrosine residues with subsequent tyrosine phosphorylation of downstream targets, including insulin receptor substrate-1 (IRS-1), IRS-2, Shc, and Crk (14). The receptor is critical for many physiologic functions including development, cell growth, transformation, and prevention of apoptosis (15, 16). Embryos from mice with a targeted disruption of the IGF-IR were 45% of normal size and nonviable at birth (17). Blockade of ligand-mediated signal transduction through the IGF-IR using a monoclonal antibody inhibits tumor growth in many model systems (18, 19). Transformation occurs when the IGF-IR is overexpressed in mouse fibroblasts but only in the presence of ligand (20). Until recently, all oncogenes investigated were found to require the presence of the IGF-IR. However, a constitutively active G-protein mutant Gt13 transforms R cells, which do not express IGF-IR. The oncogenes that require the IGF-IR to transform cells and their IGF-IR-dependent pathways are currently being elucidated.

EWS/FLI-1 fusion protein functions as an aberrant transcription factor, but as yet there is no mechanism for how this protein transforms cells. The IGF-IR is expressed in ESFT cell lines; inhibition of IGF-IR signaling, by blocking ligand stimulation, has reduced the growth of these cells (21, 22). Because investigators have shown that other transforming proteins, including SV-40 large T antigen (23), requires the presence of IGF-IR to transform cells, we sought to determine whether the presence of these receptors was required for the transforming activity of the EWS/FLI-1 fusion protein. To perform this study, we used two previously described cell lines, R and W, derived from an IGF-IR knockout mouse and a wild-type littermate, respectively (24). Both cell lines were transfected, individual clones were selected, and clones were evaluated for anchorage-independent growth. We show here that the IGF-IR
is required for the EWS/FLI-1 fusion protein to transform mouse fibroblasts. We also demonstrate that the presence of the EWS/FLI-1 fusion protein leads to altered signal transduction through the IGF-IR.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—W and R cells were a generous gift of R. Baserga (Philadelphia, PA), whereas R+ cells are transfected R cells that overexpress wild-type IGF-IR. All cells were grown in Dulbecco’s modified Eagle’s medium +10% fetal bovine serum. ESFT cell lines TC-22, CHFP-100, and R-DES have previously been shown to contain the t(11;22) translocation (25, 26) and were grown in RPMI 1640 medium +10% fetal bovine serum. All cells were maintained in humidified, 6% CO2 atmosphere at 37 °C.

Expression of EWS/FLI-1 fusion message was shown to instructions from the manufacturer using random hexamer priming and 1640 medium containing the t(11;22) translocation (25, 26) and were grown in RPMI 1640 medium +10% fetal bovine serum. All cells were maintained in humidified, 6% CO2 atmosphere at 37 °C. EWS/FLI-1 type I cDNA was a gift of C. Denny (Los Angeles, CA). Lyophilized a (Perkin-Elmer). Expression of EWS/FLI-1 fusion message was shown to instructions from the manufacturer using random hexamer priming and 1640 medium containing the t(11;22) translocation (25, 26) and were grown in RPMI 1640 medium +10% fetal bovine serum. All cells were maintained in humidified, 6% CO2 atmosphere at 37 °C. EWS/FLI-1 type I cDNA was a gift of C. Denny (Los Angeles, CA). Lyophilized a (Perkin-Elmer). Expression of EWS/FLI-1 fusion message was shown to instructions from the manufacturer using random hexamer priming and 1640 medium containing the t(11;22) translocation (25, 26) and were grown in RPMI 1640 medium +10% fetal bovine serum. All cells were maintained in humidified, 6% CO2 atmosphere at 37 °C. EWS/FLI-1 type I cDNA was a gift of C. Denny (Los Angeles, CA). Lyophilized a (Perkin-Elmer). Expression of EWS/FLI-1 fusion message was shown to instructions from the manufacturer using random hexamer priming and 1640 medium containing the t(11;22) translocation (25, 26) and were grown in RPMI 1640 medium +10% fetal bovine serum. All cells were maintained in humidified, 6% CO2 atmosphere at 37 °C. EWS/FLI-1 type I cDNA was a gift of C. Denny (Los Angeles, CA). Lyophilized a (Perkin-Elmer). Expression of EWS/FLI-1 fusion message was shown to instructions from the manufacturer using random hexamer priming and 1640 medium containing the t(11;22) translocation (25, 26) and were grown in RPMI 1640 medium +10% fetal bovine serum. All cells were maintained in humidified, 6% CO2 atmosphere at 37 °C. EWS/FLI-1 type I cDNA was a gift of C. Denny (Los Angeles, CA). Lyophilized a (Perkin-Elmer). Expression of EWS/FLI-1 fusion message was shown to instructions from the manufacturer using random hexamer priming and 1640 medium containing the t(11;22) translocation (25, 26) and were grown in RPMI 1640 medium +10% fetal bovine serum. All cells were maintained in humidified, 6% CO2 atmosphere at 37 °C.
screening did not identify clones based upon expression of the fusion message, so following initial screening, clones were expanded for extraction of RNA.

Fig. 3 shows an example of six clones evaluated for expression of the fusion message using reverse transcriptase PCR. RNA was extracted from the clones, and following this, residual DNA was eliminated by DNase digestion. Reverse transcription took place in parallel tubes, one of which contained no DNA and acted as a control to avoid amplification of genomic (incorporated plasmid) DNA sequences. The reverse transcriptase and acted as a control to avoid amplification took place in parallel tubes, one of which contained no DNA was eliminated by DNase digestion. Reverse transcription of the fusion message using reverse transcriptase PCR. Following this, residual RNA was extracted from the clones, and following this, residual RNA was eliminated by DNase digestion. Reverse transcription of the fusion message using reverse transcriptase PCR. Therefore any amplification seen in the reverse transcriptase treated RNA indicates true cDNA amplification. The bottom panel indicates the presence of β-actin and demonstrates intact mRNA in all samples.

**Functional Effect of EWS/FLI-1 Expression**—Liquid culture growth based on doubling time did not distinguish W or R clones that expressed the EWS/FLI-1 fusion protein (data not shown). Soft agar cloning was used to assay for anchorage-independent growth, a marker of transformation. Clones were tested in soft agar assays utilizing 5000 cells/35-mm² dish. A colony was defined as a cluster of cells larger than 0.12 μm in diameter after 14 days in culture. A summary of the soft agar experiments is shown in Table I. We found that all five of our clones that express both the IGF-IR and the EWS/FLI-1 fusion protein produce colonies. Neither W (expressing IGF-IRs but having EWS/FLI-1), R² (lacking IGF-IR, but expressing EWS/FLI-1, nor R (lacking both IGF-IR and EWS/FLI-1) produced colonies.

**Effect of EWS/FLI-1 on IGF-IR Signaling**—We first determined whether the EWS/FLI-1 fusion protein induced the synthesis or direct activation of IGF-IR. Cells were incubated in serum-free medium overnight to synchronize the cells and achieve basal phosphorylation of the IGF-IR. Identical cultures were lysed either before or after a 3-min exposure to IGF-I to stimulate the IGF-IR. Equal amounts of protein were immunoprecipitated with an IGF-IR antibody, followed by polyacrylamide gel electrophoresis, and blotting with anti-phosphotyrosine antibodies is shown in Fig. 4 (upper panel). The blot was then stripped and probed with IGF-IR β-subunit antibody (Fig. 4, lower panel). Densitometry was performed on all phosphotyrosine and IGF-IR blots. The fold increases in phosphorylation, after correction for IGF-IR levels, were averaged for all W and W clones (Table II). WF clones absolute IGF-IR autophosphorylation did not differ significantly from W clones, with an average 9.6-fold stimulation compared with 6.2-fold stimulation, respectively (Student’s t test, p < 0.25). Total protein lysates without immunoprecipitation were evaluated by immunoblot for IGF-IR levels and showed equal amounts of IGF-IR in both WF and W clones (data not shown).

Because neither IGF-IR protein levels nor activity was significantly altered by the EWS/FLI-1 message, we sought to determine if IGF-I-stimulated signaling distal to the IGF-IR
The IGF-IR plays a critical role in growth and development (17, 24, 33). Ligand stimulation of the IGF-IR generates a mitogenic signal, and when overexpressed, the IGF-IR can be independently transforming (20). ESFT cells express the IGF-IR, and blockade of ligand binding results in decreased cell growth (21, 22). These findings suggest that the IGF-IR signaling pathway is important for the growth of ESFT. We developed a model to evaluate the effect of the EWS/FLI-1 fusion protein upon cells in the presence and the absence of the IGF-IR. R− cells lack the IGF-IR, whereas the W cells express the IGF-IR.

We first sought to determine whether the EWS/FLI-1 fusion protein would transform primary mouse fibroblast cells (not all

FIG. 4. Evaluation of IGF-IR levels and ligand-induced activation. Serum-starved cells were stimulated with IGF-I for 3 min, followed by cell lysis. The IGF-IR was isolated by immunoprecipitation using an antibody directed at the β-subunit, and complexes were captured with protein A-Sepharose beads. Proteins were resolved in acrylamide and transferred to nitrocellulose. The upper panel was probed with a mix of anti-phosphotyrosine antibodies, 4G10 and PY-20. The blot was stripped and reprobed with an antibody directed at the β-subunit of IGF-IR (lower panel). IGF-I stimulation is indicated by +. Size markers are noted to the left of the gel. Two independent experiments were performed, and the results are combined in Table II.

| Clone | Fusion protein | IGF-IR | Number of experiments | Average |
|-------|----------------|--------|-----------------------|---------|
| WF6   | +              | +      | 7                     | 63      |
| WF22  | +              | +      | 4                     | 8       |
| WF3   | +              | +      | 6                     | 138     |
| WF7   | +              | +      | 5                     | 32      |
| WF36  | +              | +      | 2                     | 25      |
| R−F2  | +              | +      | 2                     | 0       |
| R−F17 | +              | +      | 2                     | 0       |
| R−10  | −              | −      | 2                     | 0       |
| R−4   | −              | −      | 2                     | 0       |
| R−8   | −              | −      | 2                     | 0       |
| W5    | −              | +      | 6                     | 0       |
| W6    | −              | +      | 2                     | 0       |
| W8    | −              | +      | 2                     | 1       |
| W13   | −              | +      | 2                     | 0       |

TABLE II
Summary of densitometric evaluation of immunoblots
Films shown in Figs. 4 and 6 were digitized with a CCD camera, and band densities were obtained with NIH Image software. These are the average values of duplicate experiments for two WF clones and four W clones. Values were calculated by the following equation.

\[
\text{Fold stimulation} = \frac{(\text{stimulated PY/stimulated IGF-IR or IRS-1})}{(\text{unstimulated PY/unstimulated IGF-IR or IRS-1})}
\]

where PY indicates phosphotyrosine.

| IGF-I-induced phosphorylation (fold stimulation) | Fig. 4 | Fig. 6 |
|-----------------------------------------------|--------|--------|
| IGF-IR                                       | SD     | t test | IRS-1  | SD     | t test |
| WF clones                                     | 9.6    | 8.7    | p < 0.25 | 6.0    | 4.1    | p < 0.05 |
| W clones                                      | 6.2    | 5.4    |         | 1.5    | 0.14   |         |
primary cell lines have been able to be transformed with this protein, and if so, would they transform cells from littermate animals without the IGF-IR. Most oncogenes thus far described have not transformed the R− cells, including a combination of activated ras and SV40 large T antigen (23, 29, 30). A constitutively active G-protein mutant G13 does, however, transform R− cells. This recent finding indicates that in fact not all transformation events require the presence of the IGF-IR. We show here that W cells transfected with the EWS/FLI-1 fusion protein (WF cells) do grow in an anchorage-independent fashion, whereas the R− cells transfected with the same cDNA (R−F cells) do not. Thus the answer to our initial question is that the EWS/FLI-1 requires the presence of the IGF-IR to transform cells.

To confirm that the IGF-IR pathway was activated in some way by the EWS/FLI-1 fusion protein, we selected a series of WF and W clones and analyzed the effect of ligand stimulation in the presence or the absence of the EWS/FLI-1 fusion protein. Neither IGF-IR levels nor ligand-induced receptor activation changes as a result of the presence of the EWS/FLI-1 fusion protein. When values were averaged, there was a slight difference between WF and W clones; however, this was not significant. The docking protein IRS-I, known to bind directly to the IGF-IR (14), demonstrated an apparent hypophosphorylation in WF compared with W cells preceding IGF-1 stimulation. IRS-1 phosphorylation was then specifically evaluated by immunoprecipitation and phosphotyrosine analysis. We show decreased basal levels of IRS-1 phosphorylation in the unstimulated WF clones, which contain the EWS/FLI-1 fusion protein. The observed absolute increase in phosphorylation of IRS-1 in response to ligand stimulation of WF cells is therefore due to an altered (lower) basal phosphorylation state. We corroborated our findings with the coprecipitation of Grb2, an SH2 domain-containing adapter protein that only binds to phosphorylated sites on IRS-1. In these studies, co-precipitated Grb-2 levels were lower in unstimulated WF cells compared with W cells, thus corroborating the decreased basal phosphorylation of IRS-1 in the unstimulated WF clones. When either WF or W cells were stimulated, coprecipitated Grb-2 levels rose in tandem with the rise in IRS-1 phosphorylation.

Our finding of increased ligand-stimulated IRS-1 phosphorylation in WF clones compared with W clones is likely a reflection of the communication between the fusion protein and the IGF-IR pathway. The data indicate that the communication between IGF-IR signaling and the target genes of the EWS/FLI-1 fusion protein occurs downstream of the IGF-IR, based on IGF-IR protein levels and autophosphorylation activity, which are similar in both W or WF cells. This may represent a unique interaction in the ESFT or may turn out to be characteristic of how novel chimeric transcription factors function in transformation. The precise communication between the fusion protein and the IGF-IR signaling pathway remains to be specifically elucidated.

One hypothesis we are currently testing is that Syp or another protein tyrosine phosphatase may be a downstream target of the EWS/FLI-1 fusion protein. Syp, for example, is a SHPTP2 protein that is directly activated by IGF-IR (34) and as an activated tyrosine phosphatase is mitogenic (35). Additional studies have shown that Syp is required for effective signaling through the insulin receptor (36, 37). IRS-1 is recognized as a target of Syp, and mutations in IRS-1 can be resistant to the phosphatase activity of Syp (38). In addition, this protein binds to phosphotyrosine residues within the SH2 domains of Grb2 and other growth regulatory receptors such as the platelet-derived growth factor receptor (39) and the epidermal growth factor receptor (40). Both the activation of Syp by IGF-IR, albeit not the only activator of Syp, and IRS-1 as a target of activated Syp, may explain the difference in basal IRS-1 phosphorylation. Syp may therefore play a key role in the communication between the EWS/FLI-1 protein and the IGF-IR pathway.

It is also possible that although the IGF-IR appears to be required in our system, what may really be required is the activation of a downstream target, like IRS-1. Combinations of transfections into R− cells including an oncogene, SV40 large T antigen, plus either IRS-1 or Grb2 (adapter protein downstream of many receptor tyrosine kinases, including the IGF-IR), produce cells capable of anchorage-independent growth (29, 30). Transformation that occurs by combining a known transforming oncogene, SV-40 large T Ag, with the overexpression of targets known to be downstream of the IGF-IR indicates that potentially converging pathways may allow for a bypass of the IGF-IR.

We conclude that the EWS/FLI-1 fusion protein requires the IGF-IR to transform cells. Our model shows that clones expressing the EWS/FLI-1 fusion protein had increased IRS-1 phosphorylation compared with those clones without the fusion protein. Based on this, we presume that communication occurs between the EWS/FLI-1 fusion protein and the IGF-IR signaling pathway; however, the mechanism is not yet described.

3 C. Denny, personal communication.
Further investigation is needed to elucidate alterations in signaling pathways that result from the aberrant EWS-FLI-1 fusion protein during transformation.

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