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Insulin-Like Growth Factor I Receptor Signaling in Transformation by *src* Oncogenes

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R^- cells, a line of mouse embryo fibroblasts with a targeted disruption of the insulin-like growth factor I (IGF-I) receptor genes, are refractory to transformation by several viral and cellular oncogenes. Using colony formation in soft agar as a measure of full transformation, we report here that R^- cells can be transformed by v-*src*, although they still cannot be transformed by the activated c-*src*527 (mutation at tyrosine 527 to phenylalanine), which readily transforms mouse embryo cells with a wild-type number of IGF-I receptors (W cells). Although v-*src* is a more potent inducer of tyrosine phosphorylation than c-*src*527, the extent of phosphorylation of either insulin receptor substrate 1 or Shc, two of the major substrates of the IGF-I receptor, does not seem sufficiently different to explain the qualitative difference in soft agar growth. v-*src*, however, is considerably more efficient than c-*src*527 in its ability to tyrosyl phosphorylate, in R^- cells, the focal adhesion kinase, Stat1, and $p130^{cas}$. These results indicate that v-*src*, but not c-*src*527, can bypass the requirement for a functional IGF-I receptor in the full transformation of mouse embryo fibroblasts and suggest that qualitative and quantitative differences between the two oncogenes can be used to identify some of the signals relevant to the mechanism(s) of transformation.

There is substantial evidence that the insulin-like growth factor I (IGF-I) receptor (IGF-IR) plays an important role in the establishment and maintenance of the transformed phenotype. The crucial observation was that 3T3-like cells from mouse embryos with a targeted disruption of the IGF-IR genes (5, 38), R^- cells, are refractory to transformation by simian virus 40 T antigen and/or an activated Ha-*ras* oncogene (65, 66); by bovine papillomavirus (42) and human papillomavirus E7 (69); and by overexpressed epidermal growth factor (EGF) (16), platelet-derived growth factor beta (21), or insulin (41) receptors, all conditions that readily transform embryo cells generated from their wild-type littermates, or other 3T3-like cells. Antibodies to the IGF-IR (3, 30); antisense expression plasmids or antisense oligodeoxynucleotides to either IGF-I (74), its receptor (35, 44, 46, 55–57, 67), or IGF-II (15); and dominant negative mutants of the IGF-IR (13, 19, 36, 52) can all reverse the transformed phenotype and/or inhibit tumorigenesis. Conversely, the overexpression of the wild-type (but not of a mutant) IGF-IR induces ligand-dependent transformation (16, 27, 31, 37, 39, 68) and increases the incidence of certain malignancies in transgenic mice (58). The importance of the IGF-IR in transformation and tumorigenesis, discussed in a recent review by Baserga (9), is also strengthened by the observation that tumor suppressor genes inhibit the expression of the IGF-IR gene (53, 77, 78). A mutational analysis of the human IGF-IR in R^- cells has indicated that the C terminus of the receptor is dispensable for IGF-I-mediated mitogenesis but is obligatory for its transforming activity (27, 72), thus establishing that these two properties of the receptor can be mapped to different domains.

The v-*src* oncogene of Rous sarcoma virus is a 60-kDa membrane-associated tyrosine kinase that is able to cause transformation in a variety of cell types (reviewed by Parsons and Weber in reference 45). Its cellular homolog, $pp60^{c\text{-}src}$, even when overexpressed, is considerably less transforming, unless its tyrosine residue 527 is mutated to phenylalanine (32). This activated mutant of c-*src* (hereafter designated as c-*src*527) has a kinase activity that is about 40% that of v-*src* (32). Because membrane association and tyrosine kinase activity seem to be essential for the transforming activity of v-*src*, Weber and coworkers inquired whether growth factor receptors may be involved in v-*src*-induced transformation. They reported that the IGF-IR is constitutively autophosphorylated in v-*src*-transformed cells (33) and, subsequently, that this phosphorylation is mediated through an intracellular mechanism and that insulin receptor (IR) substrate 1 (IRS-1) is also constitutively tyrosyl phosphorylated in cells coexpressing the IGF-IR and v-*src* (48, 49).

Since R^- cells have no IGF-IRs and are refractory to transformation (see above), we inquired whether v-*src* or c-*src*527 could transform R^- cells. Surprisingly, v-*src* but not c-*src*527 can transform R^- cells, while both oncogenes can readily transform cells with a physiological number of IGF-IRs (W cells). In addition, we found that two major substrates of the IGF-IR, IRS-1 and Shc, are constitutively tyrosyl phosphorylated by both oncogenes in the absence of the IGF-IR but that focal adhesion kinase (FAK), Stat1, and another protein, identified as $p130^{cas}$, are differentially phosphorylated in v-*src*-expressing R^- cells, in respect to c-*src*527-expressing R^- cells. The presence of a wild type (see also reference 66) but not of a truncated, nontransforming mutant of the IGF-IR (see above) restores the ability of c-*src*527 to transform R^- cells.

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MATERIALS AND METHODS

Plasmids. pMv-*src* and pc-*src*527 plasmids were used for the expression of the Schmidt-Ruppin A v-Src protein and the c-*src*527 mutant of the c-*src* proto-oncogene (a kind gift of D. Shalloway, described in references 29 and 32),

respectively. The plasmid expressing the human IGF-IR truncated at residue 1229 (pBPV-TC) has been described previously (27, 72).

Cell lines and transfection. R^- cells (65, 66) are mouse embryo fibroblasts with a targeted disruption of the IGF-IR genes (5, 38), routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. W cells are mouse fibroblasts with a wild-type number of IGF-IRs. Both cell lines were generated by the same protocols used to generate other 3T3 cell lines (65). Transfections were performed by a standard calcium-phosphate precipitation method. Cell lines expressing v-*src* or c-*src*527 were obtained by cotransfecting pMv-*src* or pc-*src*527, respectively, with pRSVneo (to confer the resistance to neomycin) at a molar ratio of 20:1. The establishment of cell lines expressing v-*src* or c-*src*527 is reported under Results. Plasmid pBPV-TC was cotransfected with a hygromycin resistance gene (25). BRL 3A is a rat liver cell line (ATCC CRL 1442) that expresses several growth factors and whose conditioned medium promotes the growth of R^- cells (unpublished data).

Western blotting. The expression of v-*src* or c-*src*527 in the transfected clones was analyzed by Western blotting. Cell lysates from cells exponentially growing were clarified by centrifugation, and their protein concentration was determined. Twenty micrograms of protein was resolved on a 4 to 15% polyacrylamide gradient gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted into a nitrocellulose filter. To determine the amount of Src protein, the filters were immunoblotted with a monoclonal anti-Src antibody (no. 327), followed by anti-mouse horseradish peroxidase-conjugated antibody (Oncogene Science). Visualization of the immunocomplexes was obtained by the enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham Corp.).

Growth curves. R^- cells and their derived cell lines were plated at a density of 5×10^4 in 35-mm dishes. Cells were allowed to attach to the plate in 10% serum and after 8 h were shifted to serum-free medium (SFM) or to the different concentrations of fetal bovine serum tested. Viable cells were counted after trypsinization with a hemocytometer, every day from day 0 to day 3. Cell viability was determined by trypan blue exclusion. All growth experiments were carried out in triplicate.

Coculture. R^- cells were plated at a density of 10^5 on a glass coverslip in a 35-mm plate and made quiescent in SFM for 3 days. BRL 3A, R^-/v-*src*, and R^-/c-*src*527 were grown on coverslips in SFM for 3 days until subconfluence. At day 0, two coverslips (in each case, one of the coverslips was of R^- cells) were placed adjacent in a 60-mm plate in SFM containing 0.5 μCi of [^{3}H]thymidine per ml and incubated at 37°C for 2 days. Cells were fixed and processed for autoradiography by standard procedures. Labeled R^- cells were scored at the microscope, and the percentage of labeled cells was evaluated.

IGF-IR number. This was determined both by Scatchard analysis and by Western blots, as previously described (41).

Conditioned medium assay. Conditioned medium was prepared from subconfluent cells in a 100-mm plate. After three washes in Hanks solution, cells were placed in SFM. After 12 h, the medium was removed and 10 ml of fresh SFM for the plate was added. The conditioned medium was collected after 3 days, centrifuged to remove cell debris, and filtered. R^- cells were plated and made quiescent in SFM as described under "Coculture." At day 0, cells were shifted to the different conditioned medium tested containing 0.5 μCi of [^{3}H]thymidine per ml. After 24 h, cells were fixed and processed for autoradiography by standard procedures.

Focus formation and anchorage-independent growth assay. For focus formation, cells were plated at a concentration of 5×10^5 cells per 100-mm plate. After 14 days in culture, the plates were fixed in methanol containing 0.5% crystal violet. Anchorage-independent growth of the different cell lines was investigated by the soft agar assay as described by Sell et al. (65). Cells were seeded in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.2% agarose on an underlay of 0.4% agarose. After 2 weeks at 37°C, colonies larger than 125 μm were counted.

Immunoprecipitation and immunoblot. Cells were incubated in SFM for 72 h before stimulation with the indicated growth factors, 20 μg of insulin (Sigma) per ml, or 20 ng of EGF (Gibco BRL) per ml. Cell lysates (300 μg of protein) were immunoprecipitated with the indicated antibody in HNTG buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.2 mM sodium orthovanadate, 0.2 mM phenylymethysulfonyl fluoride, 2 μg of aprotinin per ml). The immunoprecipitates were resolved on a 4 to 15% polyacrylamide gradient gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. For immunoblotting, membranes were blocked with 5% bovine serum albumin or 5% nonfat milk in TBST buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20) overnight at 4°C and probed with the indicated antibodies, followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (Oncogene Science). Blots were then developed with the enhanced chemiluminescence system according to the manufacturer's instructions (Amersham Corp.).

Antibodies. The antibodies used for immunoprecipitation or immunoblot are the following. IRS-1 and Stat1 were immunoprecipitated and immunoblotted with polyclonal anti-IRS-1 antibody (Upstate Biotechnology Inc., Lake Placid, N.Y.) or an anti-Stat1 antibody (Santa Cruz Biotechnology), respectively. Src was immunoprecipitated with the monoclonal antibody LA074 (Quality Biotech) or immunoblotted with the monoclonal antibody 327. Shc was immunoprecipitated or immunoblotted with a polyclonal or a monoclonal anti-Shc antibody (Transduction Laboratories), respectively. The IR was immunoprecipitated with a monoclonal anti-IR antibody (Ab-3; Oncogene Science) and immunoblotted with a polyclonal anti-IR β-subunit (Transduction Laboratories). FAK was immunoprecipitated or immunoblotted with an anti-FAK monoclonal antibody (Transduction Laboratories). The p85 subunit of the phosphatidylinositol 3 kinase was immunoblotted with rabbit whole antiserum anti-p85 (Upstate Biotechnology Inc.). p130^{cas} was immunoblotted with a rabbit polyclonal antibody (C-20; Santa Cruz Biotechnology). The detection of phosphorylation of immunoprecipitated proteins was performed with an antiphosphotyrosine horseradish peroxidase-conjugated antibody (PY20; Transduction Laboratories).

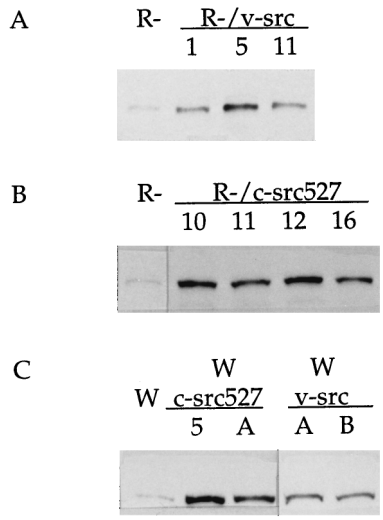

FIG. 1. Expression of *src* in stably transfected R^- and W cells. The figure shows Western blots of whole-cell lysates, with an antibody to Src. (A) v-*src* in R^- cells; (B) c-*src*527 in R^- cells; (C) both oncogenes in W cells. In all three panels, the first lane gives lysates from the parental, untransfected cell lines. The letters and numbers above the lanes designate the transfectant clones.

RESULTS

Generation of cell lines expressing *src*. 3T3 cells with or without the IGF-IR (W and R^- cells, respectively) were transfected with the plasmids expressing either v-*src* or c-*src*527 (see Materials and Methods). Clones were selected in 800 μg of G418 per ml, and some of these clones were monitored for *src* expression. Western blots of representative clones are shown in Fig. 1: in panels A and B are the R^- cells, and in panel C are the W cells. Since, as explained below, there are differences in biological behavior between v-*src*- and c-*src*527-transfected R^- cells, it is important to establish that *src* expression in R^- cells was similar with both plasmids. Figure 1A and B show that, if anything, *src* expression was higher in clones of cells transfected with c-*src*527. By densitometric measurements, with in both cases as the basis the level of expression of c-*src* in the parental R^- cells, the expression of v-*src* was increased from 4- to 9-fold, while the expression of c-*src*527 was increased from 10- to 24-fold. Even in W cells (panel C), c-*src*527 was slightly more expressed than v-*src*: by densitometry, the ratio was about threefold in favor of c-*src*527. We have not investigated further the reason(s) for these differences in the expression of the two plasmids. There were also consistent differences in morphology: R^-/v-*src* clones displayed a typical transformed phenotype, with spindle-shaped cells, in contrast to R^-/c-*src*527 cells, which had a more round shape with ruffling of the membranes.

Growth characteristics of cells overexpressing *src*. Before describing the growth characteristics of our cell lines, we would like to define our criteria of transformation, which are based on generally accepted principles, originally proposed by Pon-

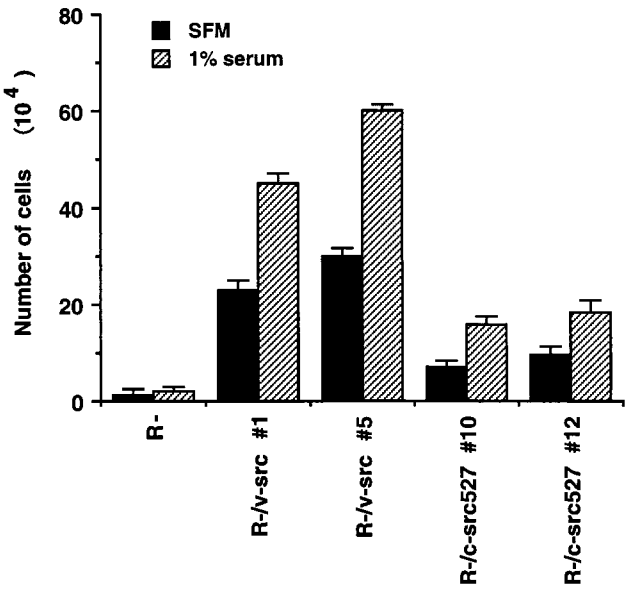

FIG. 2. Growth of R^- cells expressing *src* in SFM. Cells were grown in SFM, supplemented (striped bars) or not (closed bars) with 1% serum. The number of cells per plate was determined after 3 days of cultivation. Each experiment was done in triplicate, and the standard deviations are given.

ten (51). According to Ponten (51), the first steps in the progressive process of transformation are an increased saturation density and a lowering of the requirements for growth factors. Focus formation in monolayers is the next step, followed eventually by the ability to form colonies in soft agar and, finally, tumorigenesis in nude mice (see also reference 7). For brevity, in this paper, we will refer to the acquisition of the first two characteristics (lowering of growth factor requirement and increased cell density) as partial transformation and the ability to form colonies in soft agar (anchorage independence) simply as transformation.

All the clones that we isolated grew well in 10% serum, like the parental R^- cells (not shown). Clones of cells expressing *src* were tested in SFM or in 1% serum. Although we obtained and tested several clones of both types, for clarity we will present the results obtained with selected clones. R^-/v-*src* and R^-/c-*src*527 cells grew in both 1% serum and SFM, which was quite surprising, since R^- cells cannot grow in SFM even when supplemented with a variety of purified growth factors (65). R^- cells do not grow in 1% serum, either. The R^-/v-*src* cells grew in SFM better than the R^-/c-*src*527 cells, but there is no question that the latter cell line could also grow (Fig. 2), although its saturation density is lower. By our criteria, we can say that both v-*src* and c-*src*527 induce in R^- cells at least a partially transformed phenotype.

Since it has been suggested that v-*src*-transformed cells may secrete growth factors (2), we tested the conditioned medium of *src*-expressing R^- cells for growth-promoting activity on the parental R^- cells. Neither the conditioned medium of R^-/v-*src* nor that of R^-/c-*src*527 clones had any growth-stimulating effect on R^- cells, as measured by their ability to enter DNA synthesis (see Materials and Methods). In Table 1, column 2, the reference basis is R^- cells, which, under these conditions, gave a labeling index of 30%. Addition of conditioned medium from *src*-transfected clones did not increase the labeling index. We also tested for the presence of growth factors by coculture experiments (see Materials and Methods). R^- cells were used again, and the assay was again based on the number of cells labeled by [^{3}H]thymidine and detectable by autoradiography. The results are shown in Table 1 (column 1): DNA synthesis is not stimulated in R^- cells when they are cocultured with either R^-/v-*src* or R^-/c-*src*527 cells. As a control, we used BRL 3A cells, whose conditioned medium allows R^- cells to grow in SFM (unpublished data from our laboratory). Table 1 shows that coculture with BRL 3A cells (or conditioned medium from the same cells) does stimulate DNA synthesis in R^- cells. These experiments indicate that neither v-*src* nor c-*src*527 cells produce significant amounts of growth-promoting substances in the medium. Our results confirm indirectly the report by Peterson et al. (48) that v-*src* activates the IGF-IR pathway by an intracellular mechanism.

Focus formation in monolayers. Selected clones were then tested for the ability to form foci when grown in monolayer cultures. Results of a representative experiment are shown in Fig. 3, which compares R^- cells (plate A) with clones of R^-/v-*src* (plate C) or R^-/c-*src*527 (plate B) cells. R^-/c-*src*527 cells reach a higher saturation density than the parental R^- cells, but they do not form visible foci, which are instead numerous in the plate of R^-/v-*src* cells.

Growth in soft agar of cell lines expressing *src*. A number of R^- and W clones expressing either v-*src* or c-*src*527 were then tested for their ability to form colonies in soft agar. The results (Table 2) show that, as expected, both v-*src* and c-*src*527 could fully transform W cells, although there was variability in the ability of the single clones to form colonies in soft agar. However, with R^- cells, the results were dramatically different: while c-*src*527 behaved like the other oncogenes previously tested (16, 21, 41, 42, 65, 66) and failed to transform these cells, v-*src* was capable of transforming R^- cells, giving rise to a number of clones that formed colonies in soft agar, almost as efficiently as with W cells. Thus, while both c-*src*527 and v-*src* can induce in R^- cells the first steps of transformation (lowered growth factor requirements and increased saturation density), only v-*src* makes these cells capable of forming foci in monolayers or colonies in soft agar.

Tyrosyl phosphorylation of IRS-1 in cells expressing *src*. It is known that both v-*src* and c-*src*527 have the ability to tyrosyl phosphorylate a number of substrates, v-*src* being in general twice as effective as c-*src*527 (32). We have confirmed this in the experiment whose results are shown in Fig. 4, in which whole lysates of cells were blotted with an antiphosphotyrosine antibody (see Materials and Methods). There are more bands visible in lysates of cells expressing v-*src* (lanes 4 and 5) than in

TABLE 1. DNA synthesis in R^- cells under different conditions[a]

Cell type	% of labeled cells	
	Coculture	Conditioned medium
R^-	16	30
BRL 3A	46	84
R^-/v-*src* no. 1	ND	28
R^-/v-*src* no. 5	21	31
R^-/c-*src*527 no. 10	ND	27
R^-/c-*src*527 no. 12	16	31

[a] In every case, the test cells were R^- cells, so that the percentage of labeled cells is the percentage of R^- cells labeled with [^{3}H]thymidine under different conditions. In column 1, R^- cells were cocultured with the cell lines shown in the stub; in column 2, R^- cells were incubated in conditioned medium from the cells listed in the stub. The experimental details are given in Materials and Methods. BRL 3A is a positive control; the conditioned medium from these cells is known to stimulate growth of R^- cells (unpublished data from our laboratory). ND, not done.

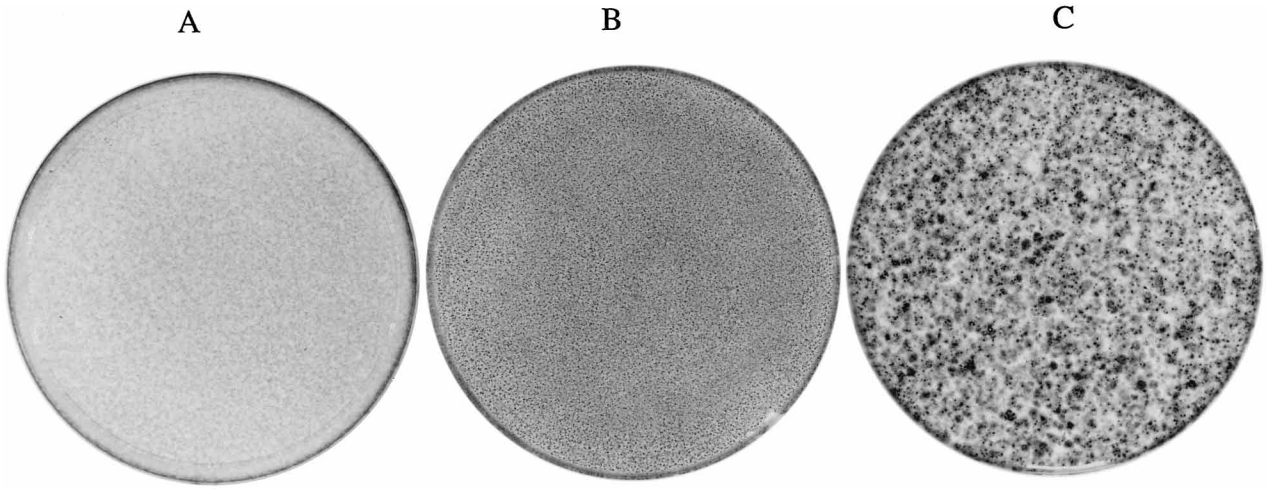

FIG. 3. Focus formation in monolayer cultures. R^- cells (A) and representative clones of R^-/c-*src*527 cells (B) and R^-/v-*src* cells (C) were grown in monolayers for 2 weeks. The plates have been stained with crystal violet.

those of cells expressing c-*src*527 (lanes 2 and 3). Under these conditions, no bands are visible in lysates of R^- cells (lane 1). Although we show only two clones, other clones were also examined, and there was a consistent difference between R^-/v-*src* and R^-/c-*src*527.

Weber and coworkers (33, 48, 49) have previously reported that both the IGF-IR and IRS-1 (70), one of the major substrates of the IGF-IR (43), were constitutively phosphorylated in *src*-transformed cells. We investigated the status of IRS-1 in *src*-transformed R^- cells, which do not have the IGF-IR (65, 66). For this purpose, lysates from cells stimulated or not with insulin (20 μg/ml) were immunoprecipitated with an antibody to IRS-1.

TABLE 2. Colony formation in soft agar of cells expressing *src*[a]

Cell line	Oncogene	No. of colonies
R^-	None	0
	Empty vector	0
	v-*src* (1)	95.7 (6)
	v-*src* (5)	175 (30.5)
	v-*src* (11)	28 (2)
	v-*src* (25)	57.5 (4.5)
	v-*src* (26)	22.5 (2.5)
	c-*src*527 (10)	1.7 (1.2)
	c-*src*527 (11)	0
	c-*src*527 (12)	4.2 (3.7)
	c-*src*527 (16)	0
W	Empty vector	0
	v-*src* (A)	26.5 (0.5)
	v-*src* (B)	148 (8.5)
	c-*src*527 (5)	27.5 (0.6)
	c-*src*527 (A)	29 (2)
p6	IGF-IR	145 (38)
R^-/c-*src*527/10	TC (1)	0
R^-/c-*src*527/10	TC (3)	7 (4)
R^-/c-*src*527/12	TC (1)	0

[a] Colony formation in soft agar was determined as described in Materials and Methods. Numbers or letters in parentheses in column 1 are the clone designations. The numbers in column 2 are the averages of several determinations (standard deviations in parentheses). All cell lines (with the exception of R^- cells) are stably transfected clones. p6 is a cell line derived from BALB/c3T3 cells that overexpresses the IGF-IR (50). TC is a truncated IGF-IR (72), with loss of the last 108 amino acids, known to be mitogenic but nontransforming.

In Fig. 5A, the immunoprecipitates were blotted with an antiphosphotyrosine antibody: in R^- cells, a band corresponding to IRS-1 is phosphorylated only in cells stimulated with insulin, and the same can be said of R^+ cells, overexpressing the IGF-IR (65), although a faint band can be detected also in unstimulated cells. However, in R^- cells stably transfected with either v-*src* or c-*src*527, IRS-1 is constitutively phosphorylated, although the extent of tyrosyl phosphorylation increases after insulin stimulation. Figure 5B shows the same filter after stripping and Western blotting with an antibody to IRS-1, confirming that the band seen in panel A is IRS-1 and that the amounts of IRS-1 are substantially the same in all lysates. If anything, IRS-1 is slightly decreased in the *src*-transfected cell lines. The same blot was also stained with an antibody to p85 and will be discussed below and with an antibody to Src (see Materials and Methods): Fig. 5D shows that Src is clearly detectable in the *src*-transformed cells, indicating that Src is coprecipitating with IRS-1, as already reported by Peterson et al. (48). The Src band is stronger in R^-/c-*src*527 cells than in R^-/v-*src* cells, but this may simply be due to the higher level of expression of c-*src*527 (Fig. 1). Interestingly, the amount of Src associated with IRS-1 increases after insulin stimulation. Finally, Fig. 5E extends these observations to two additional

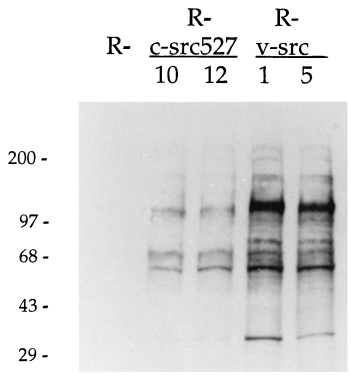

FIG. 4. Tyrosine phosphorylation blot of R^- cells and derived clones. Whole-cell lysates (20 μg) of cells in SFM for 3 days were blotted with an antiphosphotyrosine antibody. Lanes: 1, R^- cells; 2 and 3, R^-/c-*src*527 cells; 4 and 5, R^-/v-*src* cells. Numbers at left are molecular mass in kilodaltons.

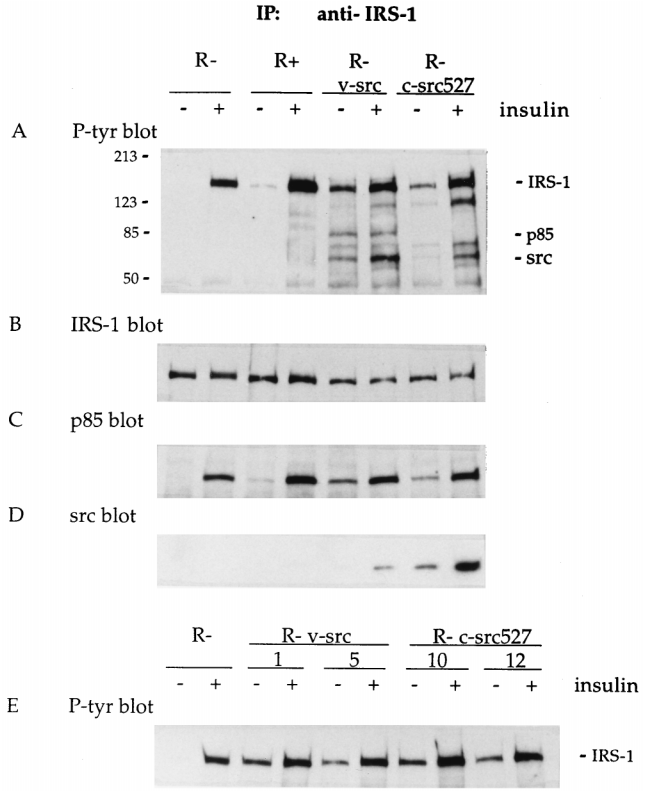

FIG. 5. Phosphorylation of IRS-1 in *src*-transfected R^- cells. Cell lysates from cells were stimulated or not with insulin (20 μg/ml for 10 min) and were immunoprecipitated (IP) with an antibody, anti-IRS-1. (A) The membrane was blotted with an antiphosphotyrosine antibody. (B to D) After stripping, the same membrane was blotted with antibodies to IRS-1 (B) and p85 (C) and an anti-Src antibody (D). (E) Antiphosphotyrosine blottings of R^- cells and two more clones each of R^-/v-*src* and R^-/c-*src*527 cells, after immunoprecipitation with an anti-IRS-1 antibody (see Materials and Methods). Molecular masses (in kilodaltons) of marker proteins are indicated.

clones of v-*src*-transformed R^- cells and two additional clones of R^- cells expressing c-*src*527. IRS-1 is again constitutively tyrosyl phosphorylated when R^- cells express *src*. There does not seem to be a clear difference in the constitutive phosphorylation of IRS-1 between c-*src* and v-*src*. In some clones (Fig. 5A), IRS-1 is more phosphorylated in v-*src* than in c-*src*527 cells, but the reverse is true in other clones (see, for instance, clones 10 and 12 versus clone 5 in Fig. 5E).

Immunoprecipitates were also obtained with lysates of W cells expressing *src* (Fig. 1C). As expected, in these cells, with a physiological number of IGF-Rs, IRS-1 was also constitutively tyrosyl phosphorylated (not shown). In other experiments, the lysates were immunoprecipitated with an antibody to Src (LA074) and then blotted with antiphosphotyrosine antibody (Fig. 6A), with anti-IRS-1 antibody (Fig. 6B), or with an anti-Stat1 antibody (Fig. 6C). IRS-1 coprecipitates in these cells with Src, the latter being visible in panel A. The lane marked pos.ctr. is a total lysate (20 μg) from a cell line (unrelated to the cell lines discussed in this paper) grossly overexpressing IRS-1, without detectable phosphorylation under the conditions used. These results, while confirming the association between IRS-1 and *src* (48) and the constitutive tyrosyl phosphorylation of IRS-1 in *src*-expressing cells, also point out that this association occurs also in the absence of the IGF-IR. However, our results also indicate that IRS-1 tyrosyl phosphorylation is independent of the degree of transformation in *src*-transfected R^- cells. Figure 6C shows that Stat1 also coprecipitates with Src, whether in R^-/c-*src*527 or in R^-/v-*src* cells. We will return to this finding later. Finally, panel D shows the same blot, restripped and blotted again with an anti-Src antibody (no. 327). The presence of bands (of higher density in c-*src*527 cells than in v-*src* cells) shows the specificity of the antibody and confirms the identity of the band in panel A.

Shc is also phosphorylated in cells expressing *src*. The Shc proteins are considered a direct substrate of the IGF-IR (61, 62), apparently interacting, like IRS-1, with the tyrosine residue at position 950 (17, 73). In these experiments, the cells were stimulated with EGF. Figure 7A shows that the Shc proteins are constitutively tyrosyl phosphorylated in R^- cells expressing v-*src* or c-*src*527. The Shc proteins also become phosphorylated after EGF treatment in R^- and R^+ cells (Fig. 7C). In panel A, they are not visible, because the extent of phosphorylation is much less than in R^- cells expressing *src*: when exposure is increased, they become visible, but then the lanes of cells expressing *src* are grossly overexposed (Fig. 7C). The extent of phosphorylation is slightly less in c-*src*527 than in v-*src*-transfected R^- cells, but this difference loses some of its significance when the protein amounts are compared (Fig. 7B). These results are in agreement with those of McGlade et al. (40) that suggest that the Shc proteins are constitutively phos-

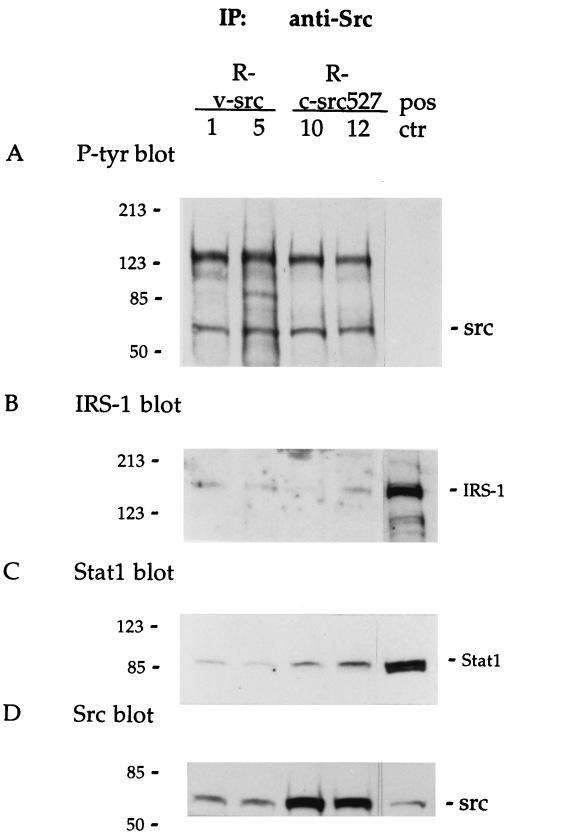

FIG. 6. IRS-1 and Stat1 coprecipitate with Src. Cell lysates were immunoprecipitated (IP) with an antibody to Src. (A) The filter was blotted with an antiphosphotyrosine antibody. (B) After stripping, the filter was blotted with an antibody to IRS-1. (C) The stripped blot was blotted again with an antibody to Stat1. (D) Finally, the blot was blotted again with an antibody to Src. The positions of Src, IRS-1, and Stat1 are indicated. The positive control (pos ctr) in panels A, B, and C is total lysate (20 μg) from a cell line overexpressing IRS-1. For the antibodies and immunoprecipitation, see Materials and Methods. Molecular masses (in kilodaltons) of marker proteins are indicated.

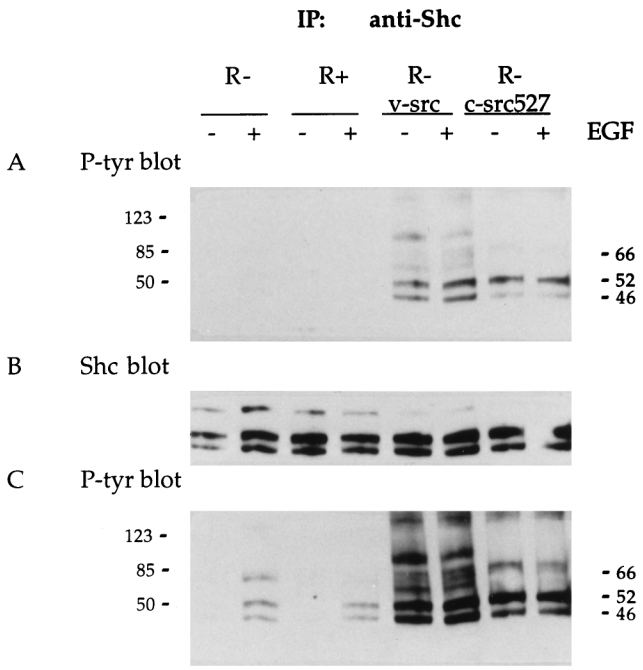

FIG. 7. Tyrosyl phosphorylation of Shc. Cell lysates were immunoprecipitated (IP) with an antibody to Shc proteins, after the cells had been stimulated or not with EGF (20 ng/ml) for 5 min. (A) Immunoblot with a phosphotyrosine antibody with an exposure of 15 s. (B) Immunoblot with anti-Shc antibody. (C) Immunoblot with a phosphotyrosine antibody with an exposure of 1 min. Numbers at right and left are molecular mass in kilodaltons.

phorylated in *src*-transformed cells. Their constitutive phosphorylation is independent of the presence of the IGF-IR.

R^- cells have a low number of IRs, roughly 5,000 receptors per cell (41), and we wanted to determine whether these receptors were constitutively autophosphorylated in *src*-transfected cells. Cell lysates were immunoprecipitated with an anti-IR antibody (see Materials and Methods) and blotted with either an antiphosphotyrosine antibody or an anti-IR antibody. We could not detect autophosphorylation of the IR, unless the cells were stimulated with insulin (data not shown).

FAK in cells expressing *src*. FAK is also known to interact with Src and to be phosphorylated by it (22, 80). We immunoprecipitated lysates of cells with an antibody against FAK and then blotted them with an antiphosphotyrosine antibody. Fig. 8A shows that FAK is phosphorylated in R^-, R^+, R^-/v-*src*, and R^-/c-*src*527 cells but that the extent of tyrosyl phosphorylation is considerably increased in R^-/v-*src* cells. When the same blot was stripped and blotted with an antibody to FAK (Fig. 8B), the protein amounts were considerably higher in R^- and R^+ cells than in R^- cells expressing *src*, confirming previous results that *src* expression causes a marked decrease in the amounts of FAK (24). Figure 8C is a Western blot of whole-cell lysates from the same cells: again, FAK expression is considerably higher in R^- and R^+ cells. When phosphorylation and protein amounts were measured by densitometry, one could calculate that FAK was eight times more phosphorylated in R^-/v-*src* cells than in the other cell lines. This experiment was repeated on the same clones and on other clones of *src*-expressing R^- cells (Fig. 8D and E), and the results were the same: FAK is more phosphorylated in v-*src*-transformed cells than in the other cell lines, but the amount of FAK is decreased in all R^- clones expressing *src*, whether v-*src* or c-*src*527.

Different results were obtained with clones of W cells stably transfected with either v-*src* or c-*src*527. FAK was hyperphosphorylated and constitutively phosphorylated equally in cells expressing c-*src*527 and in those expressing v-*src* (Fig. 9A), but the amounts of FAK were not decreased in respect to W cells (Fig. 9B).

A p85-95 protein is tyrosyl phosphorylated in R^-/v-*src* cells. In Fig. 5A, a phosphorylated band of approximately 85 to 90 kDa in size was present but only in v-*src*-transformed R^- cells. Because of its size and because it coprecipitated with IRS-1 (1, 43, 71), we suspected that it could be the regulatory subunit of phosphatidylinositol 3 kinase, a substrate of both the IGF-IR and IR (4), which is also known to bind Src (26). In Fig. 5C, it can be seen that p85 coprecipitates with IRS-1 but in R^- and R^+ cells only after insulin stimulation. In v-*src* and c-*src*527 cells, a small amount of p85 coprecipitates with IRS-1 even in the absence of insulin stimulation, although the band increases sharply after insulin stimulation. We made several attempts to confirm the identity of the phosphorylated band seen in Fig. 5A with antibodies to the p85 subunit. The results were inconclusive. Instead, we were able to demonstrate that Stat1 was

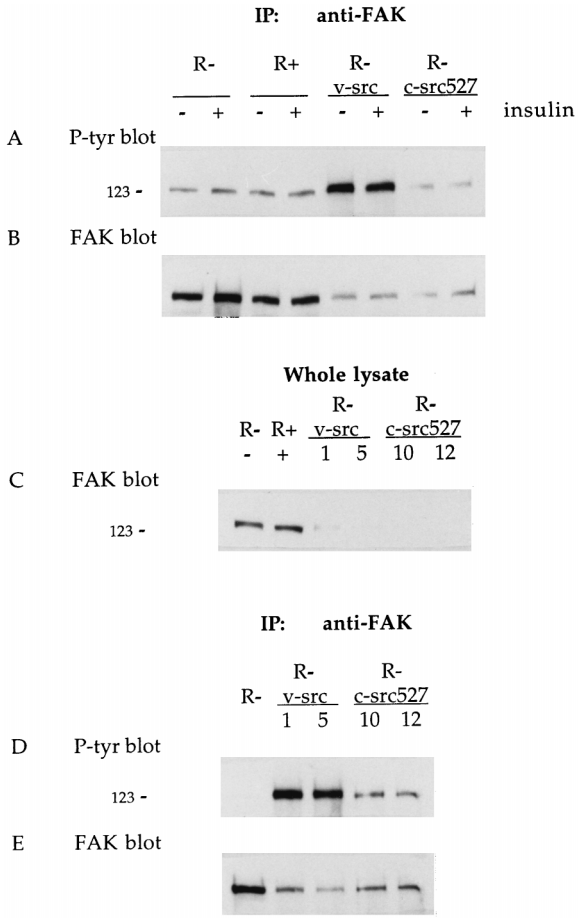

FIG. 8. Differential phosphorylation of FAK. Lysates were prepared from cells either unstimulated or stimulated with insulin for 10 min (20 μg/ml). (A and B) The lysates were immunoprecipitated (IP) with an antibody to FAK, and immunoblots were made with an antiphosphotyrosine antibody (A) or with a FAK antibody (B). (C) Direct Western blotting of cell lysates (no immunoprecipitation) with an antibody to FAK. (D and E) The same experiment as for panels A and B was repeated on two clones each of R^- cells expressing either v-*src* or c-*src*527 and on R^- cells (lane 1). Numbers at left are molecular mass in kilodaltons.

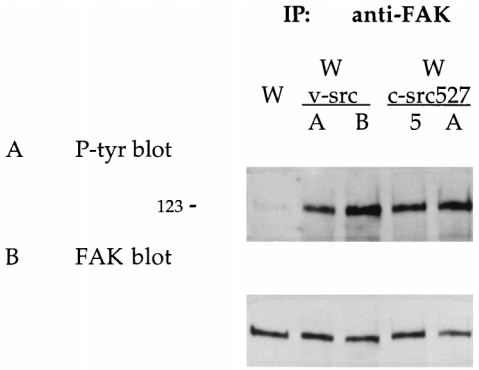

FIG. 9. FAK phosphorylation in W cells. W cells were stably transfected with either v-*src* or c-*src*527 (Fig. 1). Cell lysates were immunoprecipitated (IP) with an anti-FAK antibody and blotted with either antiphosphotyrosine antibody (A) or anti-FAK antibody (B). Lanes: 1, W cells; 2 and 3, two clones of W cells stably transfected with v-*src*; 4 and 5, two clones of W cells stably transfected with c-*src*527. Number at left is molecular mass in kilodaltons.

tyrosyl phosphorylated in R^-/v-*src* cells while its phosphorylation could not be detected in R^-/c-*src*527 cells (Fig. 10A). The bands are weak, which could be due to either of two possibilities: Stat1 is reduced like FAK in v-*src*-transformed cells or, more likely, the amount of Stat1 is modest because our lysis buffer extracts only cytoplasmic proteins, and most Stat1 may be already in the nucleus. We had already shown in Fig. 6 that Stat1 coprecipitated with an antibody to Src. The amount of coprecipitated Stat1 was somewhat higher with the cells expressing c-*src*527 than with the cells expressing v-*src*. In Fig. 10B, we can see again that the amounts of Stat1 are higher in R^-/c-*src*527 cells than in R^-/v-*src* cells. In Fig. 10A, another band of about 130 to 140 kDa is visible as highly phosphorylated in v-*src* cells and is undetectable in R^-/c-*src*527 cells. The difference is striking, and when the same blot, after stripping, is blotted again with an antibody to $p130^{cas}$, a clear band is detectable in R^-/v-*src* cells but not in R^-/c-*src*527 cells. It seems, therefore, that an antibody to Stat1 specifically coprecipitates $p130^{cas}$ in cells expressing v-*src*.

A truncated IGF-IR fails to transform R^- cells transfected with c-*src*527. Table 2 shows that, in terms of colony formation in soft agar, v-*src* can transform both R^- and W cells but c-*src*527 can transform only W cells (physiological number of receptors). In previous reports, it was shown that the C terminus of the IGF-IR is important for transformation, although it is dispensable for mitogenic stimulation transformation (27, 37, 72). A C-terminally truncated receptor, either at residue 1229 (72) or at residue 1245 (27), is incapable of transforming R^- cells, although it is perfectly competent for IGF-I-mediated mitogenesis. Reintroduction of a wild-type receptor restores transformability (65, 66). Two clones of R^-/c-*src*527 cells were transfected with the human IGF-IR truncated at residue 1229 (72). Three clones were eventually selected (Table 2), and their numbers of IGF-IRs were 80,000 (TC clone 1), 40,000 (TC clone 3), and 150,000 (TC clone 1 from R^-/c-*src*527 clone 12). When the wild-type IGF-IR is used, these receptor numbers are more than sufficient to restore transformability to R^- cells (16, 65, 66, 72). Indeed, Rubini et al. (60) have shown that as few as 30,000 receptors per cell render mouse embryo fibroblasts capable of forming colonies in soft agar. As Table 2 shows, the ability of clones expressing the truncated receptor to form colonies in soft agar is the same as that of the parental cell lines, R^-/c-*src*527, clones 10 and 12, and clearly below the ability of W/c-*src*527 cells (25,000 wild-type receptors per cell) to form colonies in soft agar. Moreover, the diameter of the few colonies made by one clone of R^-/c-*src*527 cells expressing the truncated IGF-IR never exceeded 200 μm, while all the other transformed cell lines gave colonies of up to 500 μm in diameter.

DISCUSSION

There are several novel findings in these experiments. (i) v-*src* is the first oncogene shown to readily and fully transform mouse embryo cells with a targeted disruption of the IGF-IR genes, R^- cells. (ii) c-*src* activated by a mutation at residue 527 (32) can partially transform R^- cells, making them capable of growing in SFM and of obtaining a high saturation density. However, c-*src*527 is incapable of fully transforming R^- cells (focus formation or colony formation in soft agar), although both oncogenes can transform W cells, which have a physiological number of IGF-IRs. (iii) An IGF-IR truncated at residue 1229 (mitogenic, but nontransforming) is insufficient for transformation of mouse embryo cells by c-*src*527. (iv) IRS-1 and Shc are constitutively tyrosyl phosphorylated in R^- cells expressing *src*, whether v-*src* or c-*src*527. IRS-1 phosphorylation has already been reported by Peterson et al. (48), but in our experiments, it occurs also in cells without the IGF-IR, and like Shc phosphorylation, it does not correlate with the degree of transformation. (v) Although FAK is reduced in amount in all R^-/*src*-expressing cells, it is much more phosphorylated in R^-/v-*src* cells than in all other cells, R^-, R^+, or R^- expressing c-*src*527. (vi) Curiously, we could not detect a decrease in FAK amounts in W cells transfected with either v-*src* or c-*src*527, although in both cases it was equally and constitutively tyrosyl phosphorylated. (vii) Two other bands are also preferentially tyrosyl phosphorylated in v-*src*-transformed cells, and these

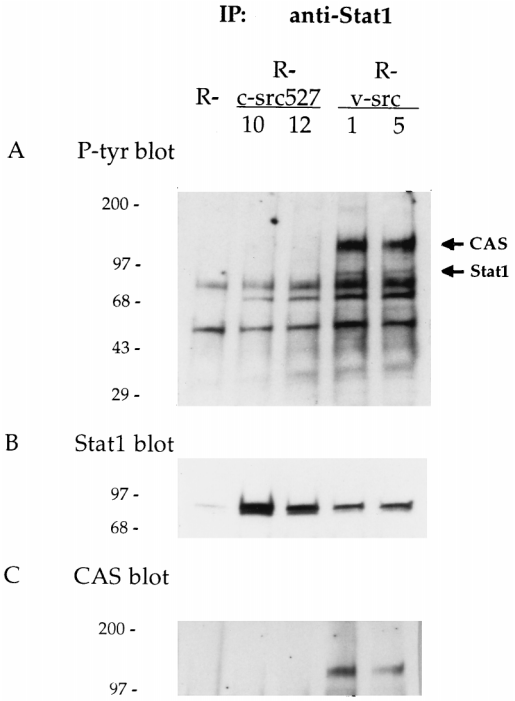

FIG. 10. Tyrosyl phosphorylation of Stat1. Lysates from the indicated cell lines were immunoprecipitated (IP) with an antibody to Stat1 and blotted with an antiphosphotyrosine antibody (A) or, after stripping, with anti-Stat1 antibody (B) or with an antibody to $p130^{cas}$ (C), as described in Materials and Methods. Numbers at left are molecular mass in kilodaltons.

bands have been identified as Stat1 and p130^{cas} (Fig. 10). These novel findings will be discussed separately.

As stated in the Introduction, R^- cells are refractory to transformation by a variety of oncogenes, and indeed, even in the present experiments, R^- cells could not be fully transformed by c-*src*527, which readily transformed W cells, with a physiological number of IGF-IRs, and which is known to be transforming in NIH 3T3 cells (32). Both R^- and W cells are mouse embryo fibroblast cell lines generated by the same protocol used to generate other 3T3 cell lines. Stable transfection with v-*src*, however, transformed R^- cells as fully and almost as efficiently as W cells, the first time we have observed that a viral or cellular oncogene can transform mouse embryo cells in the absence of the IGF-IR. It should be noted that Peterson et al. (49) failed to transform R^- cells (the same cell line from our laboratory) with the v-*src* they used, LA29. It is possible that this discrepancy may be due to the type of v-*src* used, but also (and more likely) to the differences in experimental conditions (we used stably transfected clones). Although in Table 2 we show only selected clones, other clones were also partially studied, and it was reproducibly found that v-*src* could transform R^- cells but that c-*src*527 could not. This is especially interesting because of the observation that the IGF-IR is constitutively autophosphorylated in *src*-transformed cells (33, 48). Our results are not necessarily at variance with those of Weber and coworkers, as will be explained below. The finding that v-*src* and c-*src*527 showed differences in the extent of their ability to transform R^- cells prompted us to investigate the mechanism(s) of this difference, and the present experiments are a first attempt to identify differential effects.

Constitutive tyrosyl phosphorylation of IRS-1 is not one of the differences between v-*src*- and c-*src*527-transfected R^- cells. IRS-1 is one of the known major substrates of the IR and IGF-IR (43, 59, 79). Confirming the results of Peterson et al. (48), IRS-1 is constitutively tyrosyl phosphorylated in v-*src*-transformed cells, but we find that it is also tyrosyl phosphorylated, constitutively, in c-*src*527-transfected R^- cells. The novel finding here is that IRS-1 is tyrosyl phosphorylated in the absence of the IGF-IR, which suggests a direct interaction of Src with IRS-1, without excluding a direct interaction also with the IGF-IR (48). Indeed, IRS-1 and Src coprecipitate in R^- cells transfected with either v-*src* or c-*src*527, confirming a direct interaction, as suggested by Peterson et al. (48). Interestingly, IRS-1 binds directly to at least one other oncogene, the simian virus 40 large-T antigen (84). Also novel is the demonstration that IRS-1 phosphorylation is independent of the transformed phenotype, since it is present in both v-*src* and c-*src*527 transfectants, and any difference in the extent of phosphorylation seems to be due more to clonal variation than to a difference between the two oncogenes. The activation of IRS-1 by both v-*src* and c-*src*527 is, however, consistent with the role of IRS-1 in mitogenesis (20, 59, 81), since both oncogenes stimulate the growth of R^- cells in SFM. Also consistent with the mitogenic aspect of this interaction are the reports that c-*src* interacts directly with the platelet-derived growth factor and EGF receptors (12, 23), which are mitogenic and transforming when overexpressed in other cells but are nontransforming in R^- cells (16, 21). These results are also consistent with the role of IRS-1 in transformation. Overexpression of IRS-1 causes transformation in 3T3 cells with a physiological number of IGF-IRs, and the expression of an antisense plasmid to IRS-1 causes reversal of the transformed phenotype. However, by itself, IRS-1 cannot transform R^- cells (20). In addition, v-*src* can transform 32D cells (34), and these cells are totally devoid of either IRS-1 or IRS-2 (76). Altogether, these experiments indicate that, although IRS-1 may play a role in transformation, this role is dependent on the presence of a functional IGF-IR.

Both v-*src* and c-*src*527 rendered R^- cells capable of growing in SFM, a remarkable finding, since the parental R^- cells cannot grow even in 1% serum, nor in SFM supplemented with a variety of growth factors (65, 66). Incidentally, in our system, we could not detect any evidence that *src* overexpression induced the secretion of growth-stimulating factors. The fact that c-*src*527 cannot fully transform R^- cells but can stimulate their growth in monolayers confirms once more that the IGF-IR system is more important for anchorage-independent growth than for growth in monolayers (8–10).

Tyrosyl phosphorylation of the Shc proteins (47) is markedly increased by *src* expression, confirming the results of McGlade et al. (40). Here again, the evidence that Shc may be involved in the ability of R^-/v-*src* cells to form colonies in soft agar has to be weighed against the evidence that overexpressed Shc proteins cannot transform R^- cells at all (11). Indeed, even BALB/c 3T3 cells cannot be transformed (colony formation in soft agar) by overexpressed Shc proteins (11). Our interpretation is that, at least with mouse embryo fibroblasts, IRS-1 and Shc are more important for mitogenesis than for transformation, although they may be required for the latter also (20).

A difference that stands out between R^-/v-*src* and R^-/c-*src*527 cells is the phosphorylation of FAK, a protein involved in several basic cellular processes (for a review, see reference 63). FAK amounts are decreased in both R^- derived cell lines, confirming the report by Fincham et al. (24) that v-*src* induces degradation of FAK. But FAK is clearly more phosphorylated (80) in v-*src*-transformed R^- cells than in c-*src*527 transfectants. This difference may also be related to the difference in p85 phosphorylation (54, 83). Interestingly, these differences disappear in W cells, regardless of whether the transforming agent is v-*src* or c-*src*527. In fact, in these cells, there is not even a decrease in the levels of FAK (Fig. 9), as if the presence of the IGF-IR abrogated the ability of Src to degrade FAK. Fincham et al. (24) used chick embryo fibroblasts, which do have IGF-IRs, so it is not clear why there is a discrepancy in FAK levels between our W cells and their chick embryo fibroblasts. A possible explanation can be found in a recent paper by Crouch et al. (18) suggesting that FAK proteolysis is inhibited by an antibody to an integrin, which protects chick embryo fibroblasts from c-*myc*-induced apoptosis; since the IGF-IR also protects against apoptosis, its presence may have a similar effect.

Another protein that is differentially tyrosyl phosphorylated has been identified as Stat1. Phosphorylated Stat proteins translocate to the nucleus, where they can activate transcription of a number of genes, especially cytokine-inducible genes (for a review, see reference 28). Our experiments show that Stat1 is detectable only in v-*src*-transformed R^- cells as a tyrosyl-phosphorylated protein, although its amount is actually higher in cells expressing c-*src*527. Stat1 also coprecipitates with Src, but in both types of cells; a report has already appeared that Src coprecipitates with Stat3 (14). Finally, another protein is differentially phosphorylated in R^-/v-*src* cells, and this protein has been identified as p130^{cas}. In one sense, the finding that p130^{cas} is involved in v-*src* transformation is not surprising, since this protein is known to associate with both Src and FAK (64, 75). What was less expected was the finding that, in our case, p130^{cas} was coprecipitated by antibody to Stat1, a result that, to our knowledge, has not been previously reported.

There are, therefore, some differences between R^-/v-*src* and R^-/c-*src*527 cells, in terms of both phosphorylation (FAK, Stat1, and p130^{cas}) and degree of transformation, and the

question that arises now is how these differences may be related to each other. We would like to consider here three major alternatives. The first hypothesis is based on our previous work with the transformation of R^- cells (16, 65, 72) and goes as follows: in terms of colony formation in soft agar (i.e., anchorage independence), an overexpressed IGF-IR and v-*src* are functionally equivalent, since both can transform R^- cells. Both the IGF-IR and v-*src* can activate (tyrosyl phosphorylate) IRS-1 and Shc, which are involved in mitogenesis through the Ras pathway (79). Since this is the mitogenic pathway of the IGF-IR, it is not surprising that it is also used by c-Src527, which stimulates the growth of R^- cells. But the IGF-IR also activates a transforming pathway, which is Ras independent (65) and can be mapped to the C-terminal domain of the receptor (27). An IGF-IR without the C terminus (the last 108 amino acids) is mitogenic but not transforming (27, 72), and in this respect, in R^- cells, it behaves exactly like c-Src527. Our first alternative is that the C terminus of the IGF-IR and v-Src send an additional signal, which is required for the transformation of mouse embryo fibroblasts and could be Ras independent (see also reference 6), a signal that is missing in R^- cells transfected with c-*src*527.

A second, equally plausible alternative is that there is a threshold for transformation, and, for instance, that the extent of tyrosyl phosphorylation is the determining factor. At a certain level of phosphorylation, cells may become partially transformed; at a higher level, they undergo full transformation. Indeed, v-Src is known to be a more potent tyrosine kinase than c-Src527 (32), an observation confirmed in this paper (Fig. 4). Our only objection to this interpretation is the demonstration that the truncated IGF-IR is incapable of restoring the full transformation phenotype to R^- cells transfected with c-*src*527. This receptor is mitogenic, and its activation of the IRS-1–Shc–Ras pathway is undistinguishable from that of a wild-type receptor (72). This result is significant, since c-*src*527 is fully transforming in W cells (25,000 wild-type receptors per cell) but nontransforming in R^- cells expressing the truncated receptor at levels ranging from 40 to 150,000 receptors per cell. This number of wild-type receptors is sufficient for full transformation of mouse embryo fibroblasts: Rubini et al. (60) have shown that 30,000 receptors per cell are enough for colony formation in soft agar.

There is a third alternative that is a compromise between the first two. The model is based on a recent paper by Yenush et al. (82), in which it was shown that the IR binds preferentially to the pleckstrin domain of IRS-1. However, when the IR is overexpressed, it also binds to the phosphotyrosine binding domain of IRS-1. It is possible that v-Src and c-Src527 may follow a similar pathway(s) but that they may have different effects on similar substrates.

In conclusion, our experiments show that v-Src, but not c-Src527, can bypass the requirement for a functional IGF-IR, which seems to be quasiobligatory for the transformation of mouse embryo fibroblasts (and other cell types). The use of the two *src* oncogenes in R^- cells, in combination with transforming and nontransforming mutants of the IGF-IR, offers another possibility of dissecting the mechanisms involved in *src*- and IGF-IR-mediated transformation.

ACKNOWLEDGMENT

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