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Dissecting the Activating Mutations in v-*erbB* of Avian Erythroblastosis Virus Strain R

HUI-KUO G. SHU,[1] ROBERT J. PELLEY,[2] AND HSING-JIEN KUNG[1]*

Departments of Molecular Biology and Microbiology[1] and Medicine,[2] Division of Hematology/Oncology, Case Western Reserve University, 2109 Adelbert Road, Cleveland, Ohio 44106

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The v-*erbB* oncogene isolated from the R (or ES4) strain of avian erythroblastosis virus is capable of inducing erythroleukemia and fibrosarcomas. This oncogene differs from the proto-oncogene c-*erbB*, the avian homolog of the epidermal growth factor receptor, by its lack of an intact ligand-binding domain as well as additional alterations in its cytoplasmic coding sequences. By contrast, the insertionally activated c-*erbB*, a variant oncogene, which encodes a product that also lacks the ligand-binding domain but is otherwise unaltered in its cytoplasmic coding sequences, is capable of inducing leukemia but cannot induce sarcomas. In this report, we show that the critical changes for activating the sarcomagenic potential displayed by v-*erbB* R are two point mutations within the tyrosine kinase domain and an internal deletion of 21 amino acids in the carboxyl-terminal regulatory domain. The removal of the carboxyl-terminal autophosphorylation sites is not obligatory. These activating mutations (Arg-263 to His, Ile-384 to Ser, and the deletion of residues 494 to 514), when introduced singly into the insertionally activated c-*erbB*, all dramatically increase fibroblast-transforming potential. Arg-263 resides near the highly conserved HRD motif of the kinase domain, and its mutation to His increases the autophosphorylation activity. The other two mutations do not alter the intrinsic kinase activity and presumably affect other aspects of the receptor involved in growth signaling. Therefore, the high transforming potential of v-*erbB* R is a consequence of synergism among multiple activating mutations.

Avian erythroblastosis viruses (AEVs) are acutely transforming retroviruses capable of inducing erythroleukemias and fibrosarcomas with short latencies (3 to 4 weeks) (9, 14, 18, 21). The transforming components of these oncogenic viruses were demonstrated to be v-*erbB* genes, mutated versions of the normal c-*erbB* proto-oncogene (11, 44, 51–53). The v-*erbB* of AEV strain R (AEV-R) (ES4) encodes a membrane-associated protein that ranges in size from 65 to 73 kDa owing to its glycosylation (2, 16, 17, 37–39). The avian c-*erbB* proto-oncogene product is now known to be a growth factor receptor that is homologous to the human epidermal growth factor receptor, although functional homology has not been clearly established (8, 26, 30). Recently, it was shown that the avian c-*erbB* also serves as a hematopoietic growth factor receptor and controls the self-renewal of erythroblasts (33). This may explain the propensity of erythroleukemia induction by mutated *erbB*.

In addition to AEV, the avian leukosis virus can also induce erythroblastosis in chickens, but only after a 2- to 4-month latency (12, 29). Avian leukosis virus is a nonacute retrovirus that does not carry an oncogene of its own and transforms erythroid cells by proviral insertion within the c-*erbB* locus, producing 5′-truncated transcripts that no longer encode an intact ligand-binding domain (12, 29, 40). This oncogene, termed the insertionally activated (IA) c-*erbB*, is exclusively leukemogenic and, unlike v-*erbB* of AEV-R, does not induce fibrosarcomas or transform fibroblasts (3, 29, 35, 41, 42). The IA c-*erbB* product has a truncated extracellular ligand-binding domain but has intact and unaltered sequences in the transmembrane and intracellular domains of the receptor (30). This amino-terminal truncation is presumably what is responsible for activating leukemogenic potential by producing a ligand-independent receptor that has constitutive tyrosine kinase activity (12, 29, 40). In addition to the amino-terminal truncation, v-*erbB* R, the v-*erbB* of AEV-R (or ES4), has several other alterations including six point mutations, an internal deletion of 21 amino acids, and a carboxyl-terminal truncation of 74 amino acids (Fig. 1) (6). It is these additional mutations within the remaining coding sequences of the truncated receptor which are apparently responsible for sarcomagenic potential (3, 6, 13, 34, 45, 53). The ability to transform fibroblasts can therefore be attributed to one or more of these alterations within v-*erbB* R, and determining the critical mutation(s) can then provide information regarding the residue(s) important in regulating receptor signaling in different tissues.

Previously, we showed that the 74-amino-acid carboxyl-terminal truncation of v-*erbB* R is not critical to its sarcomagenic activity (34). In this study, we extended this analysis to define the critical mutations that activates the sarcomagenic potential of v-*erbB* R. This was accomplished by introducing mutations from the transforming v-*erbB* R oncogene singly into the IA c-*erbB* (Fig. 1). After testing the transforming potential of each of these *erbB* mutants, we found that two point mutations (Arg-263 to His [R263H] and Ile-384 to Ser [I384S]) and the internal deletion of 21 amino acids within the carboxyl-terminal regulatory domain are alone capable of converting IA c-*erbB* into an oncogene with sarcomagenic potential. However, of these activated mutants, a corresponding increase in kinase activity was only observed for the R263H product.

MATERIALS AND METHODS

Plasmid construction and site-directed mutagenesis. The expression vector used in these studies is the avian replication-competent retroviral vector RCAN that carries the *gag-pol* region of the Bryan high-titer virus (20). IA c-*erbB*

* Corresponding author.

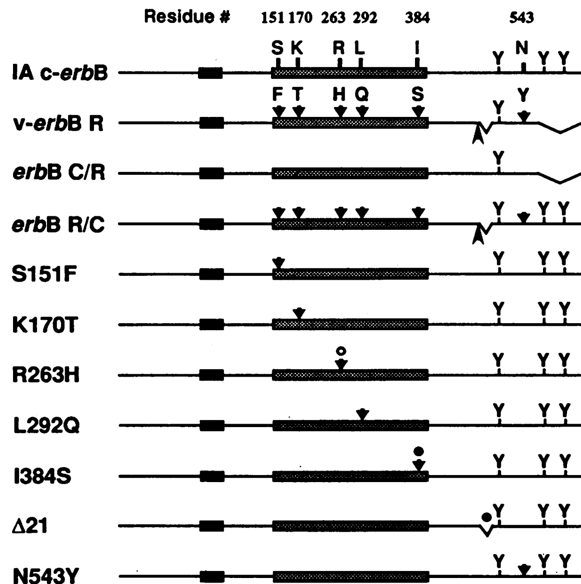

FIG. 1. Schematic representation of IA c-*erbB*, v-*erbB* R, and various *erbB* mutants that contain alterations from v-*erbB* R. The residues where v-*erbB* R differs from IA c-*erbB* are labeled with the residue number above. The mutated residues are denoted by arrows. In addition to the point alterations, v-*erbB* R contains a single amino acid deletion (residue 486, denoted by the arrowhead), a 21-amino-acid deletion (residues 494 to 514), and a 74-amino-acid carboxyl-terminal truncation (residues 572 to 645). The positions of the three major sites of autophosphorylation in the carboxyl-terminal domain are marked with Ys, representing the phosphorylated tyrosines. *erbB* R/C is a chimeric construct in which the carboxyl-terminal truncation of v-*erbB* R has been replaced with the normal wild-type sequence. *erbB* C/R is a chimeric construct in which the carboxyl-terminal truncation of v-*erbB* R has been introduced into IA c-*erbB*. The six point mutations and 21-amino-acid deletion were introduced singly into an IA c-*erbB* background. The name of the mutant describes the alteration it contains, e.g., S151F is a mutant with Ser (S)-151 changed to Phe (F). Solid bars denote the transmembrane domain, and stippled bars denote the tyrosine kinase domain. The mutations that increased both in vitro fibroblast-transforming potential and in vivo sarcomagenic potential are marked above by solid circles. The mutation which only increased in vitro fibroblast-transforming potential while not affecting in vivo sarcomagenic potential is marked above by an open circle.

and *erbB* R/C were cloned into this modified RCAN vector as described previously (34, 35). This Bryan high-titer retroviral vector achieves two- to threefold-greater levels of expression per integrated provirus than the original RCAN vector (19). The *erbB* C/R chimera is the reciprocal chimera to the *erbB* R/C chimera and was constructed similarly (34). This increased expression level is most likely responsible for the increased background soft agar colonies induced by IA c-*erbB* (34, 35). The IA c-*erbB* *Cla*I fragment was also cloned into pBluescript M13+ KS (Stratagene) in an orientation giving negative-strand rescue of the *erbB* coding sequence. This single-stranded DNA containing the IA c-*erbB* sequence was modified by using a site-directed mutagenesis kit (Amersham) (50) to create the six point mutants and the 21-amino-acid deletion mutant. Synthetic oligonucleotides were made on an Applied Biosystems DNA synthesizer and OPC cartridge purified. The oligonucleotides used to create each of the mutants are as follows (mutated codons are underlined): (i) Ser-151 to Phe, 5′-AAGGTTTTGGGCTATG GAGCTTTTGG-3′; (ii) Lys-170 to Thr, 5′-GGGAAAAGGT TACAATTCCTGTTGC-3′; (iii) Arg-263 to His, 5′-TGGAG GAACGTCACCTGGTGCACCG-3′; (iv) Leu-292 to Gln, 5′-GGCTGGCAAAGCAGCTTGGGGCAGA-3′; (v) Ile-384 to Ser, 5′-AATGCTGGATGAGTGATGCAGACAG-3′; (vi) Asn-543 to Tyr, 5′-CAAATCTACAACTACATCTCTCT CA-3′; (vii) deletion of 21 residues (residues 494 to 514), 5′-CCTGTGAGGGAAGAC//GGCTTCCTGCCTGCT-3′. The identity of the mutants were confirmed by dideoxy sequencing on double-stranded DNA templates (43).

Tissue culture and transfection. Early-passage line 0 chicken embryo fibroblasts (CEFs) were used in these studies. Line 0 CEFs were used because of their lack of endogenous virus (1), which eliminates the risk of recombination between the retroviral vector and endogenous viral sequences which might delete the *erbB* insert. The cells were cultured in a mixed medium (1:1, Dulbecco modified Eagle medium low glucose-M199) (GIBCO) supplemented with 3% (vol/vol) fetal bovine serum (GIBCO), 2% (vol/vol) chicken serum (GIBCO), 50 U of penicillin G per ml, and 50 μg of streptomycin sulfate per ml. CEFs (5×10^6 cells) were transfected by electroporation with 20 μg of plasmid DNA. Electroporation was done in cuvettes with 0.4-cm plate electrodes (Bio-Rad) under the following conditions: (i) 100-μF capacitance; (ii) 950-V/cm field strength; (iii) 2-ms pulse time. Cells were resuspended in 0.75 ml of phosphate-buffered saline with EDTA per cuvette. After electroporation, the cultures were passaged for 7 days before being assayed to ensure maximal infection of cultures by the replication-competent viruses.

Transformation assays. Transforming potential was assessed in vitro by a soft agar assay to measure anchorage-independent growth of CEFs. This assay was performed in six-well plates with a base of 2 ml of complete medium with 0.5% Noble agar (Difco). Cells were seeded in 1 ml of complete medium with 0.35% agar at high density (1×10^5 cells per ml) or low density (5×10^3 cells per ml) and layered onto the base. After 24 h, an additional 1 ml of complete medium with 0.35% agar was layered on. The plates were refed with an additional 1 ml of complete medium with 0.35% agar every 7 days. The complete medium for the soft agar assay consisted of 4.5% (vol/vol) fetal bovine serum and 0.5% (vol/vol) chicken serum instead of the normal 3% and 2% ratio. After 17 days, the number of colonies was scored in the low-density plates, with colonies of >100 cells scoring as positives. Transforming potential was assessed in vivo by measuring the rate of sarcoma induction after wingweb injection of viral supernatants. CEFs that had been transfected/infected with the RCAN-*erbB* constructs were grown to confluence in a 15-cm petri dish. Fresh complete medium was added and harvested in 16 h. This viral supernatant was then spun down to remove cellular debris and freeze-thawed twice to kill any residual cells. Two-day-old line $15I_5 \times 7_1$ or line 0 chicks were injected with 0.1 ml of the viral supernatants. Injected chickens were then assessed in the following weeks for sarcoma formation in the wingweb until they succumbed to erythroblastosis.

Immunoblotting. CEFs expressing the various *erbB* genes of interest were grown to confluence in a 10-cm petri dish. The cells were lysed with 1 ml of a boiling sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-Cl [pH 6.8], 1% SDS, 1% 2-mercaptoethanol, 0.1 M dithiothreitol, 5% sucrose, 300 μM sodium orthovanadate, 0.03% bromophenol blue) and boiled for an additional 5 to 10 min. Genomic DNA was sheared by several passages through a 26-gauge needle. Lysate (~50 μl) was resolved by 7.0% SDS-polyacrylamide

TABLE 1. Transforming potential of *erbB* mutants

Construct	No. of soft agar colonies/ 10^5 cells[a]	Soft agar colonies induced relative to IA c-*erbB*'s potential[a]	Rate of induction of wingweb sarcomas
RCAN	0	0.0	0/6
IA c-*erbB*	731	1.0	1/7[b]
erbB C/R	292	0.4	NT[c]
erbB R/C	4,245	5.8	7/7
S151F	1,562	2.1	1/7[b]
K170T	665	0.9	0/7
R263H	4,406	6.0	1/17[b]
L292Q	813	1.1	0/7
I384S	4,243	5.8	7/7
Δ21	4,405	6.0	7/7
N543Y	580	0.8	0/7

[a] Values are average from two to three independent transfections.
[b] Rare nodules form following longer latency (>4 wks) and are probably due to secondary mutation within *erbB*.
[c] NT, not tested.

gel electrophoresis (PAGE) and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore) and probed with either a rabbit polyclonal antibody specific for the *erbB* kinase domain (27) or PY20, a mouse antiphosphotyrosine monoclonal antibody (ICN). Details of the blotting procedure are described elsewhere (22). Basically, the membranes were blocked overnight with 3% (wt/vol) bovine serum albumin (Boehringer Mannheim) in Tris-buffered saline (20 mM Tris-Cl [pH 7.2], 150 mM NaCl). Membranes were then probed with either anti-*erbB* antiserum (1:750 dilution) or PY20 (1 μg/ml) in the blocking buffer. Membranes were then washed several times with Tris-buffered saline and then detected with ^{125}I-labeled protein A (Amersham) (0.5 μCi/ml) in the blocking buffer. Autoradiography was performed with an intensifying screen at −70°C. The autoradiograms were densitometrically scanned on an LKB laser densitometer.

RESULTS

As shown in Fig. 1, v-*erbB* R differs from the strictly leukemogenic IA c-*erbB* by a number of alterations including six point mutations, a 21-amino-acid deletion, and a 74-amino-acid carboxyl-terminal truncation that removed two of the major autophosphorylation sites. We have previously shown that the carboxyl-terminal truncation was not solely responsible for the sarcomagenic potential of this v-*erbB* (34). We demonstrated this by replacing the truncation with normal sequences, thereby making a v-*erbB*/c-*erbB* chimera. This chimeric construct, which contained all the mutations found in the v-*erbB* R oncogene except for the carboxyl-terminal truncation, was termed *erbB* R/C (Fig. 1). This R/C chimera maintained full sarcomagenic activity, thereby implying that other mutations within v-*erbB* R are responsible for its sarcomagenic potential. Indeed, when the reciprocal chimeric construct (*erbB* C/R, IA c-*erbB* with the 74-amino-acid carboxyl-terminal truncation) (Fig. 1) was expressed in CEFs to test for fibroblast-transforming potential, it displayed the low basal levels of transformation seen with IA c-*erbB* transfectants (Table 1). This result showed unequivocally that the carboxyl-terminal truncation was not responsible for the sarcomagenic phenotype of v-*erbB* R.

In vitro transforming potential of *erbB* mutants. Based on the above findings, the sarcomagenic activity of v-*erbB* R must be due to one or more of the remaining alterations, the six point mutations or the 21-amino-acid deletion. To test which one of these mutations was responsible for this activation, we introduced each singly into IA c-*erbB* (Fig. 1) and tested the resulting constructs for their ability to transform fibroblasts in vitro. In each case, the resulting *erbB* mutant was inserted within the avian replication-competent retroviral vector RCAN for expression (20, 34, 35, 45). Our in vitro transformation assay involved testing the ability of CEFs transfected/infected with the various *erbB* mutants to grow in soft agar (34, 45).

The six point mutants and the internal deletion mutant were tested for their ability to induce anchorage-independent growth of CEFs. We found that two of the point mutants (R263H and I384S) and the internal deletion mutant (Δ21) produced significantly larger soft agar colonies than IA c-*erbB* (data not shown). When the colonies were scored, we found that these transforming mutants induced five- to sixfold more soft agar colonies than IA c-*erbB* (Table 1). The number of soft agar colonies induced by these three activated mutants actually approached that of the *erbB* R/C chimera (which contains all the tested mutations), although the latter still exhibited more aggressive growth properties. The remaining four point mutans induced moderate to low levels of transformation, similar to IA c-*erbB* (Table 1).

Sarcomagenic potential of *erbB* mutants. These seven mutants were then tested for their sarcomagenic potential. Our in vivo transformation assay relied on sarcoma induction after injection of wingwebs with virus carrying the mutant *erbB* genes (34, 45). Consistent with the results of the in vitro transformation assay, I384S and Δ21 mutants displayed significant sarcomagenic potential, inducing large nodules in the wingweb, whereas those which scored negative in the CEF assay also failed to induce sarcoma (Table 1). The lack of sarcomagenic potential by these *erbB* mutants was not due to the inactivity of the mutant proteins because all of the wingweb-injected birds succumbed to erythroblastosis by 6 weeks postinjection (data not shown). Curiously, the R263H mutant was generally unable to induce wingweb nodules before the injected chickens succumbed to erythroleukemia despite its in vitro CEF-transforming potential (Table 1). While this is the first *erbB* mutant in which disparity between in vitro and in vivo transformation is observed, a similar phenotype has been reported for a v-*src* mutant (7, 23, 48, 49).

Kinase activity of *erbB* mutants. To try to understand the molecular basis of the increased transforming potential of the activated mutant *erbB* genes, we sought to determine the level of autophosphorylation displayed by the various *erbB* mutants. To study this, we examined the steady-state level of phosphorylation of the *erbB* products by immunoblotting with antiphosphotyrosine antibody. Lysates of CEFs expressing the various *erbB* genes were resolved in duplicate by 7% SDS-PAGE and then electrotransferred to a solid support. The filter was then probed with either the antiphosphotyrosine monoclonal antibody PY20 (Fig. 2B) or an anti-*erbB* antibody specific for the kinase domain (Fig. 2A). The anti-*erbB* immunoblot allowed a quantitation of the level of protein being expressed, while the antiphosphotyrosine immunoblot allowed a quantitation of the level of phosphorylation of the *erbB* product. This blotting was done on CEF lysates which were either untreated or treated with 10 ng of tumor growth factor α (TGF-α) for 20 min.

The anti-*erbB* blot showed that all mutants expressed comparable levels of protein except *erbB* R/C (Fig. 2A, lanes 5 and 6). The *erbB* R/C mutant protein displayed high fibroblast-transforming potential despite its relative low lev-

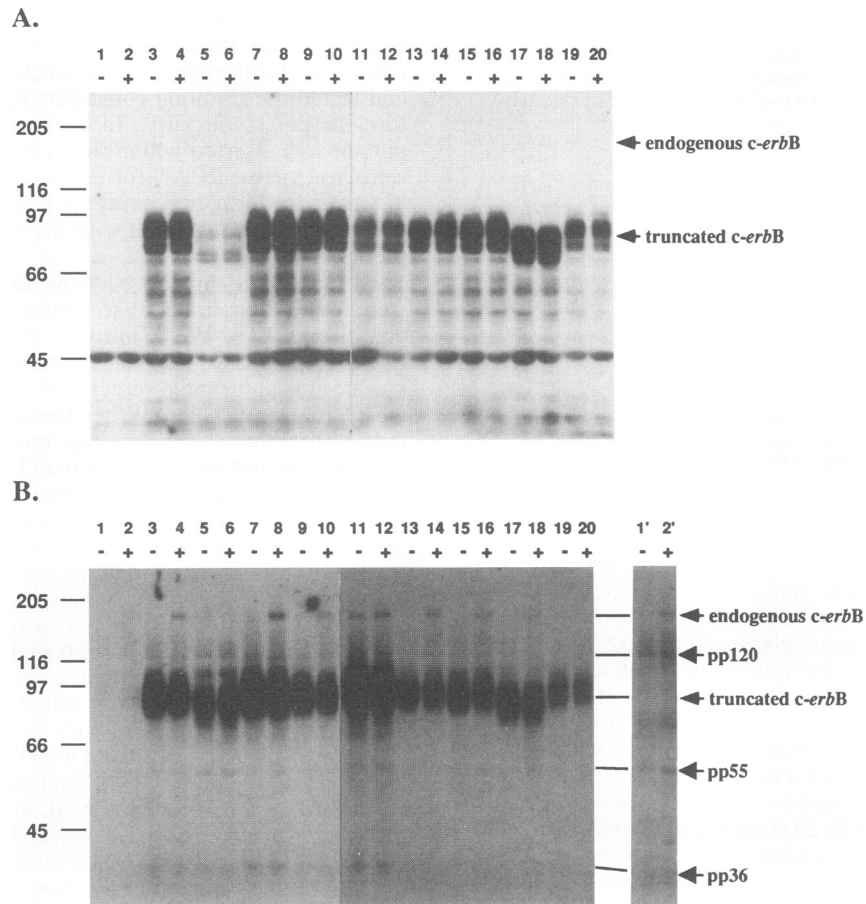

FIG. 2. Immunoblots show the level of *erbB* expression and tyrosine phosphorylation in CEFs transfected with RCAN containing no insert (lanes 1, 2, 1′, and 2′), IA c-*erbB* (lanes 3 and 4), *erbB* R/C (lanes 5 and 6), S151F (lanes 7 and 8), K170T (lanes 9 and 10), R263H (lanes 11 and 12), L292Q (lanes 13 and 14), I384S (lanes 15 and 16), Δ21 (lanes 17 and 18), and N543Y (lanes 19 and 20). Lanes 1′ and 2′ were loaded with lysates from cells that were pretreated with sodium orthovanadate to increase the sensitivity of the antiphosphotyrosine blot, thereby making the relevant bands more apparent. Total cellular lysate from 0.5×10^6 to 1.0×10^6 cells was loaded onto each lane and resolved by 7% SDS-PAGE. The resulting blots were probed with a rabbit polyclonal antibody specific for the kinase domain of the *erbB* product (A) and PY20, a mouse antiphosphotyrosine antibody (B). ^{125}I-labeled protein A was used to detect the antibody probe. The plus and minus signs above the lanes denote whether the cells were untreated or treated with 10 ng of TGF-α per ml in complete medium for 20 min before lysis. The *erbB* products migrate as an 85-kDa species. The endogenous receptor migrates as a 180-kDa species. The possible exogenous substrates migrate as 120-, 55-, and 36-kDa species (see panel B, lanes 11 and 12). Size markers on the left are in kilodaltons.

els of expression. However, although the level of R/C chimeric protein expression was severalfold less than that of the IA c-*erbB* product, the level of autophosphorylation of the R/C chimeric gene product was comparable to that of the IA c-*erbB* product (Fig. 2A and B, lanes 3 to 6). When the autoradiograms were densitometrically scanned, the *erbB* R/C chimera showed approximately 4.0- to 4.5-fold-greater levels of autophosphorylation per molecule than the IA c-*erbB* product (Fig. 3). In addition to the R/C chimera, the R263H product also showed higher levels of autophosphorylation (Fig. 2A and B, lanes 3, 4, 11, and 12). When those lanes were densitometrically scanned, the R263H protein products displayed approximately 2.5-fold-higher levels of autophosphorylation on a per molecule basis (Fig. 3). The remaining mutants, including the transforming I384S and Δ21 oncogenes, showed levels of autophosphorylation comparable to those of the IA c-*erbB* product (Fig. 3).

Figure 2B also provides information concerning in vivo phosphorylation of exogenous substrates by these mutants. Normal CEFs (Fig. 2A and B, lanes 1, 2, 1′, and 2′) expressed a low level (~10^4 to 10^5 molecules) of endogenous full-length c-*erbB* (pp180) which when stimulated by TGF-α led to rapid autophosphorylation of the receptor (indicated by arrow) and a number of other cellular substrates (pp120, pp55, pp36, etc.). These phosphorylations were relatively low and were marginally detectable under the exposure used in Fig. 2B. Most of the mutant-infected cells gave a low, constitutive phosphorylation of these substrates, and TGF-α treatment slightly enhanced such phosphorylation by the endogenous receptor. On the other hand, the R263H mutant (Fig. 2B, lanes 11 and 12), which exhibited increased autokinase activity, gave a high level of constitutive phosphorylation of cellular substrates, significantly higher than that of TGF-α-treated CEFs. It is interesting that the R263H mutant also showed an increase in its ability to phosphorylate the endogenous full-length receptor. The band representing the endogenous receptor on the antiphosphotyrosine blot was quite intense on the untreated lysates of CEFs expressing the R263H mutant construct (Fig. 2B, lane 11). This phosphorylation was severalfold more intense than that displayed

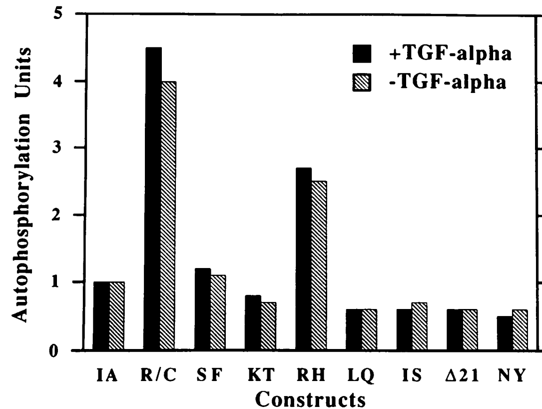

FIG. 3. Bar graph shows densitometric scan of the Western blots (immunoblots) pictured in Fig. 2. Autophosphorylation units represent the level of autophosphorylation as detected by PY20 standardized according to the level of protein present as detected by the anti-*erbB* antibody, with the IA c-*erbB* protein product phosphorylation level being arbitrarily set as 1.0 U. The solid bars denote autophosphorylation in the TGF-α-treated lanes, while the hatched bars denote autophosphorylation in untreated lanes. Analysis by Western blot of different transfection experiments gave the same qualitative results, i.e., only *erbB* R/C and R263H showed significant increases in autophosphorylation.

by the untreated lysates of CEFs expressing the IA c-*erbB* construct in which the endogenous receptor was barely evident (Fig. 2B, lanes 3 and 11). The ligand-stimulated autophosphorylation of the endogenous receptor, although still present, was no longer as pronounced as seen with CEFs expressing IA c-*erbB* (Fig. 2B, lanes 3, 4, 11, and 12). This would seem to indicate that the amino-terminally truncated *erbB* products are capable of cross-phosphorylating the full-length receptor and that this receptor cross-talk does not require the presence of extracellular ligand-binding domain.

DISCUSSION

We showed that three specific mutations within the v-*erbB* R (ES4) product are capable of making this amino-terminally truncated receptor a strong fibroblast-transforming oncogene product. These mutations include two point alterations within the kinase domain, changing Arg-263 to His and Ile-384 to Ser, and a 21-amino-acid deletion within the carboxyl-terminal domain (residues 494 to 514). The 74-residue carboxyl-terminal truncation, which encompasses two of the three major autophosphorylation sites, was previously thought to play a role in activating the fibroblast-transforming potential of v-*erbB* R (ES4). We showed that this is not the case because when this truncation alone was introduced into the IA c-*erbB* genetic background, the ability to transform fibroblasts was not achieved. Thus, unlike v-*src*, the terminal major autophosphorylation sites do not appear to control the fibroblast-transforming potential of the molecule (5, 23, 36). In our previous analysis of *erbB* mutants, the sarcomagenic potential correlated well with the ability to form large soft agar colonies. The I384S and Δ21 mutants reported here fall into this category. However, the R263H mutant, capable of forming soft agar colonies, failed to induce sarcomas in vivo. This is reminiscent of the Tyr-416-to-Phe mutant of v-*src* that has a wild-type in vitro transforming potential but a significantly reduced in vivo sarcomagenic potential (23, 48, 49). Whether this phenomenon is related to differences in the immunogenic property of the transformed cells remains to be tested.

We have yet to determine the mechanism(s) that produces the expanded tissue specificity (i.e., fibroblast and erythroblast) of these *erbB* mutants. These mutations may result in an increase in kinase activity or the accessibility of a critical substrate(s) to this enzyme. Previously, we identified the activating mutation (for fibroblast transformation) of v-*erbB* (AEV-H) to be Val to Ile at residue 157 (34, 45). This mutation resides in the highly conserved ATP-binding motif (Gly-X-Gly-X-X-Gly-X-Val) and is associated with higher levels of autophosphorylation in vivo, which is consistent with an increased V_{max} of the enzyme (45). The R263H mutant of v-*erbB* (AEV-R) has a similar phenotype. It enhances autophosphorylation as well as phosphorylation of other cellular substrates. The location of this mutation is within subdomain VI of the kinase as defined by Hanks et al. (15) and is immediately adjacent to the conserved HRD motif (Fig. 4A), which is thought to be involved in determining amino acid specificity (serine/threonine versus tyrosine) of the kinase. A single point mutation in this region of the proto-oncogene c-*kit* renders its kinase inactive and contributes to the white-spot phenotype (31). One of the activating mutations of c-*src* is Glu-378 to Gly, which resulted in an increased kinase activity (49). When aligned with the *erbB* kinase, this *src* mutation lies just three residues amino terminal to our Arg-263 mutation in *erbB*. Thus, it appears that both the glycine and HRD motifs are intimately involved in the kinase reaction and may represent parts of the active center. Consistent with these results, the recently resolved crystal structure of cyclic AMP-dependent protein kinase shows juxtaposition of the glycine and HRD motifs in the catalytic site of the kinase (24, 25).

The mechanisms of action are less clear for the other two activating mutants (I384S and Δ21), which showed no enhanced phosphorylation of *erbB* and other apparent substrates. It is conceivable that these mutants are selectively phosphorylating and activating some critical mitogenic substrates of low abundance that are undetectable by antiphosphotyrosine immunoblot or are obscured by *erbB* autophosphorylation. Conversely, this also implies that the increased phosphorylation of the abundant substrates (e.g., pp120, pp55, pp36, etc.) as seen in the R263H lanes (Fig. 2B, lanes 11 and 12) is not obligatory for transformation. Based on the binding sites of several phosphotidylinositol-3′-kinase (PI-3) kinase-associated proteins, a consensus binding sequence was proposed (4). We note that the Ile-384-to-Ser mutation is in close proximity to a putative binding site in the *erbB* kinase based on this consensus sequence (Fig. 4B). The recently cloned regulatory subunit (p85) of PI-3 kinase was found to contain two *src* homology 2 domains (10, 32, 47). Also, Margolis et al. (28) showed that the carboxyl-terminal phosphotyrosines of the intact human epidermal growth factor receptor are involved in high-affinity interactions with *src* homology 2 domains (of phospholipase C-γ), thus implicating these residues as important for the binding of PI-3 kinase as well. The importance of PI-3 kinase in the mitogenic signaling is not known. However, it remains possible that in the truncated form of the receptor (such as the *erbB* mutants in our studies), the above-mentioned motif in the kinase domain participates in binding to the p85 subunit of PI-3 kinase and that the Ile-384-to-Ser mutation increases the affinity between the molecules, leading to increased mitogenic signaling. Given its location outside the kinase domain

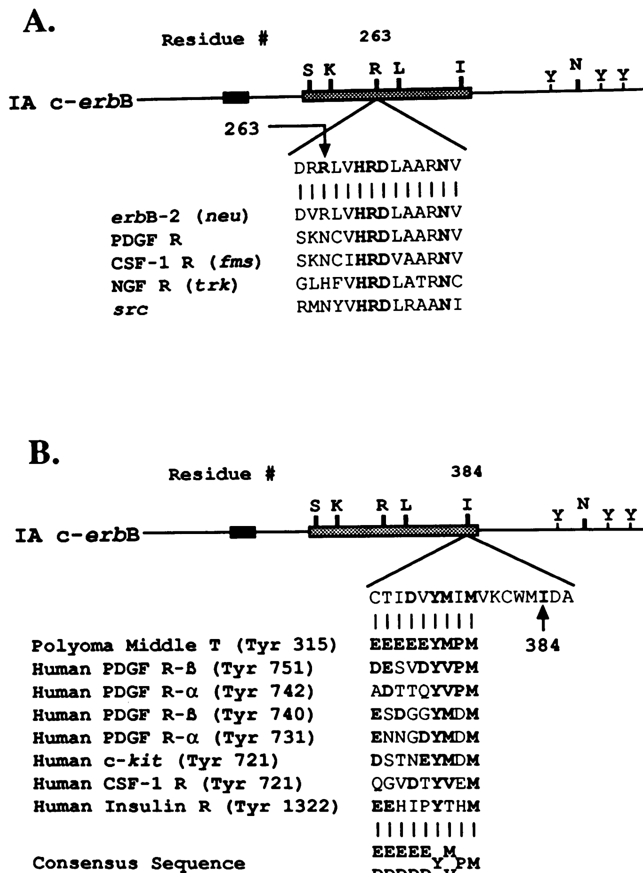

FIG. 4. Schematic of IA c-*erbB* is pictured with positions of the point mutations in v-*erbB* R labeled. (A) Position within *erbB* of the Arg-263-to-His mutation. A close-up of the sequence in this region shows the proximity of residue 263 to the conserved HRD motif within subdomain VI of the kinase. A comparison with other tyrosine kinases indicates a high degree of homology within this area. The position of the mutated residue, though, is not highly conserved among the kinases. (B) Position within *erbB* of the Ile-384-to-Ser mutation. A close-up of the sequence here indicates the proximity of residue 384 to a putative site of PI-3 kinase binding. This sequence is compared with sequences within other PI-3 kinase-associated proteins such as middle T antigen and a number of receptor tyrosine kinases. A consensus sequence for PI-3 kinase binding is pictured below the sequence comparison. PDGF R, platelet-derived growth factor receptor; CSF-1 R, colony-stimulating factor 1 receptor; NGF R, nerve growth factor receptor.

and lack of effect on the kinase activity, the Δ21 mutation is also likely to affect substrate binding. Interestingly, there are two other independent AEV isolates that carry *erbB* genes with internal deletions encompassing this region. Both are sarcomagenic and do not exhibit enhanced kinase activity (46). One possibility is that this region is involved in binding a negative regulatory substrate. It is also possible that deletions in this region will modulate the overall conformation of the receptor, thereby affecting substrate binding or aspects of receptor processing.

In summary, we identified three mutations in v-*erbB* responsible for the induction of sarcomas by AEV-R. We postulate that AEV-R originated from insertion of an intact provirus within the c-*erbB* locus with subsequent capture of IA c-*erbB* by the helper viral genome. This progenitor virus would be primarily leukemogenic. But with further passaging, other mutations including the activating ones accumulated, leading to the genesis of the present-day AEV-R with both fibroblast- and erythroblast-transforming potential. The activating mutations identified to date are either point mutations within the kinase domain or internal deletions within the regulatory domain. These will provide useful reference points for understanding how different domains of the receptor work in concert to transmit tissue-specific mitogenic signals.

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