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# The Baculovirus Anti-apoptotic p35 Protein Promotes Transformation of Mouse Embryo Fibroblasts\*

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The baculovirus p35 protein is a potent inhibitor of programmed cell death induced by a variety of stimuli in insects, nematodes, and mammalian cell lines. The broad ability of p35 in preventing apoptosis has led us to investigate its effect on mouse embryo fibroblasts *in vitro* and *in vivo*. For this purpose, we have used R<sup>-</sup> cells (3T3-like fibroblasts derived from mouse embryos with a targeted disruption of the insulin-like growth factor I receptor (IGF-IR) genes) and R508 cells (derived from R<sup>-</sup> and with 15 × 10<sup>3</sup> IGF-IRs per cell). Both cell lines grow normally in monolayer, but they do not form colonies in soft agar, and they are non-tumorigenic in nude mice. We show here that, in addition to its anti-apoptotic effect, p35 causes transformation of R508 cells, as evidenced by the following: 1) decreased growth factor requirements, 2) ability to form foci in monolayer and colonies in soft agar, and 3) ability to form tumors in nude mice. Since R<sup>-</sup> cells stably transfected with p35 do not transform, our observations suggest that in addition to its effect as an inhibitor of apoptosis, the baculovirus p35 protein has transforming potential that requires the presence of the IGF-IR. The possibility that these two properties could be separated was confirmed by demonstrating that R508 cells expressing another anti-apoptotic protein, Bcl-2, could not form tumors in nude mice.

The baculovirus gene product p35 is a potent suppressor of programmed cell death induced by different stimuli in insects, nematodes, and mammalian cell lines (1–11). The broad ability of p35 to block apoptosis induced by different signals in diverse organisms is generally considered to be due to inhibition of ICE,<sup>1</sup> ICH-1, ICH-2, CPP32, and the *Caenorhabditis elegans* CED-3 (12, 13).

It is now well established that the IGF-IR plays a crucial role in transformation and protects cells from apoptosis. Fibroblasts derived from mouse embryos with a targeted disruption of the IGF-IR genes (14, 15) cannot be transformed by a variety of viral and cellular oncogenes, such as SV40 large T antigen, an

activated Ha-*ras* or a combination of both (16, 17), by the E5 protein of the bovine papilloma virus (18), by an activated *c-src* (19), and by overexpressed growth factor receptors (reviewed in Ref. 20), all conditions that readily transform fibroblasts with wild-type levels of IGF-IR. Conversely, tumor cells with a down-regulation of the IGF-IR (by antisense strategies or by expression of dominant negative mutants) are no longer tumorigenic (21–26).

Overexpression of the IGF-IR protects cells from apoptosis (27–31), whereas a functional impairment of the IGF-IR (either by decreasing its number or by certain mutations) renders the cells more susceptible to undergo apoptosis (21–26, 30, 32). Although the mechanisms by which the targeting of the IGF-IR results in apoptosis remain to be elucidated, several reports have shown that IGF-I protects cells from apoptosis through activation of the phosphatidylinositol 3-kinase/Akt pathway (33–37). In addition, IGF-I can suppress ICE-mediated cell death in Rat-1 fibroblasts (38).

On this basis, we decided to investigate the relationship between these two anti-apoptotic proteins, p35 and the IGF-IR, using mouse embryo fibroblasts stably transfected with a plasmid coding for the baculovirus p35 protein derived from the *Autographa californica* nuclear polyhedrosis virus (Refs. 3–5 and 8–10). In this study, we evaluated the effects of the baculovirus p35 protein on mouse embryo fibroblasts *in vitro* and *in vivo*. We show that the expression of p35 in R508 cells, but not in R<sup>-</sup> cells, results in anchorage-independent growth. R508.p35 cells were able to form foci in monolayer and colonies in soft agar and to develop tumors in nude mice. In parallel experiments, R508 cells expressing another anti-apoptotic protein, Bcl-2, were not tumorigenic in nude mice, indicating that in mouse embryo fibroblasts transformation and protection from apoptosis can be separated. Our observations also indicate that, in addition to its effect as an inhibitor of apoptosis, the baculovirus p35 protein has a transforming potential that requires the presence of the IGF-IR.

## EXPERIMENTAL PROCEDURES

**Cell Lines**—R<sup>-</sup> cells are cells derived from mouse embryos with a targeted disruption of the IGF-IR genes (14, 15). These cells, totally devoid of IGF-IRs, have been previously characterized and described in detail (16, 17). R508 cells are R<sup>-</sup> cells stably transfected with pMRIG-FIR12 (39), a derivative of CVN-IGFIR that expresses both the hygromycin B phosphotransferase gene of *Escherichia coli* and the human IGF-IR cDNA under the control of the rat IGF-IR promoter. These cells have been previously characterized regarding the number of IGF-I binding sites, mitogenicity, and clonogenicity in soft agar (39).

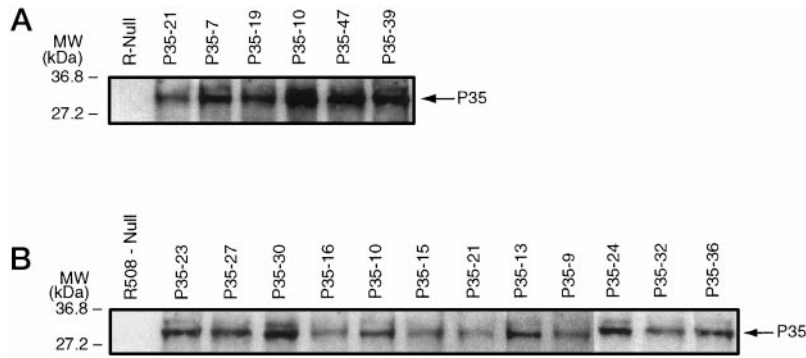
**Plasmids**—The plasmid pPRM-35KORF coding for the p35 protein derived from the *A. californica* nuclear polyhedrosis virus (3) was subcloned into the *Bam*HI site of the mammalian expression vector pcDNA (Invitrogen), as described previously (9). The plasmid encoding for human wild-type Bcl-2 subcloned in pcDNA was a kind gift of Dr. S. Haldar (Thomas Jefferson University).

**Stable Transfections**—R<sup>-</sup> and R508 cells were co-transfected with

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<sup>1</sup> The abbreviations used are: ICE, interleukin-1 $\beta$  converting enzyme; IGF-IR, insulin-like growth factor I receptor; IGF-I, insulin-like growth factor I; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; ICH-1, ICE homologue; poly-HEMA, poly-(2-hydroxyethylmethacrylate).



**FIG. 1. Characterization of cells expressing p35.** A, expression of p35 in R<sup>-</sup> cells was determined by Western blot analysis, as described under "Experimental Procedures." An empty vector clone together with representative clones expressing high levels of p35 are shown. A total of 6 empty vector clones and 48 clones expressing p35 were characterized. B, expression of p35 in R508 cells was determined by Western blot analysis, as above. A representative empty vector clone together with clones expressing different levels of p35 are shown. A total of 6 empty vector clones and 36 clones expressing p35 were characterized.

the plasmids encoding for p35, or Bcl-2, or the empty vector and a plasmid (pPDV6+) coding for the puromycin resistance gene (40). Puromycin-resistant clones, selected in medium containing 2.5  $\mu\text{g}/\text{ml}$  puromycin, were screened by Western blot analysis for expression of the baculovirus p35 protein (using a rabbit antiserum anti-p35, as described in Ref. 3) or of human Bcl-2 (using a monoclonal antibody anti-human Bcl-2 from Santa Cruz Biotechnology).

**Western Blot Analysis**—Proteins from cell lysates (20  $\mu\text{g}$ ) were separated in a 4–15% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After transfer, the filters were blocked for 1 h with 10% non-fat milk in PBS, 0.1% Tween 20 and incubated with the rabbit antiserum anti-p35 (1/5000) for 1 h at room temperature followed by a 1-h incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1/3000). Detection was performed using the ECL reagent (Amersham Pharmacia Biotech). Detection of human Bcl-2 was performed using a monoclonal antibody anti-human Bcl-2 followed by incubation with a goat anti-mouse antibody conjugated with horseradish peroxidase and the ECL reagent, as described above.

**Cell Growth**—The cells were seeded at a density of  $3 \times 10^4$  cells per 35-mm plate in DMEM supplemented with 5% fetal bovine serum and 5% calf serum. After 6 h, the cells were carefully washed and transferred to serum-free medium (DMEM supplemented with 0.1% bovine serum albumin fraction V (Sigma) and 0.1  $\mu\text{M}$  ferrous sulfate). Two days later, the quiescent cells were stimulated with 50 ng/ml IGF-I (Bachem), and the growth response was evaluated after 48 h by cell counting.

**Focus Formation**—For this assay, the cells were plated at a density of  $4 \times 10^3$  cells/cm<sup>2</sup>. After 14 days, when the cells became confluent and began to form foci, the cultures were fixed in methanol containing 0.5% crystal violet stain, as described previously (17).

**Anchorage-independent Growth**—Anchorage-independent growth was determined by the ability of the cells to form colonies in soft agar. Clonogenicity in soft agar was assayed by scoring the number of colonies formed in medium (DMEM supplemented with 5% fetal bovine serum and 5% calf serum) containing 0.2% agarose with a 0.4% agarose medium underlay. Cells were seeded at a density of  $10^3$  cells per 35-mm plate in duplicate. The number of colonies and their size were determined after 3 weeks.

**Tumorigenesis in Nude Mice**—The cells ( $10^6$  cells suspended in 0.1 ml of PBS, calcium- and magnesium-free) were injected subcutaneously above the hind leg of 7-week-old male Balb/c nude mice (Charles River Breeders). The animals were monitored daily for tumor development, and they were sacrificed following the development of bulky tumors. Three mice were used in each experimental condition.

**Survival in Poly-HEMA Plates**—Petri dishes (Falcon) were coated with a film of poly-(2-hydroxyethylmethacrylate) (poly-HEMA; Sigma), following the protocol reported by Folkman and Moscona (41). Cells were seeded at  $10^3/\text{ml}$ . Cell number was determined after 24 h; the results are expressed as percentage recovery of surviving cells from initial seeding.

**Statistics**—The data represent the mean of three independent determinations, each performed in duplicate. Statistical significance was determined by Student's *t* test.

## RESULTS

**Generation and Characterization of Cell Lines Expressing the Baculovirus p35 Protein**—Mouse embryo fibroblasts (3T3-like

cells), totally devoid of IGF-IRs (R<sup>-</sup> cells) or expressing  $15 \times 10^3$  IGF-IRs per cell (R508 cells), were stably co-transfected with the plasmids encoding for p35 or the empty vector and the puromycin resistance gene, as described under "Experimental Procedures." Puromycin-resistant clones, selected in 2.5  $\mu\text{g}/\text{ml}$  puromycin, were then screened for expression of p35 by Western blot analysis using a rabbit antiserum anti-p35 (3). The expression levels of p35 in representative clones of R<sup>-</sup> cells are shown in Fig. 1A, whereas for clones of R508 cells, the results are shown in Fig. 1B.

**Growth Characteristics of Cells Expressing p35**—R508 cells (untransfected or expressing the empty vector) are not able to grow in serum-free medium supplemented with 50 ng/ml IGF-I (Ref. 39 and Table I), although they remain viable under these conditions (Ref. 39 and see below). Expression of p35 resulted in growth in serum-free medium and increased mitogenic response to IGF-I stimulation (Table I).

As expected, in R<sup>-</sup> clones expressing p35 no mitogenic response to IGF-I stimulation was observed (Table I). However, it is also interesting that these cells do not grow in serum-free medium, suggesting a requirement for a functional IGF-IR in the growth stimulation by p35.

**Focus Formation in Monolayer**—R508 cells are contact inhibited in monolayer, even in the presence of 10% serum (Ref. 39 and Fig. 2A). R508 cells expressing the empty vector behaved as untransfected cells and were not able to form foci after 2 weeks in monolayer (Fig. 2B). Expression of p35 resulted in formation of multiple and large foci (Fig. 2, C and D). However, clones derived from R<sup>-</sup> cells, despite the high expression levels of p35, were not able to form foci in monolayer and remained contact inhibited as the controls (Fig. 2G). This suggested that, in order to transform mouse embryo fibroblasts, the baculovirus p35 requires the presence of the IGF-IR, as reported for other cellular and viral oncogenes (see Introduction). These observations were confirmed by determining the ability of these cells to form colonies in soft agar.

**Anchorage-independent Growth of Cells Expressing p35**—The ability of cells expressing p35 to grow under anchorage-independent conditions was evaluated by colony formation in soft agar. R508 cells, seeded at  $10^3$  cells per 35-mm plate, do not form colonies in soft agar (Ref. 39 and Table II). Clones expressing the empty vector behaved as untransfected R508 cells. Expression of p35 conferred clonogenic potential to R508 cells. The number of colonies and their size seemed to be dependent on the levels of expression of p35; clones expressing low levels of p35 were able to form an average of  $4 \pm 1$  colonies of 25  $\mu\text{m}$  in diameter, whereas clones expressing high levels of p35 formed an average of  $22 \pm 5$  colonies, ranging from 60 to 100  $\mu\text{m}$  in diameter (Table II and Fig. 3, C and D).



TABLE I  
Growth characteristics of R<sup>-</sup> and R508 cells expressing p35

The cells were seeded at a density of  $3 \times 10^4$ /cells per 35-mm plate in triplicate, as described under "Experimental Procedures." Cell number was determined 2 days after addition of IGF-I (50 ng/ml) to the medium.

Cell type	Cell number ( $\times 10^{-4}$ )	
	SFM	IGF-I
R508	$3.3 \pm 0.2$	$3.6 \pm 0.3$
R508-Null-1	$3.6 \pm 0.2$	$3.7 \pm 0.2$
R508-35-23	$6.6 \pm 0.1$	$8.0 \pm 0.1$
R508-35-27	$6.5 \pm 0.1$	$8.5 \pm 0.2$
R <sup>-</sup>	$2.2 \pm 0.3$	$2.1 \pm 0.2$
R <sup>-</sup> 35-39	$3.3 \pm 0.2$	$3.5 \pm 0.2$
R <sup>-</sup> 35-47	$3.2 \pm 0.2$	$3.4 \pm 0.2$

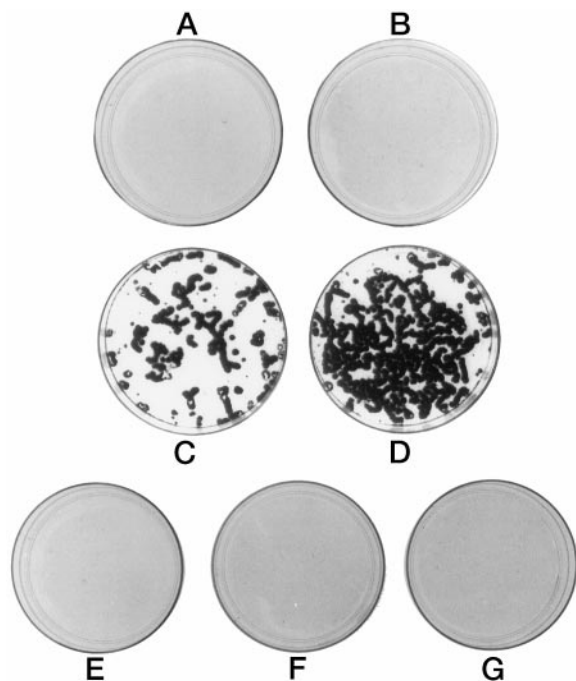


FIG. 2. **Focus formation assay.** This assay was done as described under "Experimental Procedures." A, R508 untransfected cells. B, a representative clone of R508 cells expressing the empty vector. C and D, 2 representative clones of R508 cells expressing p35. E, R<sup>-</sup> untransfected cells. F, a representative clone of R<sup>-</sup> cells expressing the empty vector. G, a representative clone of R<sup>-</sup> cells expressing high levels of p35.

The absence of the IGF-IR prevented the transforming potential of p35 in R<sup>-</sup> cells. Clones of R<sup>-</sup> cells expressing high levels of p35 were unable to form colonies in soft agar, although single cells remained viable for at least 3 weeks after seeding (Fig. 3B).

**Tumorigenesis in Nude Mice**—The ability to form tumors in nude mice is the most rigorous test for transformation of cells in culture. We therefore tested R508 cells expressing p35 for their ability to form tumors when injected subcutaneously in nude mice. R508 cells expressing p35 developed subcutaneous tumors, which became palpable 10 days after injection of  $10^6$  cells (Fig. 4A). The controls receiving  $10^6$  R508 cells expressing the empty vector remained tumor-free for as long as followed (40 days, not shown). Similarly, R<sup>-</sup> cells stably transfected with p35 did not produce palpable tumors when injected into nude mice (not shown).

**Bcl-2 Protects from Apoptosis but Does Not Transform R508 Cells**—We then asked whether transformation and protection from apoptosis were two aspects of the same phenomenon or whether they could be dissociated. To elucidate this, we decided

TABLE II  
Anchorage-independent growth of R508 cells expressing p35

Anchorage-independent growth was determined by colony formation in soft agar. For this purpose, R508 cells and its derivative clones were seeded at a density of  $10^3$  cells per 35-mm plate as described under "Experimental Procedures." The number of colonies and their size were determined after 3 weeks.

Cell type	Clonogenicity in soft agar	
	No. of colonies	Average size $\mu\text{m}$
R508	0; 0	
R508-Null-1	0; 0	
R508-Null-2	0; 0	
R508-p35-23	20; 18	80
R508-p35-27	22; 24	80
R508-p35-30	21; 27	80
R508-p35-24	13; 16	50
R508-p35-32	16; 10	50
R508-p35-36	12; 14	50
R508-p35-9	10; 12	40
R508-p35-10	9; 11	40
R508-p35-13	8; 9	40
R508-p35-15	5; 4	25
R508-p35-16	4; 3	25
R508-p35-21	2; 4	25

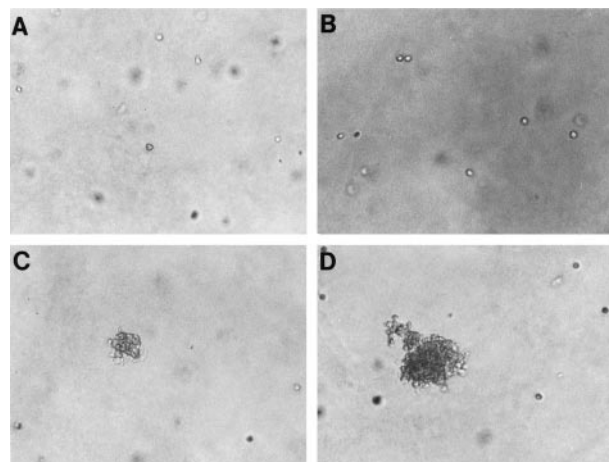
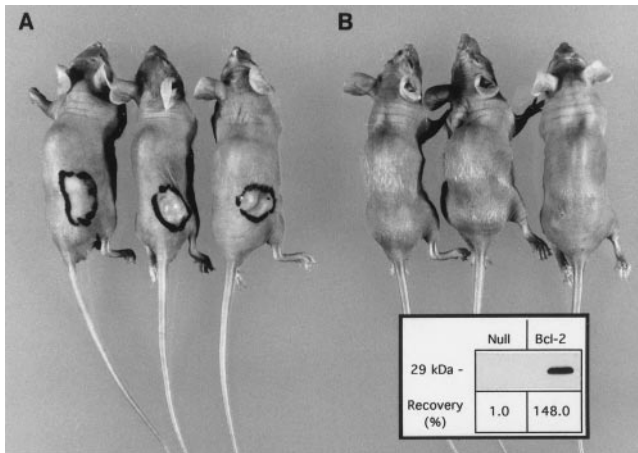


FIG. 3. **Clonogenicity in soft agar.** Microphotographs of soft agar assays are shown. A, R<sup>-</sup> cells. B, a representative clone in R<sup>-</sup> cells expressing high levels of p35 (clone 47). C and D, 2 representative clones of R508 cells expressing p35 (clones 15 and 24).

to express another anti-apoptotic protein in R508 cells, such as Bcl-2, which is a potent inhibitor of apoptosis, and it is known to lack transforming ability (42–45). A plasmid coding for human Bcl-2 was then co-transfected with a plasmid coding for the puromycin resistance gene, and puromycin-resistant clones were screened for Bcl-2 expression by Western blot. Clones expressing Bcl-2 were then tested for survival in poly-HEMA plates and tumorigenesis in nude mice. The results shown in Fig. 4 demonstrate that R508 cells expressing human wild-type Bcl-2, although fully protected from apoptosis (see Fig. 4, inset), do not form tumors in nude mice (Fig. 4B).

#### DISCUSSION

The novel finding in this report is that the baculovirus p35 protein, besides its well established ability to protect cells from apoptosis, has a previously unidentified property, which is its ability to induce a transformed phenotype in mouse fibroblasts. Our conclusion that p35 has transforming potential, above and beyond its anti-apoptotic activity, is supported by the following evidence: 1) R508 cells expressing p35 were able to grow in serum-free medium and in serum-free medium supplemented only with IGF-I (Table I), thus displaying reduced growth fac-



**FIG. 4. Tumorigenesis in nude mice.**  $10^6$  cells suspended in 0.1 ml of PBS, calcium- and magnesium-free, were injected subcutaneously above the hind leg of 7-week-old male Balb/c nude mice. Three mice were used in each experimental condition. *A*, mice injected with R508 cells expressing p35. *B*, mice injected with R508 cells expressing human wild-type Bcl-2. *Inset: top*, expression levels of human Bcl-2 detected by Western blot analysis, as described under "Experimental Procedures." Representative empty vector and Bcl-2 clones are shown. A total of 3 clones expressing human Bcl-2 were characterized. *Bottom*, survival in poly-HEMA plates was determined as described under "Experimental Procedures." Representative clones expressing the empty vector and human Bcl-2 are shown. A total of 3 clones expressing Bcl-2 were analyzed.

tor requirements; 2) they were able to form foci in monolayer (Fig. 2) and colonies in soft agar (Table II and Fig. 3, *C* and *D*); and 3) they developed tumors in nude mice (Fig. 4*A*). None of these properties was shared by the parental cell line R508 or by cells expressing the empty vector. The transforming potential of p35 required the presence of the IGF-IR for p35 clones derived from R<sup>-</sup> cells remained contact inhibited when grown in monolayer, even in the presence of 10% serum (Fig. 2*G*). Moreover, they were not able to grow under anchorage-independent conditions (Table II and Fig. 3*B*) nor develop tumors in nude mice (data not shown). These observations are in agreement with previous reports showing that R<sup>-</sup> cells cannot be transformed by a variety of other viral oncogenes, such as SV40 large T antigen, an activated Ha-*ras*, or a combination of both (16, 17), or by the E5 protein of the bovine papilloma virus (18), or by an activated *c-src* (19) all of which can readily transform cells with wild-type levels of IGF-IR.

We believe that the transforming and anti-apoptotic activities of p35 can be dissociated for the following reasons: 1) R508 cells are not able to grow in serum-free medium supplemented solely with 50 ng/ml IGF-I (Ref. 39 and Table I), but they remain viable under these conditions and do not undergo apoptosis (Ref. 39 and data not shown). Expression of p35 allows R508 cells to respond to IGF-I stimulation (Table I), decreasing the requirements for growth factors (a characteristic of transformed cells), under conditions in which the parental cell line does not undergo apoptosis; 2) R508 cells expressing p35 are transformed, but R<sup>-</sup> cells expressing p35 are not (Figs. 2 and 3*B*); and 3) most important, R508 cells expressing another anti-apoptotic protein, such as Bcl-2, are protected from apoptosis (Fig. 4, *inset*), but they are not able to form tumors in nude mice (Fig. 4*B*).

The mechanisms by which the baculovirus p35 protein induces transformation of mouse embryo fibroblasts remain to be elucidated. The use of mouse embryo fibroblasts totally devoid of IGF-IRs has allowed us to dissociate the effects of p35 on cell survival and transformation. The baculovirus p35 protein requires the presence of the IGF-IR in order to transform mouse

embryo fibroblasts, as shown for a variety of other viral oncogenes (16–18). One possible mechanism could be due to cooperation between IGF-IR and p35; the hypothesis we favor is that the IGF-IR transmits a permissive signal that allows p35 and other viral oncogenes to transform mouse embryo fibroblasts. To date, the only viral oncogene shown to bypass the requirement of IGF-IR for transformation is *v-src* (19). Therefore, we can conclude that the mechanism by which the baculovirus p35 protein induces transformation of mouse embryo fibroblasts is different from the one induced by *v-src*. One possibility that is attractive is that p35 may up-regulate the IGF system. Experiments along these lines are in progress.

We believe that the transforming ability of p35 is not simply due to caspase inhibition. Our hypothesis is supported by another report in the literature (46) showing that peptide inhibitors of ICE-related proteases delay but do not prevent cell death in Rat-1 fibroblasts following etoposide treatment, Bak expression, *c-myc* activation, or E1A expression. In the presence of these inhibitors, the cells are not able to divide or to form colonies in soft agar (46). Therefore, the transforming ability of p35 observed in the presence of the IGF-IR cannot be explained only by its effect as a caspase inhibitor and should be due to an additional activity of p35.

Similar observations have been reported for SV40 large T antigen. It can suppress ICE-mediated cell death (47), but it still requires the presence of the IGF-IR in order to transform mouse embryo fibroblasts (16, 17). In addition, SV40 large T antigen is known to transcriptionally activate the IGF-I promoter and induce production of IGF-I (48). A similar effect could be postulated for the baculovirus p35 protein. Future experiments, requiring appropriate mutants of p35, in which the anti-apoptotic domain can be dissociated from the transforming domain, will address this issue.

In summary, in addition to its effect as an inhibitor of apoptosis, we now report for the first time that the baculovirus p35 protein has transforming potential that requires the presence of the IGF-IR.

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