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Association of Insulin Receptor Substrate 1 with Simian Virus 40 Large T Antigen

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Mouse embryo cells expressing a wild-type number of insulin-like growth factor I receptors (IGF-IR) (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 of 3T3-like fibroblasts, designated, respectively, R^- (receptor minus) and W (wild-type) cells (46, 47). R^- 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^- 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^- 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^- 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]). A number of reports have indicated that IRS-1 plays an important role in insulin-mediated (43, 58) and IGF-I-mediated (31) mitogenesis. We recently reported (8) that an overexpressed IRS-1 cannot transform R^- cells. However, when the same IRS-1 plasmid was expressed in R^-/T cells (i.e., R^- cells expressing SV40 large T antigen [47], which, by itself, fails to transform R^- cells), the cells became capable of forming colonies in soft agar. Since an overexpressed IGF-IR can transform cells by itself (see above), the question arises of how T antigen and IRS-1, in combination but not separately, can substitute for the transforming activity of IGF-IR. For this purpose, we have investigated the possible association of T antigen and IRS-1 in immunoprecipitates of cell lysates. We show here that SV40 T antigen and IRS-1 are coimmunprecipitated from cell lysates and that their association is necessary for the transformation of cells lacking IGF-IR.

MATERIALS AND METHODS

Plasmids and transfection. pRSVBneoT (a kind gift of A. Srinivasan, University of Pittsburgh) was the plasmid used to express wild-type SV40 T antigen. It contains the full-length coding sequence of SV40 T antigen under the control of a Rous sarcoma virus promoter as well as the neomycin resistance gene. The truncated SV40 T-antigen expression plasmid pCAVT251-708 (a kind gift of M. J. Tevethia, Pennsylvania State Medical School) is a modified CAV vector containing, under the control of a cytomegalovirus promoter, a T-antigen sequence encoding a truncated SV40 T antigen missing the amino-terminal 250 amino acids (53).

The mouse IRS-1 cDNA was cloned from 3T3-L1 adipocytes (21). The entire sequence, in either the sense or antisense direction, was cloned into the *Hin*dIII site of the pRc/CMV expression vector (Invitrogen). The orientation of the insert was monitored by restriction analysis with *Bgl*II, and the resulting plasmids were designated CMV-IRS-1 or CMV-anti-IRS-1, respectively. Both IRS-1 plasmids encode also neomycin resistance (inherent to pRc/CMV).

pLHL4 contains the *Escherichia coli* hygromycin B phosphotransferase gene, which confers resistance to hygromycin B (15). Plasmid pBSpacDp encodes a puromycin resistance gene (10).

Transfections were performed by using the Transfectam reagent (Promega) in serum-free Dulbecco's modified Eagle medium (DMEM).

Cell lines. (i) Derived from R^- and W cells. R^- cells have been described in detail elsewhere (7, 9, 25, 28, 29, 46, 47) and were routinely cultured in DMEM supplemented with 10% fetal bovine serum. R^-/T and W/T cells were derived, respectively, from R^- and W cells (the latter originating from wild-type mouse

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embryos, littermates to the R^- embryos) by cotransfection with pLHL4 (hygromycin 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 (*ts*A)R^- and (*ts*A)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 into a 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 pBSpacDp (10) and with plasmid pRSVBneoT (wild-type T antigen) and pCAVT251-708 (truncated T antigen), respectively. Selection was carried out in puromycin (5 μg/ml). W/T/anti-IRS-1 cells were developed by cotransfection of plasmid CMV-anti-IRS-1 and the plasmid encoding puromycin resistance (see above). These cells lines have been described and characterized in a previous report (8).

(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 pRSVBneoT 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). p6/T cells were derived from p6 cells, which overexpress human IGF-IR (35), by transfection with the *ts*A58 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 (a kind gift from Bruno Calabretta), a myeloid progenitor cell line, was grown and maintained in DMEM medium 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, PAb901 (provided by M. J. Tevethia), which recognize a T-antigen carboxy-terminal epitope between amino acids 682 and 708 (16, 30).

Immunoprecipitation and Western blotting (immunoblotting). Cells, grown in 10% serum or starved for 24 h followed by treatment with IGF-I (20 ng/ml) or insulin (10 μg/ml), were rinsed twice with phosphate-buffered saline and lysed with HNIG buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl, 1 mM EDTA, 10 mM sodium PP_i, 100 μM sodium orthovanadate, 100 mM NaF, 10 μg of aprotinin per ml, 1 mM phenylymethylsulfonyl fluoride). After the insoluble material was removed by centrifugation, the protein concentration in the clarified lysate was determined by Bio-Rad protein assays (Bio-Rad Laboratories, Richmond, Calif.). Cell lysates containing equal amounts of protein were subject to immunoprecipitation with the indicated antibodies. The precipitated proteins were resolved on a 4 to 15% gradient sodium dodecyl sulfate (SDS) polyacrylamide gel, and then transferred to a nitrocellulose filter by electroblotting for analysis.

For immunoblotting, membranes were blocked with 5% nonfat milk in TBST buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 0.1% Tween 20) and probed with the indicated antibodies. Blots were then developed with the ECL system as instructed by the manufacturer (Amersham Corp).

Antibodies. For the detection of IRS-1 association with T antigen, cell lysates (1 mg of protein) were immunoprecipitated with antibody PAb901 (see above) and then subjected to Western blotting with an anti-IRS-1 polyclonal antibody (Upstate Biotechnology, Inc.) or with an antiphosphotyrosine monoclonal antibody conjugated with horseradish peroxidase (Transduction Laboratories). Conversely, cell lysates (300 to 500 μg of protein) were immunoprecipitated with the anti-IRS-1 antibody and Western blotted with the anti-T-antigen antibody PAb901. We also used a second antibody against T antigen, PAb101 (Santa Cruz Biotechnology, Inc.), which is a monoclonal mouse immunoglobulin G2a antibody recognizing a carboxy-terminal epitope of T antigen; for controls, we used three different antibodies against unrelated proteins, (glutamine synthetase [Transduction Laboratories], proliferating cell nuclear antigen [Oncogene Science], and E1A [Transduction Laboratories]), all of the same class as the antibody against T antigen.

Coprecipitation of IGF-IR with T antigen was detected by immunoprecipitation with the anti-IGF-IR antibody αIR3 (Oncogene Science) and then Western blotting with antibody PAb901. We used a lesser amount of lysates from IFG-IR-overexpressing cells (p6 and p6/T) (100 μg of protein) than from the cells with only endogenous IGF-IR (T6, F2, and F21) (500 μg of protein).

Detection of the association of Shc with T antigen was performed in a way analogous to that used for IRS-1. The cell lysates (500 μg of protein) were immunoprecipitated with antibody PAb901 or with a polyclonal antibody against Shc (Transduction Laboratories). Both T-antigen and Shc immunoprecipitates were then immunoblotted with an anti-Shc monoclonal antibody (Transduction Laboratories). The same procedure was also used to investigate a possible association of Grb2 and Sos with T antigen; the Grb2 and the Sos antibodies were monoclonal antibodies (Transduction Laboratories).

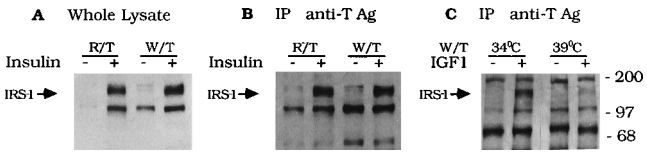

FIG. 1. Coprecipitation of p185 with SV40 T antigen. Cells were made quiescent and then stimulated for 5 min with either insulin (A and B) or IGF-I (C). Cell lysates and the immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis. Tyrosine-phosphorylated proteins were detected with 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 PAb901; (C) lysates prepared from W/T cells, at either 34 or 39°C, immunoprecipitated with PAb901. The position of p185 (presumed IRS-1) is indicated by arrows. Sizes are indicated in kilodaltons.

Growth assays. (i) Growth in medium containing 1% serum. Cells were plated at a density of $0.2 \times 10^4/cm^2$ in DMEM supplement with 10% serum. After an overnight incubation to allow cell attachment, the medium was changed to DMEM containing 1% fetal bovine serum. Cell numbers were determined 6 days later by trypsinizing cells from each well and counting them in a hemocytometer.

(ii) Anchorage-independent growth. The ability of transfected cell lines to divide in the absence of anchorage was determined by scoring the number of colonies formed in 0.2% agarose supplemented with 10% fetal bovine serum (with a 0.5% agarose underlay). Colonies larger than 0.12 mm were counted after 2 weeks in culture.

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 after stimulation with either insulin or IGF-I (21, 31). Figure 1 shows that such a p185 tyrosyl-phosphorylated protein band can be seen in whole cell lysates from R^-/T and W/T cells (Fig. 1A), but only after stimulation with insulin. IGF-I does not induce phosphorylation of the p185 band in R^- cells, which lack IFG-IR (not shown). If the lysates are immunoprecipitated with a specific antibody against SV40 T antigen, PAb901 (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 *ts*A58 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). These experiments, therefore, indicate that an antibody to SV40 T antigen coprecipitates a protein of approximately 185 kDa that is tyrosyl phosphorylated by either insulin or IGF-I (the latter only in cells with IGF-IR).

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 Biotechnology) and subsequent immunoblotting with PAb901 (Fig. 2). The original W and R^- cells (no T antigen) were used and compared with the 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-IR (7, 47), the experiments indicate that under the conditions used,

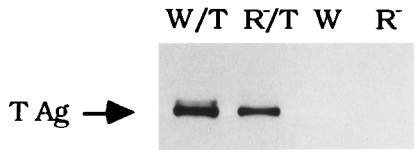

FIG. 2. Presence of SV40 T antigen in cell lysates after immunoprecipitation with an antibody to IRS-1. Lysates from W/T and R⁻/T cells grown in 10% serum were immunoprecipitated with an antibody to IRS-1 (see Materials and Methods) and immunoblotted with an anti-T-antigen (Ag) antibody, PAb901. Lysates from the parental W and R⁻ cells (no T antigen) served as controls. The position of T antigen is indicated by an arrow.

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 pRSVBneoT (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).

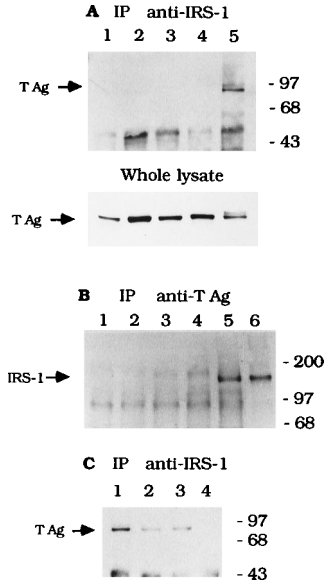

FIG. 3. Association of SV40 T antigen with IRS-1 is dependent on IRS-1 levels. 32D/T cells (32D cells expressing wild-type T antigen [Ag]) were used. (A) Cell lysates from either 32D/T cells (lanes 1 to 4) or T6 cells (lane 5) were immunoprecipitated (IP) with an antibody to IRS-1 and immunoblotted with an antibody to T antigen (PAb101) (top), and whole lysates were immunoblotted with an antibody to T antigen (bottom). (B) The lysates used for panel A were immunoprecipitated with an antibody against T antigen (Ag) and immunoblotted for IRS-1. The lanes are the same as in panel A (see below); in addition, a recombinant IRS-1 protein (Upstate Biotechnology) (UBI) was used to monitor the activity of the IRS-1 antibody (lane 6). (C) Lysates from cells expressing an antisense RNA to IRS-1 were immunoprecipitated with an antibody to IRS-1, as for Fig. 2, and immunoblotted with PAb901. Lanes: 1, W/T cells; 2 and 3, two clones of W/T cells expressing the IRS-1 antisense RNA; 4, parental W cells (no T antigen) as controls. The positions of T antigen and IRS-1 are indicated by arrows; sizes of marker proteins are indicated in kilodaltons.

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-I, 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

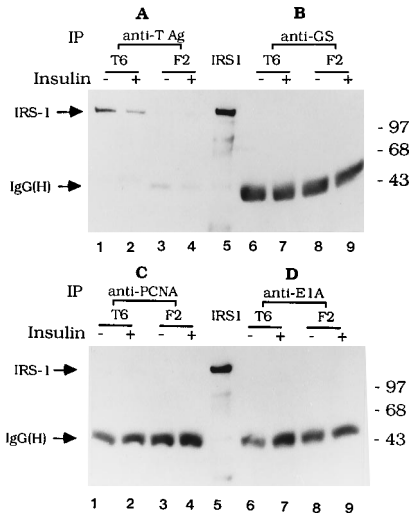

FIG. 4. An amino-terminally truncated T antigen does not coprecipitate IRS-1. Cells were made quiescent and then stimulated for 5 min with insulin (10 μg/ml). (A) Lysates from T6 cells (wild-type T antigen [Ag]) or F2 cells (T antigen with amino-terminal truncation) were immunoprecipitated (Ip) with 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 glutamine 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.

the ability of SV40 T antigen to associate with IRS-1 is correlated with its mitogenic and transforming potential. For this purpose, we used the cell lines used for the studies described above: cell lines expressing either wild-type or truncated T antigen and derived either from BALB/c 3T3 cells, which have physiological amounts of IGF-IRs, or from R^-/IRS-1 cells, which are devoid of IGF-IR but overexpress IRS-1 (8).

It is well established that SV40 T-antigen-expressing cells are capable of growing in 1% serum. The amino-terminal region of T antigen has been shown to be necessary for its mitogenic ability in low concentrations of serum (53). We

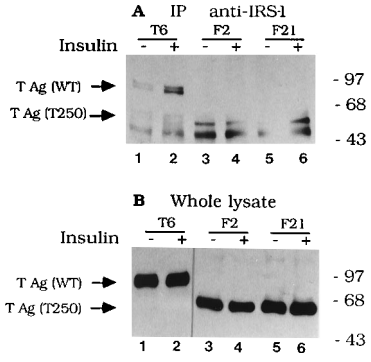

FIG. 5. An antibody to IRS-1 does not coprecipitate a truncated SV40 T antigen. Lysates were prepared from T6 cells or F2 and F21 cells (two clones expressing an amino-terminally truncated T antigen [T250]). (A) The lysates were immunoprecipitated (IP) with an antibody to IRS-1 and stained with an antibody 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 two 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

Cell line[a]	Colony formation (%)[b]
BALB/c 3T3	0
T6 (BALB/c 3T3 with T antigen)	69.7
F2 (BALB/c 3T3 with truncated T antigen)	13.9
F21 (same as F2)	22.3
R^- (no IGF-IR)	0
T/R^- (R^- cells with T antigen)	0
R^-/IRS (R^- cells with IRS-1)	0
R^-/IRS/T	
Clone 10	12.0
Clone 13	28.6
R^-/IRS/trunc. T, clones F, L, and P	0

[a] 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 phosphorlated by SV40 T antigen. The lysates were immunoprecipitated with an anti-IRS-1 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.

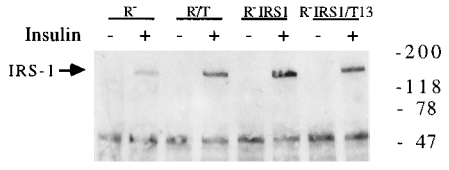

FIG. 6. IRS-1 is not tyrosyl phosphorylated by SV40 T antigen. Quiescent cells were left unstimulated (−) or were stimulated for 5 min with insulin (+). Cell lysates were immunoprecipitated with an antibody to IRS-1 and immunoblotted with an antiphosphotyrosine antibody. Lanes: 1 and 2, R^- cells; 3 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.

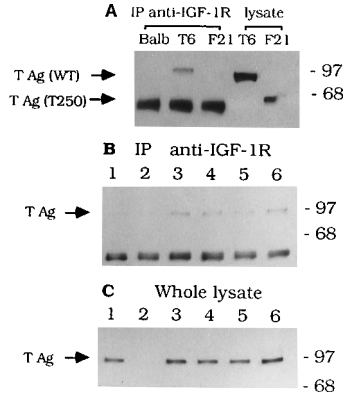

FIG. 7. Association of SV40 T antigen with IGF-IR. Cells were grown in 10% serum. (A) Lysates (500 μg of protein) from BALB/c (parental), T6, and F21 cells were immunoprecipitated (IP) with an antibody to IGF-IR and probed with the antibody PAb901. The last two lanes are whole lysates only (15 μg of protein), prior to immunoprecipitation with the anti-IGF-IR antibody. T Ag (WT), wild-type T antigen; T Ag (T250), amino-terminally truncated (B) Lysates (100 μg of protein) from BALBA58 (lane 1), p6 (lane 2), and various clones of p6 cells expressing SV40 T antigen (lanes 3 to 6) were immunoprecipitated with an antibody to IGF-IR and probed with PAb901. (C) Whole lysates (15 μg of protein) from the same cells, immunoblotted with the PAb901. Sizes of marker proteins are indicated in kilodaltons.

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 BALBA58 cells, which express a 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 BALBA58 cells.

SV40 T antigen does not associate with Shc and other signal-transducing molecules. Since Shc is considered a substrate of IGF-IR (45, 60, 61), we investigated whether T antigen would coprecipitate with Shc. 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 Shc by immunoblotting with an anti-Shc antibody (Fig. 8A), although Shc proteins (p52 and p66) are clearly present in Shc 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 immunoprecipiated with an anti-Grb2 antibody, a small amount of T antigen was coprecipitated (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

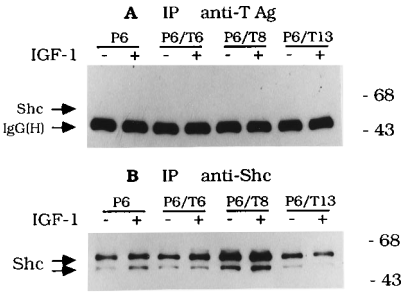

FIG. 8. Lack of association of T antigen with Shc. p6 cells and three clones of p6 cells expressing SV40 T antigen were made quiescent and then stimulated with IGF-I. Cell lysates (500 μg of protein) were immunoprecipitated (IP) with either an antibody to T antigen (Ag) (A) or an antibody to Shc (B) and immunoblotted with an anti-Shc antibody. Arrows indicate expected positions of Shc proteins. IgG(H), immunoglobulin G heavy chain. Sizes of marker proteins are indicated in kilodaltons.

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 R^- 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 R^- cells, whereas in combination, they can (reference 8 and this report). R^- 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

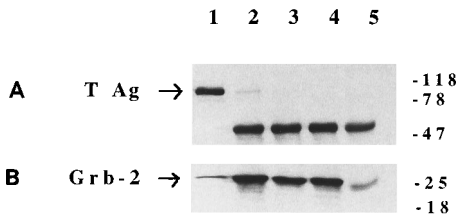

FIG. 9. Lack of association of SV40 T antigen with Grb2. Cell lysates were prepared from W/T cells (lanes 1 and 2), 32D/T cells (lane 3), or W cells (lane 4). Immunoprecipitation was carried out with an anti-Grb2 monoclonal antibody (lanes 2 to 5). The immune complexes or the whole lysates (lane 1) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and stained with an anti-T-antigen (Ag) antibody (A) or an anti-Grb2 antibody (B). Lane 5 is a control (no lysates), showing the position of the antibody heavy chain. Sizes of marker proteins are indicated in kilodaltons.

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 (Fig. 3C). (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 antibody 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 R^- 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 polyomavirus 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-IR 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, DiMaio and coworkers (32, 33) showed that the receptor was consitutively phosphorylated in cells transformed by the virus. Similarly, both Shc and polyomavirus 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 immunprecipitates 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, not 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 (13), and Deppert et al. (11) have reported that underphosphorlation 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-

dent 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^- 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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