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Failure of the Bovine Papillomavirus To Transform Mouse Embryo Fibroblasts with a Targeted Disruption of the Insulin-Like Growth Factor I Receptor Genes

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Mouse embryo cells with a targeted disruption of the insulin-like growth factor I receptor (IGF-IR) genes (R^- cells) are refractory to transformation by the simian virus 40 large T antigen and/or an activated and overexpressed Ras, both of which readily transform cells from wild-type littermate embryos and other 3T3-like cells. R^- cells are also refractory to transformation induced by overexpressed epidermal growth factor receptor and platelet-derived growth factor receptor β. Since the platelet-derived growth factor receptor β is required for transformation by bovine papillomavirus, we inquired whether the IGF-IR was also required for transformation by bovine papillomavirus E5 oncoprotein. We show here that R^- cells are refractory to transformation by E5; reintroduction into R^- cells of a human IGF-IR restores the susceptibility to transformation.

R^- cells (35, 36) are 3T3-like fibroblasts originating from mouse embryos with a targeted disruption of the insulin-like growth factor I receptor (IGF-IR) genes (2, 21). We have previously reported that R^- cells are refractory to transformation by the simian virus 40 (SV40) large T antigen, by an activated and overexpressed Ras protein, or by a combination of both (35, 36), factors that readily transform W cells (i.e., cells originating from wild-type littermate mouse embryos). Reintroduction into R^- cells of a wild-type (but not a mutant) human IGF-IR results in abrogation of the growth deficits of R^- cells, including their resistance to transformation, indicating that the growth and transformation phenotypes of R^- cells are due to the absence of the IGF-IR.

DiMaio and coworkers (25–27) and Meyer et al. (23) have shown that the E5 transforming protein of the bovine papillomavirus (BPV) interacts with the platelet-derived growth factor receptor (PDGFR) β and phosphorylates it and that this interaction is crucial to the E5 transforming process, since BPV cannot transform cells with no PDGFR β molecules. Overexpression of the PDGFR β also causes ligand-dependent transformation (15, 42); however, we recently showed that an overexpressed PDGFR β fails to transform R^- cells, this failure being again promptly corrected by the reintroduction of a wild-type human IGF-IR (12). We therefore examined whether the BPV E5 transforming protein could still transform mouse embryo cells in the absence of a functional IGF-IR.

MATERIALS AND METHODS

Cell cultures. Mouse embryo fibroblast cell lines from embryos with a targeted disruption of the IGF-IR genes (2, 21) and from their wild-type littermates have been described by Sell et al. (35, 36). R^- and W cells were both obtained by a 3T3-like protocol. p6 cells are BALB/c 3T3 cells overexpressing the IGF-IR (28). All cell lines were passaged in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Plasmid transfections. Both R^- and W cells were transfected with the Transfectam reagent (Promega Corp.) in serum-free Dulbecco modified Eagle medium. Plasmid pBPV-A6H, containing the wild-type BPV genome (a kind gift of Dan DiMaio, Yale University), was digested with *Bam*HI. The 8-kb viral fragment was purified and cotransfected with plasmid pPDV6+, expressing the puromycin resistance gene (12, 13). Clones were selected in medium containing 2 μg of puromycin per ml. The plasmids used to express the human IGF-IR were CVN Igf-Ir (40), containing the full-length coding sequence of the human IGF-IR cDNA and the neomycin resistance gene, both under the control of the SV40 early promoter, and pBPV-IGF-IR, containing the full-length coding sequence of the human IGF-IR cDNA under the control of the murine sarcoma virus enhancer and the metallothionein I promoter (17). pBPV-IGF-IR was cotransfected with plasmid pSV2-Neo, expressing the neomycin resistance gene. All clones stably transfected with the human IGF-IR were selected in medium containing 800 μg of neomycin per ml.

Antiserum, immunoprecipitation, and Western blot (immunoblot) analysis. The E5 protein was immunoprecipitated with αE5, a rabbit antiserum raised to the 16 C-terminal amino acids of the BPV E5 protein (5), also a kind gift of Dan DiMaio. Cell lysates were prepared in 1 ml of cold radioimmunoprecipitation assay buffer (20 mM morpholinepropanesulfonic acid [MOPS], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride) and cleared of cell debris by spinning in a microcentrifuge for 5 min. Five hundred-microgram aliquots of lysates from R^- and W transfected and nontransfected cells were immunoprecipitated with 5 μl of αE5 antiserum overnight at 4°C with rotation; 20 μl of protein A-agarose beads (Oncogene Science) was then added to the mixture, which was rotated for 1.5 h at 4°C. The beads were pelleted and washed four times with cold radioimmunoprecipitation assay buffer, and the immune complexes were resuspended in Laemmli sample buffer with 5% β-mercaptoethanol and 100 mM dithiothreitol. After boiling for 5 min, the samples were electrophoresed on an SDS–15% polyacrylamide gel and electroblotted on an Immobilon membrane. The filter was blocked for 1 h at room temperature in 5% nonfat milk–TNET buffer (10 mM Tris [pH 7.5], 2.5 mM EDTA, 50 mM NaCl, 0.1% Tween 20) and incubated for 2 h in 1:500-diluted αE5 antiserum at room temperature. Bound antibodies were detected by the appropriate protein A-horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence reagent (Amersham).

Transformation assay. Anchorage-independent growth and focus formation were analyzed as previously described (35, 36). One thousand cells were seeded in soft agar, and the colonies (>125 μm) were counted after 3 weeks in culture.

Determination of receptor numbers. The number of IGF-IRs was determined by IGF-I binding assays, using monoiodinated [^{125}I]IGF-I as already described (24), while the number of PDGFR β molecules was determined by Scatchard analysis, using the ligand replacement protocol as previously described (12).

RESULTS

W and R^- cells have similar numbers of PDGFRs but differ greatly in the number of IGF-IRs (Table 1), with the R^- cells showing as before no binding sites for IGF-I (11, 35, 36). Both cell lines were cotransfected with the BPV genome and the puromycin resistance gene as described in Materials and Methods, producing R^-/BPV and W/BPV cells.

Clones growing in puromycin were screened by PCR for the presence of the BPV genome (not shown), and selected clones

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TABLE 1. Number of receptors in W and R^- cells

Cell line	Receptor	No. of receptors (10^4)[a]
W	IGF-IR	3
	PDGFR	3.6
R^-	IGF-IR	0
	PDGFR	3.6

[a] Determined as described by DeAngelis et al. (12) in unstimulated cells in serum-free medium.

were then examined for expression of E5. Results for three of the W clones selected are shown in Fig. 1, lanes 2 to 4; clones 4, 5, and 17 clearly express substantial amounts of E5 protein, while the parental cells (lane 1) are negative. Results for four of the selected R^- clones (lanes 6 to 9) and the parental cell line (lane 5) are also shown. R^-/BPV clones 9 and 10 show levels of E5 expression that are comparable to those of the three W/BPV clones. R^-/BPV clones 2 and 4 show lower levels of expression.

These same clones were then tested for focus formation in monolayers and for colony formation in soft agar. Figure 2 shows representative plates from four different cell types. The parental cells, W and R^-, being 3T3-like cells, do not form foci in monolayer (Fig. 2, plates A and C). R^-/BPV cells also fail to form foci (plate D), while W/BPV cells form clearly detectable foci (plate B).

The inability of BPV to transform R^- cells was confirmed by the soft agar assay. The W/BPV clones all formed 30 to 50 colonies in soft agar (Table 2), fewer than the number produced by p6 cells (grossly overexpressing the IGF-IR) but nevertheless substantial. Four different clones of R^-/BPV failed to form colonies in soft agar (Table 2), although clones 9 and 10 expressed levels of E5 protein similar to those expressed by the transformed W/BPV clones.

In previous reports, we have shown that the resistance of R^- cells to transformation by SV40 large T antigen (36), by an activated Ras (35), by an overexpressed epidermal growth factor (EGF) receptor (11), or by an overexpressed PDGFR (12) was abrogated if the cells were stably transfected with a human wild-type IGF-IR. We selected clone 4 of the R^-/BPV cells and transfected it with plasmid CNV Igf-Ir (see Materials and Methods), coding for a full-length human IGF-IR. Two clones, 4-5 and 4-7, were selected, and these two clones could now form colonies in soft agar (Table 2). The number of colonies was somewhat less than in W/BPV cells, but these new clones were derived from cells that had a lower level of E5 protein expression. We therefore repeated the experiment with R^-/BPV clone 2, which expresses more E5 protein although still somewhat less than W/BPV cells. Two clones now expressing the human IGF-IR cDNA were selected, and these clones formed many colonies in soft agar (Table 2). Since the wild-type IGF-IR, overexpressed, can transform by itself, one can raise the criticism that these clones now form colonies in soft agar through the receptor rather than the E5 protein. We tested three clones for IGF-IR levels by Scatchard analysis;

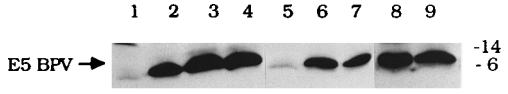

FIG. 1. Expression of E5 BPV protein in W and R^- cells. E5 BPV protein was immunoprecipitated as described in Materials and Methods. Lanes: 1, W cells; 2 to 4: W/BPV clones 4, 5, and 17, respectively; 5, R^- cells; 6 to 9: R^-/BPV clones 2, 4, 9, and 10, respectively.

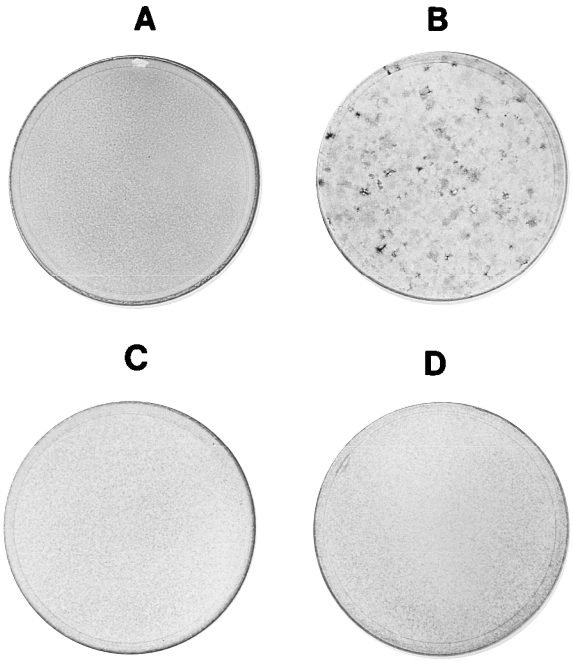

FIG. 2. Focus formation in W/BPV and R^-/BPV cells. The assay was performed as described in Materials and Methods. (A) W cells; (B) W/BPV cells; (C) R^- cells; (D) R^-/BPV cells.

clone 4-7 had 8,000 receptors per cell, and clone 2-1 had 25,000. Both of these numbers are within the range of IGF-IR levels for 3T3 cells and W cells, which do not form colonies in soft agar. Clone 2-3 had 130,000 receptors per cell and yet did not form more colonies than clone 2-1.

DISCUSSION

Overexpression or constitutive activation of the IGF-IR in a variety of cell types leads to ligand-dependent growth in se-

TABLE 2. Growth in soft agar of W/BPV and R^-/BPV cells[a]

Cell line	No. of colonies in soft agar
W (wild type)	0, 0
W/vector alone	0, 0
W/BPV	
Clone 4	39, 29
Clone 5	40, 53
Clone 17	25, 32
R^-	0, 0
R^-/vector alone	0, 0
R^-/BPV	
Clone 2	0, 0
Clone 4	0, 0
Clone 9	0, 0
Clone 10	0, 0
R^+/BPV	
Clone 4-5	17, 26
Clone 4-7	18, 19
Clone 2-1	250, 255
Clone 2-3	185, 180
p6	150, 164

[a] One thousand cells were seeded, and the colonies were counted after 3 weeks. p6 cells were used as a positive control. Results from two assays are presented.

rum-free medium and to the establishment of a transformed phenotype (11, 16, 20, 22, 35). Conversely, 3T3-like mouse embryo cells with a targeted disruption of the IGF-IR genes (2, 21) are refractory to transformation by SV40 T antigen, an activated Ras, or a combination of both, factors that easily transform cells from wild-type littermate embryos or other 3T3 cells (35, 36). The important role of the IGF-IR in transformation is also supported by other findings, indicating that antisense oligodeoxynucleotides or antisense expression plasmids against either IGF-II (7), IGF-I (38, 39), or the IGF-IR (4, 31, 32, 36), antibodies to the IGF-IR (1), and dominant negative mutants of the IGF-IR (19, 30) can all reverse the transformed phenotype and/or inhibit tumorigenesis.

The original purpose of this investigation was purely to examine whether, in addition to SV40 T antigen and activated Ras, another oncoprotein would fail to transform R^- cells. The rationale for this inquiry has been discussed in a recent review by Baserga (3), where it was pointed out that viral and nonviral oncogenes often use growth factors and their receptors to implement their transforming potential. Examples include the interaction of the gp55 glycoprotein of Friend spleen focus-forming virus (44, 45), the association of polyomavirus middle T antigen with Shc (6, 14) and phosphatidylinositol 3-kinase (43), and the induction of IGF-I by SV40 T antigen (29). For this study, we chose the E5 oncoprotein of BPV because of its close association with the PDGFR β and the requirement of this receptor for transformation (25–27). As mentioned above, we (12) have shown that the mitogenic and transforming activities of an overexpressed PDGFR β were blocked in R^- cells, indicating that the PDGFR β requires a functional IGF-IR for its growth-promoting activities.

Our present results are a confirmation of both the studies by DiMaio and coworkers (25–27) and our own. W and R^- cells have similar numbers of PDGFRs, but only W cells can be transformed by the E5 oncoprotein of BPV, again indicating that the PDGFR, even when directly activated by an oncoprotein, still needs a functional IGF-I system.

The mechanism is probably multifactorial and is not well understood, but one mechanism by which the IGF-IR regulates the growth-promoting potential of the PDGFR can be pointed out. It has been known for several years that PDGF increases the secretion of IGF-I and also increases the number of IGF-I binding sites on cell surfaces (8–10, 41). More recently, it has been reported that PDGF (and EGF) activate the IGF-IR promoter (33) as well as the IGF-I promoter (18). Thus, while the activated PDGFR undoubtedly has several different actions, one of them is the activation of the IGF-I autocrine loop. The results do not imply any hierarchical differences: just as the PDGFR needs a functional IGF-IR, the IGF-IR is nonmitogenic unless the cell has been previously primed with PDGF (and/or EGF [34, 37]).

It is also interesting that the number of colonies in soft agar, in cells expressing both E5 and the wild-type IGF-IR, is proportional to the levels of E5 protein expression.

Our data emphasize again the importance of the IGF-IR in transformation (see above). We can now say that R^- cells are refractory to transformation by SV40 large T antigen (36), by an activated and overexpressed Ras (35), by BPV (this report), and by overexpressed PDGFR (12) and EGF receptor (11). Clearly, the requirement for a functional IGF-IR extends to more than one oncogene and, coupled with the reversal of the transformed phenotype in tumor cells with a decreased IGF-IR function (see above), again implicates this receptor as a key component in the transformation process.

In conclusion, our findings that the E5 oncoprotein, while activating the PDGFR, requires a functional IGF-IR for transformation point out an interesting cross talk among growth factor receptors and between them and viral oncogenes.

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