Mouse embryo cells expressing a wild-type number of insulin-like growth factor I receptors (IGF-I) (W cells) can be transformed either by simian virus 40 large T antigen (SV40 T) or by overexpressed insulin receptor substrate 1 (IRS-1), singly transfected. Neither SV40 T antigen nor IRS-1, individually, can transform mouse embryo cells with a targeted disruption of the IGF-IR genes (R cells). However, cotransfection of SV40 T antigen and IRS-1 does transform R cells. In this study, using different antibodies and different cell lines, we found that SV40 T antigen and IRS-1 are coprecipitated from cell lysates in a specific fashion, regardless of whether the lysates are immunoprecipitated with an antibody to SV40 T antigen or an antibody to IRS-1. The same antibody to SV40 T antigen, however, fails to coprecipitate another substrate of IGF-IR, the transforming protein Shc, and two other signal-transducing molecules, Grb2 and Sos. Finally, an SV40 T antigen lacking the amino-terminal 250 amino acids fails to coprecipitate IRS-1 and also fails to transform R cells overexpressing mouse IRS-1. These experiments indicate that IRS-1 associates with SV40 T antigen and that this association plays a critical role in the combined ability of these proteins to transform R cells. This finding is discussed in light of the crucial role of the IGF-IR in the establishment and maintenance of the transformed phenotype.

The insulin-like growth factor I (IGF-I) receptor (IGF-IR) (56) plays a crucial role in the establishment and maintenance of the transformed phenotype. Antibodies to IGF-IR (1, 18), antisense expression plasmids to either IGF-I (55), IGF-IR (4, 40, 41, 48), or IGF-II (6), and dominant negative mutants of IGF-IR (25, 37) can all reverse the transformed phenotype and/or inhibit tumorigenesis. Conversely, overexpression of the wild-type (but not a mutant) IGF-IR induces transformation (7, 19, 25, 28, 46), while overexpression of IGF-II in transgenic mice increases the incidence of certain malignancies (42).

Recently, we have generated from mouse embryos homozygous for a targeted disruption of the IGF-IR genes and from their wild-type littermates (2, 26) cell lines (3T3-like fibroblasts, designated, respectively, R\textsuperscript{−} (receptor minus) and W (wild-type) cells) (46, 47). R\textsuperscript{−} cells grow in 10% serum (albeit more slowly than W cells) but do not grow at all in serum-free medium supplemented with the growth factors that sustain the growth of W cells, and of other 3T3-like cells, such as platelet-derived growth factor (PDGF), epidermal growth factor, IGF-I, IGF-II, insulin at supraphysiological concentrations, and fibroblast growth factors (7, 46). In addition, R\textsuperscript{−} cells are refractory to transformation by simian virus 40 (SV40) T antigen (47) or by other oncoproteins, such as an activated Ha-Ras (46, 52), singly or in combination. The growth deficits of R\textsuperscript{−} cells, including their resistance to transformation, are abrogated by the stable transfection of a plasmid expressing a wild-type (but not a mutant) human IGF-IR cDNA (7, 9, 25, 28, 46, 47), indicating that the growth phenotype of R\textsuperscript{−} cells is due to the absence of IGF-IR.

Insulin receptor substrate 1 (IRS-1) (20, 50, 51) is a major substrate for both the insulin receptor and IGF-IR. Substantial evidence indicates that it acts as a docking protein for both receptors, transmitting the receptor signal to downstream transducing proteins (see the review by White and Kahn [60]).
embryos, littermates to the R-embryos) by cotransfection with pHL4A (hydrogymcin resistance) and plasmid ptsA, which contains a temperature-sensitive mutant of SV40 T antigen (36, 38, 39). R-/T and W/T cells, formerly designated (tsA)100 and (tsA)W cells, have also been described in detail by Sell et al. (46). R-/IRS-1 cell lines were developed by transfection with the CMV-IRS-1 vector (C) in a cotransfected subclone of R- cells that had lost the neomycin resistance marker, and subsequent selection in 800 μg of G418 per ml (8). R-/IRS-1/T and R-/IRS-1/trunc T cell lines were generated by cotransfection of R-/IRS-1 cells with pHPSVNeoT (wild-type T antigen) and pCAVT251-708 (encoding a truncated T antigen missing the first 250 amino acids). In either case, they were cotransfected with plasmid pLHL4 encoding the hygromycin resistance gene. Clones were selected in hygromycin.

(ii) Derived from BALB/c 3T3 cells. The parental cell line, 3T3, has been grown in our laboratory for several years. The cells display an untransformed phenotype, do not grow in 1% serum, and need for growth at least two growth factors, usually PDGF and IGF-I (34, 35). T6 is a cell line derived from 3T3 cells by stable transfection with plasmid pRSVNeoT encoding wild-type SV40 T antigen. F2 and F21 are two clones of cell lines obtained by transfecting 3T3 cells with pCAVT251-708 (encoding a truncated T antigen missing the first 250 amino acids). In either case, they were cotransfected with plasmid pLHL4 encoding the hygromycin resistance gene (15). p6T cells were derived from p6 cells, which overexpress human IGF-IR (35), by transfection with the αTS5 mutant of SV40 large T antigen (36), cloned in a plasmid that also carried the hygromycin resistance gene. Clones were selected in hygromycin.

(iii) Derived from 32D cells. 32D is a kind gift from Bruno Calabretta), a myeloid progenitor cell line, was grown and maintained in DMEM supplemented with 10% inactivated fetal bovine serum and 15% conditioned medium (containing interleukin-3) from WEHI cells. The cells were transfected by electroporation with the SV40 T-antigen expression plasmid pRSVBneoT (see above). Clones expressing T antigen were selected in medium containing 800 μg of G418 per ml.

Immunostaining for T antigen. For a quick screening of selected clones, expression of wild-type or truncated SV40 T antigen was detected with a specific mouse antibody, PA901 (provided by M. J. Tevethia, which recognize a T-antigen carboxy-terminal epitope between amino acids 682 and 708 (16, 30).

FIG. 1. Coprecipitation of p185 with SV40 T antigen. Cells were made quiescent and then stimulated for 5 min with either insulin (A and B) or IGF-I (C). Cell lysates and the immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis. Tyrosine-phosphorylated proteins were detected with an antiphosphotyrosine antibody. (A) Whole lysates (15 μg of protein) from either R-/T or W/T cells; (B) the same lysates immunoprecipitated (IP) with anti-T-antigen (Ag) antibody PA901; (C) lysates prepared from W/T cells, at either 34 or 39°C, immunoprecipitated with PA901. The position of p185 (presumed IRS-1) is indicated by arrows. Sizes are indicated in kilodaltons.

RESULTS

SV40 large T antigen coprecipitates a p185 tyrosyl-phosphorylated protein. The IRS-1 protein was originally demonstrated, by immunoblotting with a phosphotyrosine antibody, as a band of approximately 185 kDa that is phosphorylated upon stimulation with either insulin or IGF-I (21, 31). The original W and R T cell lines. T antigen is clearly detectible and subsequently immunoblotting with antibody PA901 (Fig. 1B), a similar band can be detected in both cell lines, the intensity of the band again increasing after insulin stimulation. As W/T cells express the αTS5 mutant of SV40 T antigen (7, 47), a mutant that loses most of its functions at the restrictive temperature of 39°C (38, 39), we investigated the association of the p185 band with T antigen in W/T cells incubated at either 34 or 39°C. As shown in Fig. 1C, the p185 band is more pronounced at 34°C than at 39°C, although in both cases, IGF-I stimulation increases the intensity of the band. We have not been able to identify the extra bands visible in Fig. 1, especially Fig. 1B and C, despite repeated efforts with antibodies against possible candidate proteins (not shown).

An antibody to IRS-1 coprecipitates SV40 T antigen. The association of IRS-1 with T antigen was then investigated in a reverse way in the same W/T and R-/T cells, using immunoprecipitation with an antibody to IRS-1 (Upstate Biotechnol- ogy) and subsequent immunoblotting with PA901 (Fig. 2). The original W and R cells (no T antigen) were used and insulin antibody PA901 with W/T and R-/T cells. T antigen is clearly visible in lysates of W/T or R-/T cells after immunoprecipitation with an antibody to IRS-1, while no T antigen is detectable in W and R- cells. Since R-/T cells do not have IGF-IIR (7, 47), the experiments indicate that under the conditions used,
SV40 T antigen is coimmunoprecipitated by an antibody to IRS-1 in the absence of IGF-IR.

**Specificity of the association between IRS-1 and SV40 T antigen.** The specificity of the SV40 T-antigen association with IRS-1 was confirmed in two ways. In the first approach, we took advantage of the fact that 32D cells have been shown to be completely devoid of IRS-1 (57). We transfected 32D cells with the expression plasmid pRSVNeoT (encoding wild-type SV40 T antigen) to generate 32D/T cells, which express SV40 T antigen (as monitored by immunostaining; see Materials and Methods) but are still devoid of IRS-1. Two experiments were carried out with these cells. In the first experiment, lysates of four independent cell lines of 32D/T cells were immunoprecipitated with an antibody to IRS-1 and Western blotted for T antigen (Fig. 3A). No T antigen can be seen in these immunoprecipitates (lanes 1 to 4), although it is detectable in T6 cells (lane 5), which are derived from BALB/c 3T3 cells and express IRS-1 (see below). Figure 3A also shows that T antigen was present in whole cell lysates (no immunoprecipitation) of all four clones of 32D/T cells and, except for clone 1, in amounts similar to that in T6 cells. In the reverse experiment, the lysates of 32D/T cells were immunoprecipitated with an antibody to T antigen (PAb101; see below), and the gels were stained for IRS-1 (Fig. 3B, lanes 1 to 4). No bands are visible in the IRS-1 region under these conditions, while IRS-1 is immunoprecipitated in lysates of T6 cells, which have IRS-1 (lane 5).

In the second approach, we used W/T cells expressing an antisense RNA to IRS-1 (8). The cells used were W/T cells stably transfected with a plasmid expressing an antisense RNA to IRS-1 RNA; the expression plasmid, the cell lines stably transfected with it, and the decreased expression of endogenous IRS-1 have all been described in a previous report (8). The lysates were immunoprecipitated with an anti-IRS-1 antibody as for Fig. 2 and immunoblotted with PAb901. SV40 T antigen is visible again in W/T cells (Fig. 3C, lane 1), but its amount is barely detectable in the cells with a reduced amount of IRS-1 (Fig. 3C, lanes 2 and 3). Lane 4 represents the negative control, W cells without SV40 T antigen.

These results confirm that an antibody to IRS-1 coprecipitates SV40 T antigen (and vice versa) and indicate that this coprecipitation is dependent on the presence and/or the amount of IRS-1.

**A truncated SV40 T antigen does not associate with IRS-1.** Results of the experiments performed with W/T and R’/T cells were confirmed and extended by using cell lines derived from BALB/c 3T3 cells (see Materials and Methods) expressing either wild-type T antigen (T6) or T250 (F2 and F21), which is a truncated T antigen lacking the amino-terminal 250 amino acids (see Materials and Methods). We used these different cell lines also to establish that the coimmunoprecipitation of T antigen and IRS-1 is not a peculiarity of R’ cells. The lysates were immunoprecipitated with PAb101 (Santa Cruz Biotechnology), a monoclonal antibody which recognizes a carboxy-terminal epitope of SV40 T antigen. After immunoprecipitation with this antibody and blotting with an IRS-1 antibody, a band is visible in cells expressing the wild-type T antigen (Fig. 4A, lanes 1 and 2) but not in cells expressing the T250 antigen (Fig. 4A, lanes 3 and 4). The other panels show the same lysates after immunoprecipitation with three different antibodies, anti-glutamine synthetase (Fig. 4B), anti-proliferating cell nuclear antigen (Fig. 4C), and anti-E1A (Fig. 4D), all belonging to the same antibody class as PAb101 (see Materials and Methods). All of them were negative for the presence of IRS-1. It seems, therefore, that the 250 amino-terminal amino acids of T antigen are necessary for coimmunoprecipitation of IRS-1.

These results were confirmed by using an antibody to IRS-1 (Fig. 5). After immunoprecipitation with an antibody to IRS-1 followed by blotting with PAb901, T-antigen bands were detected in T6 cells (expressing wild-type T antigen). The intensity of the T-antigen band was increased by stimulation with IGF-1, suggesting that T antigen binds more strongly to a phosphorylated IRS-1. In contrast, F2 cell immunoprecipitates fail to show the presence of T antigen (Fig. 5A lanes 3 and 4). Both wild-type and truncated T antigen were detectable in the same cell lysates by direct immunoblotting with anti-T-antigen antibody PAb901. These experiments confirm the association of SV40 T antigen with IRS-1 and show that the amino-terminal 250 amino acids of T antigen are required for this association.

**Lack of association with IRS-1 correlates with the transforming defect of truncated T antigen.** We examined whether
FIG. 4. An amino-terminally truncated T antigen does not coprecipitate IRS-1. Cells were made quiescent and then stimulated for 5 min with insulin (10 μg/ml). (A) Lysates from T6 cells (wild-type T antigen [Ag]) or F2 cells (T antigen with amino-terminal truncation) were immunoprecipitated (IP) with antibody PAb101, which recognizes a carboxy-terminal epitope of SV40 T antigen, and the blots were stained with an antibody to IRS-1. (B to D) The same lysates were immunoprecipitated with different antibodies (against glutamin synthetase [GS], proliferating cell nuclear antigen [PCNA], and EIA) belonging to the same antibody class as PAb101 (see Materials and Methods). Fifteen nanograms of recombinant IRS-1 protein was used to control the specificity of Western immunodetection. The positions of IRS-1 and immunoglobulin G heavy chain [Ig(H)] are indicated by arrows; sizes of marker proteins are indicated in kilodaltons.

FIG. 5. An antibody to IRS-1 does not coprecipitate a truncated SV40 T antigen. Lysates were prepared from T6 cells or F2 and F21 cells (two clones expressing an amino-terminally truncated T antigen [T250]). (A) The lysates were immunoprecipitated (IP) with an antibody to IRS-1 and stained with an antibody to T antigen, PAb901. The arrows indicate the expected positions of the wild-type (WT) and truncated T antigen (Ag). (B) Whole lysates stained with the same antibody to T antigen. The T antigens are indicated by arrows; sizes of marker proteins are indicated in kilodaltons.

Table 1. Colony formation in soft agar of various cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Colony formation (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c 3T3</td>
<td>0</td>
</tr>
<tr>
<td>T6 (BALB/c 3T3 with T antigen)</td>
<td>69.7</td>
</tr>
<tr>
<td>F2 (BALB/c 3T3 with truncated T antigen)</td>
<td>13.9</td>
</tr>
<tr>
<td>F21 (same as F2)</td>
<td>22.3</td>
</tr>
<tr>
<td>R− (no IGF-1R)</td>
<td>0</td>
</tr>
<tr>
<td>T/R− (R− cells with T antigen)</td>
<td>0</td>
</tr>
<tr>
<td>R−/IRS1 (R− cells with IRS-1)</td>
<td>0</td>
</tr>
<tr>
<td>R−/IRS/T</td>
<td>0</td>
</tr>
<tr>
<td>Clone 10</td>
<td>12.0</td>
</tr>
<tr>
<td>Clone 13</td>
<td>28.6</td>
</tr>
<tr>
<td>R−/IRS/trunc T, clones F, L, and P</td>
<td>0</td>
</tr>
</tbody>
</table>

* See Materials and Methods for more detailed characterization. Expressed as the percentage of cells seeded (two seeding densities were used) at 2 weeks.

determined the growth of various cell lines in 1% serum-containing medium. Confirming previously published data (53), T6 cells (expressing wild-type T antigen) grow in 1% serum, whereas F2 and F21 cells (expressing truncated T antigen) do not (data not shown).

More important, we tested the transforming potential of these cell lines by a soft agar assay. The results are shown in Table 1. T6 cells formed many colonies (69% of seeded cells) in soft agar after 14 days. The potential of cell lines expressing the truncated T antigen, F2 and F21, was much lower (14 and 22%, respectively), and the colonies were smaller. R−/IRS1/T cell lines (clones 10 and 13) did form colonies, thus confirming the data of D’Ambrosio et al. (8), although the colony number was less than that of T6 cells, while R−/IRS1/trunc T cells (clones F, L, and P) failed to grow in soft agar. This finding indicates that IRS-1 can cooperate with SV40 T antigen in transforming R− cells, which cannot be transformed by SV40 T antigen only or by IRS-1 only. Significantly, the amino-terminally truncated T antigen, which cannot bind IRS-1, also fails to cooperate with IRS-1 in transforming R− cells.

SV40 T antigen does not tyrosyl phosphorylate IRS-1. Since it has been previously shown that polyomavirus middle T antigen and Shc phosphorylate each other (5, 12), we examined whether IRS-1 may be tyrosyl phosphorylated by its association with SV40 T antigen. The results (Fig. 6) show that despite the association, IRS-1 is not tyrosyl phosphorylated by SV40 T antigen. The lysates were immunoprecipitated with an anti-IRS1 antibody and stained for phosphotyrosine. In all cell lines, tyrosyl phosphorylation of IRS-1 was detectable only after stimulation with insulin; there was no constitutive phosphorylation of IRS-1 in the two cell lines (R−/T and R−/IRS-1/T) expressing T antigen.

FIG. 6. IRS-1 is not tyrosyl phosphorylated by SV40 T antigen. Quiescent cells were left unstimulated (−) or were stimulated for 5 min with insulin (+). Cell lysates were immunoprecipitated with an antibody to IRS-1 and immunoblotted with an antiphosphotyrosine antibody. Lanes: 1 and 2, R− cells; 3 and 4, R− cells expressing T antigen; 5 and 6, R− cells overexpressing IRS-1; 7 and 8, R− cells overexpressing both IRS-1 and T antigen. The position of IRS-1 is indicated by the arrow; sizes of marker proteins are indicated in kilodaltons.
proteins are indicated in kilodaltons. Protein (WT), wild-type T antigen; T Ag (T250), amino-terminally truncated (B) Lysates of p6 cells expressing SV40 T antigen (lanes 3 to 6) were immunoprecipitated with an antibody to IGF-IR and probed with the antibody PAb901. The last two lanes are whole lysates only (15 μg of protein), prior to immunoprecipitation with the anti-IGF-IR antibody. T Ag with the antibody PAb901. The last two lanes are whole lysates only (15 μg of protein), prior to immunoprecipitation with the anti-IGF-IR antibody. T Ag

Association of SV40 T antigen with IGF-IR. Having established that SV40 T and IRS-1 are coimmunoprecipitated in a specific fashion from cell lysates, we investigated whether SV40 T antigen can also coprecipitate with IGF-IR itself. A variety of cell lines were used for this purpose, the approach involving immunoprecipitation of cell lysates with an antibody to IGF-IR followed by immunoblotting with PAb901. Figure 7A shows the results for BALB/c (parental cells), no T antigen, T6, and F21 cells. T antigen is detectable only in T6 cells; the last two lanes show that the whole lysates have detectable wild-type and truncated T antigen, respectively. For Fig 7B, we used p6 cells, which grossly overexpress IGF-IR (34, 35). No T antigen is, of course, detectable in p6 cells (Fig. 7B, lane 2), but it is detectable in p6 cells expressing T antigen (Fig. 7B, lanes 3 to 6) after immunoprecipitation with an antibody to IGF-IR. SV40 T antigen, under these conditions, is barely detectable in BALB/c cells, which express an normal amount of IGF-IR (36). Figure 7C shows the T-antigen staining of whole lysates, indicating that substantial amounts of T antigen are present also in BALB/A58 cells.

SV40 T antigen does not associate with She and other signal-transducing molecules. Since She is considered a substrate of IGF-IR (45, 60, 61), we investigated whether T antigen would coprecipitate with She. For this purpose, we used some of the same cells as used for Fig. 7; the results are shown in Fig. 8. Immunoprecipitation with PAb901 does not result in the presence of recognizable She by immunoblotting with an anti-Shc antibody (Fig. 8A), although She proteins (p52 and p66) are clearly present in She immunoprecipitates (Fig. 8B). There was also no specific coprecipitation of T antigen and Grb2 (Fig. 9A). In this experiment, lysates were prepared from cells expressing T antigen. Lane 1 shows whole lysates of W/T cells blotted for T antigen, which is present in abundant amounts; when lysates from the same cells were immunoprecipitated with an anti-Grb2 antibody, a small amount of T antigen was co-immunoprecipitated (lane 2). However, when lysates from 32D/T cells were immunoprecipitated with an antibody to Grb2, no T antigen was detectable (lane 3), although these cells expressed copious amounts of T antigen (Fig. 3). This result indicates that in the absence of IRS-1, no T antigen coprecipitates with Grb2. Lane 4 represents a control with W cells (devoid of T antigen), and lane 5 represents the whole reaction without lysates, showing that the band detectable around the 47-kDa marker is the antibody heavy chain. Figure 9B shows that these cells expressed Grb2 (lane 5 again is without lysates, and the light chain of the antibody is about the same size as Grb2, but lanes 2 to 4 have a much stronger signal than lane 5). Finally, we used similar techniques to examine whether T antigen would coprecipitate Sos. No Sos was detectable in the PAb901 immunoprecipitates, although it was present in either whole lysates or the Sos immunoprecipitate (not shown).

DISCUSSION

While both SV40 large T antigen and IRS-1 have several and varied functions, in this study we focused on their possible association and their cooperation in establishing a transformed phenotype in 3T3-like cells, including R2 cells, which are completely devoid of IGF-IR. The rationale for these experiments was based on the observation that neither SV40 T antigen nor IRS-1 alone can transform R2 cells, whereas in combination, they can (reference 8 and this report). R2 cells overexpressing IRS-1 can grow in serum-free medium supplemented solely with insulin but do not transform (8). In addition, Fohrman and Imperiale (14) had previously reported that a protein band of approximately 185 kDa (not further characterized) coprecipitated with T antigen in one of their cell lines. The combination of their observation and ours prompted us to investigate...
a possible association between SV40 large T antigen and IRS-1 in cell lysates. Our data indicate that such an association exists, and the validity of this statement is based on the following facts. (i) Two antibodies against T antigen coprecipitate IRS-1. (ii) An antibody against IRS-1 coprecipitates wild-type T antigen. (iii) In cells devoid of IRS-1 (32D cells), an antibody to IRS-1 fails to coprecipitate T antigen. In fact, the amount of T antigen that is coprecipitated by an anti-IRS-1 antibody is roughly related to the amount of IRS-1. (iv) Control, unrelated antibodies of the same class (for T antigen) fail to coprecipitate IRS-1. Interestingly, T antigen cannot coprecipitate another substrate of IGF-IR, Shc, nor other signal-transducing molecules such as Grb2 and Sos (60).

More importantly, the association between IRS-1 and SV40 T antigen is not casual but rather seems to be required for the cotransformation of the 3T3-like cells that we examined. Thus, a truncated T antigen lacking the 250 amino-terminal amino acids fails to coprecipitate IRS-1 and also fails to transform R2 cells overexpressing IRS-1. Since neither IRS-1 nor T antigen can transform cells lacking IGF-IR individually but can do so in combination, the failure of the truncated T antigen to cooperate with IRS-1 in transforming R- cells is an indication that the association is required for the cotransformation of these cells.

Commenting further on our findings, the first question that can be raised is how T antigen, a nuclear protein, can physically associate with IRS-1, clearly a cytosolic protein. Although SV40 T antigen is a predominantly nuclear protein, a small portion of it is found in the cytoplasm (44). A transport-defective mutant of SV40 T antigen (cytoplasmic T antigen) has transforming potential (23). Since there is no evidence that IRS-1 is translocated to the nucleus, we assume that the T antigen that interacts with IRS-1 is the cytoplasmic one. One can calculate, very roughly, the percentage of T antigen that associates with IRS-1 by quantitating the amount of T antigen coprecipitated by an IRS-1 antibody and the amount present in whole cell lysates. Taking into consideration the amount of proteins used, one can calculate that roughly 5% of the total cellular T antigen associates with IRS-1. The reverse calculation is even more difficult, but from the data in Fig. 1, it seems that most of IRS-1 may be bound to T antigen. A physical association between an oncogene and growth factor receptors or their immediate substrates has been reported previously and reviewed recently by Baserga (3). For instance, the polyoma virus middle T antigen is physically associated with both Shc (5, 12) and phosphatidylinositol 3-kinase (62); the gp55 glycoprotein of Friend murine leukemia virus binds to the erythropoietin receptor (63, 65), the transforming protein of bovine papillomavirus binds to the PDGF receptor (32, 33), and IGF-IR is constitutively phosphorylated in v-src-transformed cells (22). Our data extend this oncogene association to the IGF-1R system, a very important point because of the crucial role of IGF-1R in the establishment and maintenance of transformation (see the introduction).

In the case of the association between the PDGF receptor and the transforming protein of bovine papillomavirus, Dimao and coworkers (32, 33) showed that the receptor was constitutively phosphorylated in cells transformed by the virus. Similarly, both Shc and polyoma virus middle T antigen were found to be tyrosyl phosphorylated in polyomavirus-transformed cells (5, 12). It does not seem that T antigen constitutively causes much tyrosyl phosphorylation of IRS-1, since a band is not visible or barely visible in R- T cells not stimulated with insulin (Fig. 1A and B). A band is visible in W/T cells not stimulated with insulin, but one must remember that T antigen markedly increases the secretion of IGF-I (36), which in turn activates IGF-IR. This interpretation is confirmed by the results in Fig. 6, showing that in cells expressing SV40 T antigen, there is no constitutive tyrosyl phosphorylation of IRS-1. This apparent difference from other growth factor-related proteins may also depend on the fact that IGF-IR, by itself, can transform (7, 19), whereas the transforming activities of both the epidermal growth factor and PDGF receptors require a functional IGF-IR (7, 9).

A second question is whether IGF-IR itself binds to T antigen. We can tell from our experiments that T antigen binds to IRS-1 independently of IGF-IR (experiments with R- cells), but we cannot tell whether it also binds directly to IGF-IR. An antibody to IGF-IR immunoprecipitates T antigen, but this may happen because of the association between IGF-IR and IRS-1 and the possibility of the formation of ternary complexes, already described in other circumstances involving the insulin receptor (24). 32D cells are not suitable for arriving at a definitive answer because they contain too low a level of IGF-IR. For the moment, therefore, this question must go unanswered.

A third question, namely, the mechanism by which a T antigen lacking the amino-terminal 250 amino acids fails both to bind IRS-1 and to cotransform with IRS-1, may be more important. The truncated T antigen is transforming, if only weakly (53, 64), and that function is completely abrogated in R- cells overexpressing IRS-1. The various functional domains of SV40 large T antigen have been reviewed several times (13, 53, 54). The 250 amino-terminal amino acids include, among other domains, those for binding the Rb protein, for stimulating cell DNA synthesis, and for nuclear localization. Indeed, an obvious explanation is that the truncated T antigen fails to transform R- IRS-1 cells because of its inability to bind the Rb protein (27). However, we would like to point out the following: (i) the truncated T antigen transforms 3T3 cells with IGF-IR (F2 and F21 cells); (ii) wild-type T antigen does not transform R- cells (46, 47); (iii) wild-type T antigen transforms cells overexpressing IRS-1 (reference 8 and this report); and (iv) the truncated T antigen cannot transform R- IRS cells (Table 1). A reasonable interpretation is that binding to IRS-1 is required for T transformation of cells lacking IGF-IR. Clearly, when IGF-IR is present, binding is no longer required (F2 and F21 cells), and the other transforming mechanisms of T antigen enter into play.

The precise identification of the T-antigen domain required for IRS-1 binding is obviously important, but so is the identification of the IRS-1 domain required for binding of T antigen, an easy problem, considering the multiple potential phosphorylation sites of the latter molecule (49). These two questions are presently among our priorities, but one comment is appropriate at this point. The N-terminal domain of SV40 T antigen contains several serine/threonine phosphorylation sites of the latter molecule (13), and Deppert et al. (11) have reported that underphosphorylation of these sites decreases the transforming activity of SV40 T. These sites, in addition to other T-antigen functions, will have to be considered seriously.

To explain our findings and previously reported data, we would like to formulate the following hypothesis, stressing the fact that it is a hypothesis. An overexpressed wild-type IGF-IR can transform mouse embryo cells, using at least two different pathways, one of which is Ras independent (46). SV40 T antigen also has more than one transformation pathway, as suggested by the requirement of multiple domains for full transforming activity (11, 13, 53). One of these pathways is IGF-IR dependent, via IRS-1: in the absence of IGF-IR, mouse embryo fibroblasts cannot be transformed by SV40 T antigen (46, 47). An overexpressed IRS-1 can replace the IGF-IR-depen-
fectant function (this report), presumably by amplifying the much weaker insulin signal. The connection between IRS-1 and SV40 T antigen is seemingly dependent on the N-terminal region of SV40 T: without it, but with wild-type levels of IGF-IR, SV40 T antigen is weakly transforming (presence of the other transforming domains of SV40 T antigen plus a weak signal from the wild-type IGF-IR). For full transforming activity, one needs wild-type SV40 T antigen and an IRS-1 activated by IGF-IR. Again, since SV40 T antigen and Ras can cooperate in transforming wild-type cells (59) but fail to cooperate when cotransfected into R\textsuperscript{cells} (46, 52), we have to postulate a Ras-independent pathway, with the Ras pathway being necessary but not sufficient (59). An important question is whether these different pathways eventually converge or remain divergent. It is also possible that the unique IGF-IR pathway (not shared with other receptors) is totally different from any known pathway; there is some evidence that the activation of IGF-IR may lead not only to transcriptional activation but also to pre-mRNA processing (29, 66), which would constitute a totally new signaling pathway.

In conclusion, we have demonstrated an interesting association between SV40 large T antigen and one of the two major substrates of IGF-IR, IRS-1, and that this association is important in transforming 3T3-like cells when these cells are devoid of IGF-IR. This finding is of considerable interest when one considers the important role of IGF-IR and its signaling pathway in mitogenesis and transformation (see the introduction) and in apoptosis (4, 5, 17).

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