Mutagenesis of the Avian Erythroblastosis Virus *erbB* Coding Region: An Intact Extracellular Domain Is Not Required for Oncogenic Transformation

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Avian erythroblastosis virus (AEV) is an oncogenic retrovirus of birds. The AEV-encoded *erbB* polypeptide, a transmembrane glycoprotein bearing an N-terminal domain exposed on the surface of virally transformed cells, plays a crucial role in AEV-mediated oncogenesis. We report here a characterization of a mutated form of the AEV *erbB* protein which lacks over two-thirds of the extracellular region of this oncogenic protein. This mutant *v-erbB* protein, although lacking the three possible extracellular sites of N-linked protein glycosylation, appears unimpaired in the ability to transform cells to an oncogenic phenotype.

The *v-erbB* oncogene confers on the avian erythroblastosis retrovirus (AEV) the ability to induce fibrosarcomas and erythroblastosis in susceptible birds and to transform both fibroblasts and erythroid progenitor cells in culture to an oncogenic state (1, 2, 5, 6, 11, 17, 20, 26, 30). The protein product of the *v-erbB* locus is a glycosylated transmembrane protein possessing tyrosine-directed protein kinase activity (8, 13, 14, 16, 21-23, 29). We were interested in relating the structural and biochemical features of the *v-erbB* protein to its oncogenic properties. The possible role of the glycosylated extracellular domain of the *v-erbB* protein is of particular interest. What is the significance of the retention of a short extracellular domain in the *v-erbB* protein relative to the presence of a much larger extracellular domain found in the cellular progenitor of this viral oncogene, the epidermal growth factor (EGF) receptor (3, 4, 18, 27)? Is glycosylation of this region of the *v-erbB* protein essential to its function? Is the presence of an extracellular domain suggestive of a novel mechanism of oncogenesis operating in *v-erbB*-transformed cells? We report here the creation and analysis of a mutated form of the *v-erbB* protein which bears a deletion of over two-thirds of the extracellular domain and lacks the associated sites of N-linked protein glycosylation.

Generation of an N-terminal deletion mutant of *v-erbB*. A plasmid subclone, pAE-SalRI, of the coding domain for the AEV *erbB* protein was isolated from the molecularly cloned AEV strain ES-4 genome (28) (Fig. 1A). The region between an *StuI* restriction site (located immediately after the first base of codon 15 of the *v-erbB* coding region) and a *ThaI* restriction site (located immediately after the first base of codon 67) was deleted by *StuI* to *ThaI* restriction endonuclease cleavage and religation (29). This deletion removed over two-thirds of the *v-erbB* extracellular domain, but preserved the extreme N terminus and the transmembrane domains of the *v-erbB* polypeptide. The pAE-SalRI *StuI* to *ThaI* deletion was subsequently reconstructed into pAEV-11-3R, an infectious molecular clone representing the entire AEV genome (Fig. 1A). The AEV genomic plasmid bearing the *StuI* to *ThaI* deletion, pAEV-delS/T (referred to as delS/T), was introduced into chicken fibroblasts by a calcium phosphate coprecipitation technique (26). A molecular clone of the Rous-associated virus type 1 genome (RAV-1) was

included in the transfection as a helper virus to complement the replication-defective AEV genome and permit integration and replication (25, 26).

AEV encoding the deleted *v-erbB* protein retained the ability to transform fibroblasts to an oncogenic state. Chicken fibroblasts transfected by the RAV-1 helper alone, by the helper virus plus the delS/T mutant, and by the helper virus plus the wild-type AEV plasmid were propagated and were examined for morphological alteration. After four (1:5) passages in culture, fibroblasts transfected by either the delS/T mutant or by the wild-type AEV plasmid developed a distinctive spindle-shaped, highly refractile appearance and grew in a crisscrossed and disorganized pattern (Fig. 2 middle and bottom) characteristic of fibroblasts infected by wild-type AEV (11, 24). In contrast, fibroblasts exposed to the RAV-1 plasmid alone exhibited no visible change in morphology, remaining in flat organized monolayers and retaining an appearance virtually identical to that of uninfected cells (Fig. 2 top).

We next tested the ability of fibroblasts transfected by the delS/T mutant to grow in soft agar. Anchorage-independent growth is a fairly stringent test of oncogenic transformation. Fibroblasts transformed by a number of different agents (including wild-type AEV) can propagate in soft agar me-

TABLE 1. Transformation parameters of chicken embryo fibroblasts transfected by the AEV delS/T mutant^a

Transfection agent	Colonies in soft agar (>1 mm diam/0.5-1 mm diam) ^b	Hexose uptake (cpm) ^c	Caseinolytic plaques ^d
RAV-1 only	0 (0)	1,350	0
RAV-1 + AEV delS/T mutant	42 (>200)	11,400	37
RAV-1 + AEV wild type	36 (>200)	9,000	51

^a Each data point represents the average of two independent experiments.

^b In the assay for anchorage-independent growth, fibroblasts were trypsinized, counted, and plated into soft agar medium as previously described (26). The plates were scored after a 2-week incubation at 39°C. Soft agar colony-forming efficiency ranged from 2 to 4% for both the wild-type AEV and AEV delS/T mutant transfected cells.

^c Hexose uptake was determined by the method of Royer-Pokora et al. (24).

^d Plasminogen-activator protease secretion was assayed by the casein-agarose method described previously (9, 19, 24).

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FIG. 1. (A) Plasmids used in construction of the delS/T mutant. pAEV-11-3R is an infectious DNA clone of the entire wild-type AEV genome. The long terminal repeat (LTR), deleted *gag*, *v-erbA*, and *v-erbB* coding regions are shown. pAE-SalRI is a plasmid subclone of the 2.3-kilobase *Sal*I to *Eco*RI fragment from the pAEV-11-3R plasmid, which consists largely of a *v-erbB* sequence. The N-terminal *v-erbB* deletion described in the text and below was initially created in the pAE-SalRI subclone. The mutated pAE-SalRI plasmid was subsequently cleaved by *Sal*I and *Eco*RI restriction endonucleases, and the resulting *Sal*I to *Eco*RI fragment (bearing the *v-erbB* mutation) was used to replace the equivalent wild-type sequences in the infectious pAEV-11-3R molecular clone, yielding the infectious mutant clone pAEV-delS/T. *Sal*, *Sal*I; *RI*, *Eco*RI. (B) Construction of the deletion. A deletion was generated between the *Stu*I site and the *Tha*I site within the *v-erbB* gene by appropriate restriction endonuclease cleavage and religation, as shown and as described in the text. The resulting deletion removes 156 base pairs of DNA sequence and 52 amino acid codons in the extracellular N-terminal domain of the *v-erbB* protein. The protein sequences (uppercase, standard one-letter designations) of the N termini of the wild-type and delS/T *v-erbB* proteins are presented for comparison. The predicted protein sequence is shown, starting at the first methionine codon within the *v-erbB*-specific oncogene sequence. The nucleic acid sequences (lowercase letters) immediately surrounding the restriction sites used in the construction are also shown. cho, Potential sites of N-linked protein glycosylation; transmembrane, hydrophobic transmembrane domain; n-, putative N terminus of the *v-erbB* protein.

dium and give rise to macroscopic colonies, whereas normal fibroblasts cannot (19, 24–26). Our results are shown in Table 1 and Fig. 3. Fibroblasts transfected by the helper virus alone yielded no visible colonies in the soft agar assay. Fibroblasts transfected by an AEV mutant carrying a non-sense codon in the *v-erbB* gene also failed to give rise to colonies in this assay, confirming the requirement of a functional *v-erbB* protein for AEV-induced fibroblast transformation (data not shown). In contrast, cells bearing the delS/T mutation in the *v-erbB* gene gave rise to fibroblast colonies indistinguishable in morphology and number from

those arising from the wild-type AEV plasmid (Table 1 and Fig. 3 middle and bottom).

Fibroblasts infected by wild-type AEV are known to exhibit several additional parameters of oncogenic transformation, including an elevated uptake of hexoses and an increased secretion of plasminogen-activator protease, when compared with untransformed cells (19, 24). Fibroblasts transfected by the AEV delS/T mutant were therefore assayed for these additional manifestations of the transformed state (Table 1). Fibroblasts transfected by the delS/T mutant demonstrated significantly elevated levels of 2-deoxyglucose

uptake, equal to or greater than the levels seen for the wild-type virus and many times higher than the levels seen in fibroblasts transfected with the RAV-1 helper alone. Similarly, levels of plasminogen-activator protease were low in fibroblasts transfected by the RAV-1 helper alone but were greatly increased in fibroblasts transfected by either wild-type AEV or by the delS/T mutant (Table 1).

The AEV delS/T mutant transformed erythroid cells to an oncogenic state. In addition to being able to transform fibroblasts, wild-type AEV is also capable of transforming immature erythroid cells to an oncogenic state (5, 11). The requirement of a functional *v-erbB* protein product in AEV erythroid transformation has been previously established (6, 26). We therefore tested the delS/T *v-erbB* mutant for the ability to transform erythroid cells by a bone marrow colony assay (Table 2) (7, 10, 12). As determined by this assay, the delS/T mutant was capable of transforming chicken bone marrow cells and of producing colonies of erythroid cells similar in number and appearance to those produced by wild-type AEV. These erythroid colonies were absent in bone marrow cultures infected by the RAV-1 helper virus alone.

The delS/T proviruses retained the genetic lesion and synthesized a truncated *v-erbB* polypeptide. A Southern blotting analysis on DNAs isolated from RAV-1-, delS/T-, and wild-type AEV-transfected fibroblasts confirmed that the proviruses in the delS/T-transformed fibroblasts retain the genetic lesion engineered into the molecular clone (data not shown) and that the transformed phenotype is not therefore due to a detectable reversion or contamination with wild-type virus. We next examined the polypeptide products encoded by the wild-type AEV and delS/T viruses. Fibroblasts transfected by the different viral genomes were metabolically radiolabeled with [³⁵S]methionine for 2 h, and the *erbB*-related polypeptides were immunoprecipitated and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). As previously ascertained, the wild-type virus synthesized two predominant *v-erbB* protein species during this 2-h pulse label, gp65^{*v-erbB*} and gp68^{*v-erbB*} (14, 23) (Fig. 4, lane 1). This heterogeneity in the apparent molecular weight of the wild-type *v-erbB* polypeptide is due to glycosylation (14, 23). In contrast, the delS/T mutant synthesized a smaller polypeptide of homogeneous size (approximately 56,000 in apparent molecular weight [56K polypeptide]) (Fig. 4, lane 3) that was not recognized by nonimmune serum (Fig. 4, lane 4) and was not present in cells transfected with RAV-1 helper virus alone (data not shown). The size of the delS/T 56K *v-erbB* polypeptide is consistent with the molecular weight expected of an unglycosylated *v-erbB* protein possessing a 52-amino-acid deletion (approximately 61,000 - 5,200) (14, 23). Preliminary experiments with tunicamycin also suggest that the 56K protein encoded by the delS/T mutant is not glycosylated (data not shown), consistent with the fact that the delS/T mutation removes all three putative extracellular glycosylation sites found in the *v-erbB* sequence. Subcellular fractionation experiments (data not shown) demonstrate that the delS/T-encoded *v-erbB* protein fractionates with mem-

FIG. 2. Fibroblasts infected by the AEV delS/T mutant. Chicken embryo secondary cells were transformed by the RAV-1 helper plasmid alone (top), by the helper plus the pAEV-delS/T plasmid (middle), or by the helper plus the wild-type pAEV-11-3R plasmid (bottom) and were propagated for four (1:5) passages. Representative photomicrographs of the resulting adherent fibroblast cultures are presented. Bar, approximately 50 μ m.

TABLE 2. Erythroid cell transformation by the AEV delS/T mutant^a

Virus	No. of transformed erythroid colonies	
	Expt 1	Expt 2
RAV-1 only	0	0
RAV-1 + AEV delS/T mutant	7	5
RAV-1 + AEV wild type	24	12

^a Virus stocks were isolated from the cell culture supernatants of transfected fibroblasts and were used to infect bone marrow cells obtained from 7- to 14-day-old chickens (SPAFAS, Inc.). A 0.5 ml portion of each stock was used to infect 2×10^6 erythrocyte-depleted bone marrow cells. The infected cells were then suspended in methylcellulose medium (12) and were incubated for 3 weeks at 39°C. The AEV delS/T mutant and wild-type virus stocks used demonstrated approximately equal fibroblast-transforming activity.

branes, in parallel with the wild-type polypeptide, indicating that deletion of the extracellular domain did not significantly alter the subcellular localization of this protein.

Nature and properties of the delS/T mutation. The deletion of *v-erbB* coding sequences described here resulted in the precise elimination of 52 amino acids from (approximately two-thirds of) the extracellular domain of this oncogenic polypeptide. The reading frame of the remainder of the *v-erbB* protein was preserved, no novel amino acid codon was generated, and all three Asn-X-Ser/Thr sequences that are thought to represent potential sites of glycosylation of the *v-erbB* protein were eliminated (see Figure 1B) (15).

The delS/T mutant appeared to be fully capable of oncogenic transformation of fibroblasts by all criteria tested: morphological alteration, substrate-independent growth, elevated hexose uptake, and elevated secretion of plasminogen-activator protease. Similarly, the delS/T mutant virus was able to transform bone marrow cells in vitro, giving rise to proliferating erythroid colonies identical in appearance to those infected by wild-type AEV. It should be noted that the experiments were performed in a *v-erbA*-positive genetic background (a functional *v-erbA* protein was also encoded by the pAEV plasmids used in these studies). Although the AEV *erbA* protein is not itself capable of oncogenic transformation of either erythroid or fibroblast cells, the *v-erbA* protein potentiates the ability of *v-erbB* (and certain other tyrosine-directed protein kinases) to transform erythroid cells (6, 25).

The large extracellular domain of the host cell EGF receptor is involved in the binding of growth factor and is therefore intimately related to the transduction of the growth factor-mediated mitogenic response (4, 27). Our data suggest that a significant portion of the residual extracellular sequences found in the *v-erbB* protein is not necessarily of functional significance. Instead, the extracellular domain retained in the viral protein may simply reflect a balance between two opposing influences: the necessity of a membrane-association site (the transmembrane domain) for func-

FIG. 3. Substrate-independent growth by fibroblasts infected by the AEV delS/T mutant. Chicken embryo secondary cells transfected by the RAV-1 helper plasmid alone (top), by the helper plus the pAEV-delS/T plasmid (middle), or by the helper plus the wild-type pAEV-11-3R plasmid (bottom) were seeded into soft agar media and were incubated at 39°C for 2 weeks as previously described (26). Representative photomicrographs of these soft agar cultures are shown. Bar, approximately 1 mm. These results are presented quantitatively in Table 1.

FIG. 4. *erbB*-related protein synthesized in delS/T-infected fibroblasts. Fibroblasts infected by the AEV delS/T mutant or by wild-type AEV were incubated for 2 h in Dulbecco modified Eagle medium containing 100 μ Ci of [35 S]methionine per ml (3,000 Ci/mmol). The cells were washed and lysed, and the lysates were subjected to an immunoprecipitation analysis as previously described (23). One-half of each lysate was precipitated with anti-*erbB* serum (E), and the other half was precipitated with normal rabbit serum (N). The resulting immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the radiolabeled proteins were visualized by autoradiography. Indicated are the gp65^{v-*erbB*} (gp65) and gp68^{v-*erbB*} (gp68) glycosylated protein species synthesized by wild-type AEV and a 56K *erbB*-related polypeptide synthesized in the delS/T mutant-infected cells. Apparent molecular weights were calculated relative to protein standards resolved in adjacent lanes of the electrophoretograms (not shown).

tion of the v-*erbB* protein and the necessary deletion of N-terminal EGF-binding and -regulatory sequences to permit activation of the EGF receptor as an oncogenic protein (13, 18). A transduction event that meets both these criteria, perhaps additionally constrained by the need for an appropriately placed splice acceptor site and an initiation codon or signal sequence, would be likely to result in the retention of at least some extracellular sequences in the resulting viral gene product (18).

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