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Tissue-specific transformation by epidermal growth factor receptor: A single point mutation within the ATP-binding pocket of the *erbB* product increases its intrinsic kinase activity and activates its sarcomagenic potential

(cellular transformation/erythroblastosis/retrovirus/sarcoma/tyrosine kinase)

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ABSTRACT **Avian c-*erbB* is activated to a leukemia oncogene following truncation of its amino-terminal, ligand-binding domain by retroviral insertion. The insertionally activated transcripts encode protein products that have constitutive tyrosine kinase activity and that can induce erythroleukemia but not sarcomas. We have found that a single point mutation within the ATP-binding pocket of the tyrosine kinase domain in this truncated molecule can increase the ability of this oncogene to induce anchorage-independent growth of fibroblasts *in vitro* and fibrosarcoma formation *in vivo*. Associated with this increased transforming potential is a corresponding increase in the kinase activity of the mutant *erbB* protein product. The mutation, which converts a valine to isoleucine at position 157 of the insertionally activated c-*erbB* product, is at a residue that is highly conserved within the protein kinase family. To our knowledge, this is the first demonstration of a point mutation in the ATP-binding pocket that activates a tyrosine kinase.**

Avian c-*erbB* encodes a growth factor receptor that is structurally homologous to the mammalian epidermal growth factor receptor (EGFR) (1–3), although their functional homology has not been clearly established. Like EGFR, it contains intrinsic tyrosine kinase activity (4, 5). The avian c-*erbB* protein can be activated by binding the ligand mammalian EGF or transforming growth factor α (TGF-α). Unlike the EGFR, the avian receptor binds TGF-α with 200-fold greater affinity than EGF (3). This receptor–ligand interaction presumably initiates a mitogenic signal resulting in cellular proliferation.

Avian leukosis virus can induce erythroleukemia in chickens after a long latent period (4–6 months) by invariably inserting within the c-*erbB* locus (6–8). This insertion results in the production of truncated growth factor receptor transcripts that maintain coding sequences for transmembrane and intracellular domains but that lack an intact ligand-binding domain (2, 6, 7). The resultant oncogene, the insertionally activated (IA) c-*erbB*, encodes a product that has constitutive tyrosine kinase activity and is exclusively leukemogenic (8–11). In contrast, acutely transforming viruses that induce erythroblastosis [avian erythroblastosis viruses (AEVs)] contain mutated versions of c-*erbB* within their genome (12, 13). In particular, the R (ES4) and H strains induce erythroleukemias and, interestingly, are also able to induce fibrosarcomas (13–15). Like IA c-*erbB*, the viral *erbB* (v-*erbB*) genes of AEVs encode truncated proteins that lack the ligand-binding domain. In addition, they contain sequence alterations including point mutations and deletions within the cytoplasmic domain (Fig. 1*A*) (2, 17, 18). Therefore, the expanded oncogenic potential of AEVs can be attributed to one or more of the various alterations within the v-*erbB* coding sequences. Both *erbB* R and *erbB* H products (Fig. 1*A*) have carboxyl-terminal truncations that encompass one or more major autophosphorylation sites (17, 18) in a manner similar to v-*src* and other kinase oncogene products (for example, see refs. 19 and 20). The carboxyl-terminal 72 amino acids are deleted from *erbB* R, whereas the last 34 amino acids are deleted from *erbB* H. We previously showed (16) that in each case, when these deletions were repaired with 3′ c-*erbB* sequences (chimeric *erbB* R/C and *erbB* H/C), sarcomagenic potential was retained. Thus, carboxyl-terminal truncation is not solely responsible for the fibroblast-transforming potential of v-*erbB* genes. Indeed, when the sarcomagenic chimeric *erbB* H/C and the leukemogenic IA c-*erbB* were compared, only two amino acid residues were found to differ. One mutation ($Trp^{40} \rightarrow Arg$) lies within the residual extracellular domain sequences, and the second ($Val^{157} \rightarrow Ile$) lies within the ATP-binding pocket of the kinase domain (16–18). This valine residue is part of the Gly-Xaa-Gly-Xaa-Xaa-Gly-Xaa-Val motif found in nearly all tyrosine and serine/threonine kinases (21). Here we report that a single point mutation at the valine residue is sufficient to confer fibroblast-transforming potential on the *erbB* oncogene product. Interestingly, the enhanced oncogenic potential of *erbB* is accompanied by a significant increase in the *in vivo* autokinase activity.

MATERIALS AND METHODS

Plasmid Construction and Site-Directed Mutagenesis. The modified replication-competent retroviral vector RCAN (22), carrying the *gag–pol* region of the Bryan high-titer virus, was used for expression. IA c-*erbB* was cloned into the modified RCAN vector as described (11). The IA c-*erbB Cla* I fragment was also cloned into pBluescript M13+ KS in an orientation giving negative-strand rescue of the *erbB* coding sequence. A site-directed mutagenesis kit (Amersham) (23) was used to convert the Val^{157} codon to an isoleucine codon. The oligonucleotide 5′-GCTTTTGGCACTATTTATAAGGGAC-3′ was used (codon 157 is underlined). Mutations to leucine, methionine, arginine, and tryptophan were achieved using the oligonucleotide mixture 5′-GGAGCTTTTGGCACTNNNTATAAGGGACTTTGG-3′, with degenerate bases

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Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; IA c-*erbB*, insertionally activated c-*erbB*; AEV, avian erythroblastosis virus; CEF, chicken embryo fibroblast.

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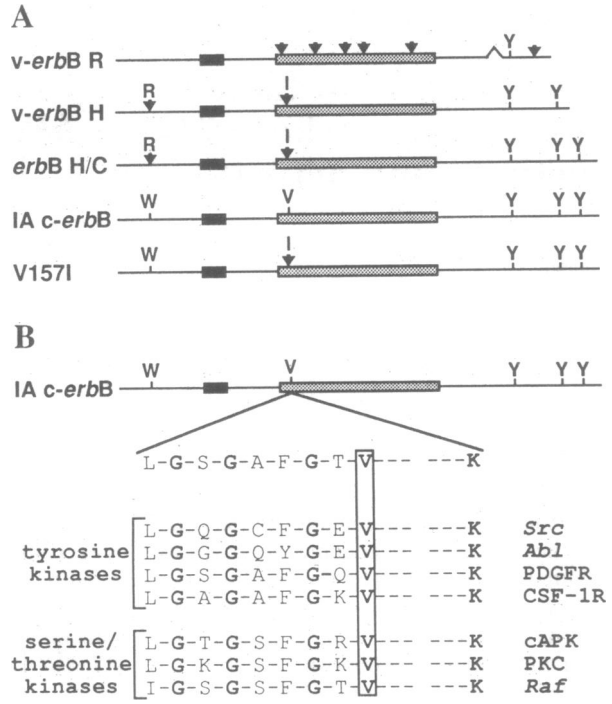

FIG. 1. (*A*) Schematic representation of the protein products encoded by five variant *erbB* genes, v-*erbB* R, v-*erbB* H, *erbB* H/C, IA c-*erbB*, and IA c-*erbB* V157I. All these variants have truncation of their amino-terminal, ligand-binding domain. v-*erbB* R is derived from the R strain of the acutely transforming retrovirus AEV (12). v-*erbB* H is derived from the H strain of AEV (13). *erbB* H/C is a chimeric fusion of v-*erbB* H and the carboxyl-terminal sequences from IA c-*erbB* (16). The IA c-*erbB* product has only the amino-terminal truncation and is otherwise identical in sequence to the normal full-length c-*erbB* receptor (2, 3). IA c-*erbB* V157I was derived from IA c-*erbB* by substituting an isoleucine codon for the Val^{157} codon by site-directed mutagenesis. Arrowheads denote point mutations, the solid bar denotes the transmembrane domain, and the stippled bar denotes the tyrosine kinase domain. (*B*) Schematic of the IA c-*erbB* product with detailed amino acid sequence of the ATP-binding pocket of the kinase. This motif is compared to protein sequences from several other tyrosine and serine/threonine kinases: the *src* oncogene product, the *abl* oncogene product, platelet-derived growth factor receptor (PDGFR), the c-*fms* product [the receptor for colony-stimulating factor 1 (CSF-1R)], cAMP-dependent protein kinase (cAPK), protein kinase C (PKC), and the *raf* oncogene product. Strictly conserved amino acids are highlighted in bold letters. The position of the critical mutation in *erbB* H/C (*A*) that codes for the conserved valine is boxed.

(25% of each nucleotide) at the valine codon. The mutants were confirmed by double-stranded dideoxy sequencing (24).

Cell Culture, Electroporation, and Transformation Assays. Early-passage, line 0 chicken embryo fibroblasts (CEFs), which lack endogenous virus, were used (25). They were cultured as described (26). DNA (30 μg) was introduced into CEFs (10^7 cells) by electroporation (100-μF capacitance, 950-V/cm field strength, 2-msec pulse time). Following electroporation, cells were passaged for 7 days before being assayed to assure maximal infection of cultures by replication-competent viruses. Transformation of infected CEFs was assayed by anchorage-independent growth in soft agar (26). Colonies of >100 cells were scored after 17 days. Sarcomagenic potential of the virus was assayed by wing-web inoculation of 2- to 4-day-old chicks (16).

***In Vitro* Immune Complex Kinase Assay.** Immune complex autophosphorylation was assayed as described (27) with an antibody specific for the carboxyl-terminal domain of the *erbB* product (28) and protein A-Sepharose beads. For determination of the K_m of ATP, beads were divided into aliquots. Serial dilutions of [γ-^{32}P]ATP (NEN) were added and the reactions were allowed to proceed for 1 min at 30°C. Samples were resolved by SDS/10% PAGE. Following autoradiography, *erbB* bands were excised and ^{32}P incorporation was determined by counting Cerenkov radiation in a β counter. For determination of autophosphorylation activity, the same method was used except that equal amounts of lysate total protein were used for immunoprecipitation and 100 μM [γ-^{32}P]ATP was used in each reaction.

Immunoblotting. CEFs expressing the *erbB* gene of interest were grown to confluence in a 10-cm Petri dish and lysed with 1 ml of boiling 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. Genomic DNA was sheared by several passages through a 26-gauge needle. Lysate (≈50 μl) was resolved by 7.5% PAGE and electrophoretically transferred to a poly(vinylidene difluoride) membrane (Immobilon, Millipore) and probed with a rabbit polyclonal antibody specific for the carboxyl-terminal domain of the *erbB* product (28) and with PY20, a mouse anti-phosphotyrosine monoclonal antibody (ICN), as described (29). ^{125}I-labeled protein A (Amersham) was used to detect the antibody probe on the membrane.

RESULTS

***In Vitro* and *in Vivo* Transforming Potentials of IA c-*erbB* and V157I Mutant.** The enhanced *in vivo* sarcomagenic and *in vitro* fibroblast-transforming potentials of *erbB* H/C can be attributed to either the Trp → Arg change in the extracellular domain, the Val → Ile change in the kinase domain, or both (16). To determine which mutation is critical, we first converted Val^{157} of the IA c-*erbB* product to isoleucine. We chose this mutation initially because Bassiri and Privalsky (30) had demonstrated that the extracellular portion of the v-*erbB* R product is dispensable for transforming potential. In addition, Val^{157} is highly conserved among the protein kinase family and may therefore play a significant role in kinase activity (Fig. 1*B*) (21). After site-directed mutagenesis of codon 157 (Fig. 1*A*), the mutant *erbB*, V157I, was expressed in the replication-competent ALV vector RCAN (11, 22) and analyzed for its transforming properties *in vitro* and *in vivo*. The constructs were tested for their ability to induce anchorage-independent growth in soft agar following infection of CEFs and to induce sarcomas following injection of infectious virus into the wing web of 2- to 4-day-old chicks.

The Val^{157} → Ile mutation alone proved capable of activating the sarcomagenic potential of the IA c-*erbB* product. In soft agar, V157I and *erbB* H/C produced >10-fold more colonies than IA c-*erbB* (Table 1). Importantly, these mutant colonies were significantly larger than those produced by IA c-*erbB* (Fig. 2 *A* and *B*). *erbB* H/C also showed a similar phenotype to V157I, giving colonies that were significantly larger than IA c-*erbB*-produced colonies (data not shown). Consistent with these data, the V157I and *erbB* H/C viruses

Table 1. Sarcomagenic potential of *erbB* mutant constructs

Construct	Genotype		Colonies in soft agar, no. per 10^5 CEFs	Sarcoma incidence
	Codon 40	Codon 157		
RCAN	—	—	0	0/13
IA c-*erbB*	Arg	Val	660	1/21
V157I	Arg	Ile	7400	13/13
erbB H/C	Trp	Ile	7200	13/13
V157L	Arg	Leu	2800	NT
V157M	Arg	Met	650	NT
V157R	Arg	Arg	0	NT
V157W	Arg	Trp	0	NT

NT, not tested.

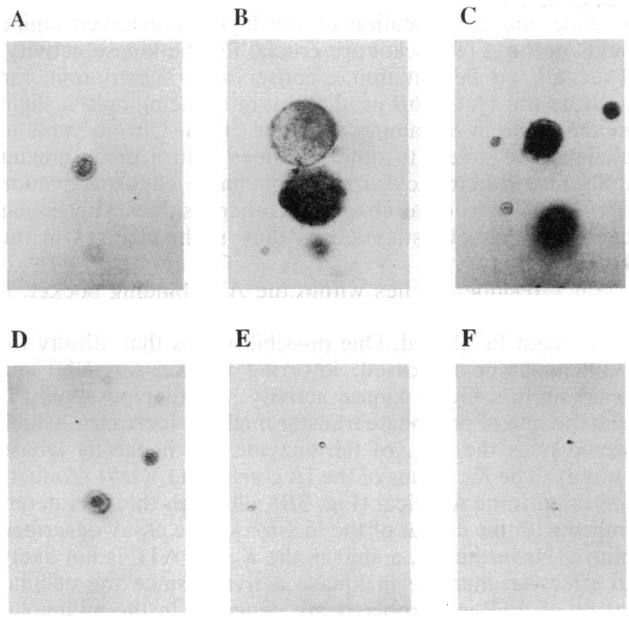

FIG. 2. Ability of various *erbB* genes to induce anchorage-independent growth of CEFs in soft agar. The expression vector containing *erbB*, engineered as described, was transfected by electroporation into early-passage CEFs. These transfected cells were then assayed in soft agar as described. Pictured are 17-day-old colonies of CEFs transfected with IA c-*erbB* (*A*), IA c-*erbB* V157I (*B*), IA c-*erbB* V157L (*C*), IA c-*erbB* V157M (*D*), IA c-*erbB* V157R (*E*), and IA c-*erbB* V157W (*F*). (×30.)

reproducibly induced significant wing-web sarcomas whereas the IA c-*erbB* virus was not sarcomagenic. In one instance, the IA c-*erbB* virus produced a small nodule after a long latency just before the bird succumbed to leukemia (Table 1). As a control, a revertant mutation was made, altering Ile^{157} in the *erbB* H/C product to valine. This *erbB* H/C I157V construct lost its transforming potential and was comparable to IA c-*erbB* in activity both *in vitro* and *in vivo* (data not shown).

***In Vitro* Immune Complex Kinase Assay of IA c-*erbB* and V157I Mutant.** Since the activating mutation in codon 157 lies within the tyrosine kinase domain, we examined the kinase activity of IA c-*erbB* and V157I products in an immune complex kinase assay to determine whether an increase in this activity was associated with the increased sarcomagenic potential. An antibody specific for the carboxyl-terminal domain of the *erbB* product was used to immunoprecipitate the two proteins (28). Following immunoprecipitation, protein levels were assayed by Western blot analysis and equal amounts of protein were reacted with [γ-^{32}P]ATP and resolved by SDS/PAGE. Autoradiography showed that both the IA c-*erbB* and V157I products were autophosphorylated (Fig. 3*A*, lanes 1 and 2). They did not show any appreciable difference in their kinase activity in this assay as evidenced by the similar intensity of the two *erbB* bands. Likewise, the degrees of phosphorylation of the exogenous substrate enolase by these two *erbB* products were also similar (data not shown).

Since Val^{157} lies within a highly conserved sequence that contains the putative ATP-binding pocket, we examined whether mutation of this codon affected affinity for ATP. In the *in vitro* kinase assay, the *erbB* products were incubated with various concentrations of ATP. A double reciprocal plot of the data gave an estimated K_m of $\approx$2 μM ATP for both the IA c-*erbB* and V157I products (Fig. 3*B*). Therefore, mutation of Val^{157} to isoleucine does not appear to significantly alter ATP affinity of the *erbB* kinase.

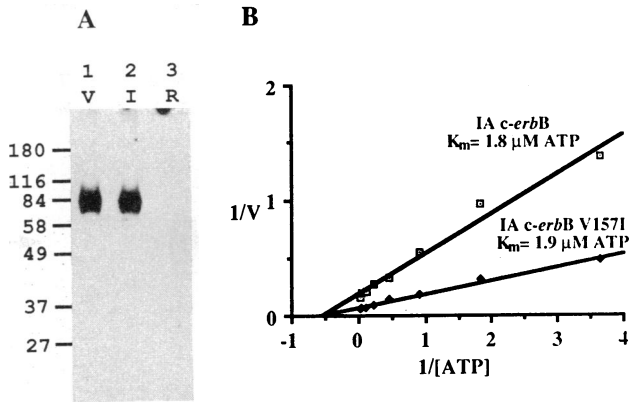

FIG. 3. *In vitro* immune complex kinase assay of *erbB* variants. (*A*) Autoradiogram of IA c-*erbB* (lane 1), IA c-*erbB* V157I (lane 2), and IA c-*erbB* V157R (lane 3) products that were resolved by SDS/10% PAGE following labeling in the *in vitro* kinase assay. Size markers are in kilodaltons. (*B*) Lineweaver–Burk plot of autophosphorylation of *erbB* products at various concentrations of ATP in the *in vitro* kinase assay to determine K_m. The *y* axis represents the reciprocal of Cerenkov counts in the *erbB* band and the *x* axis represents the reciprocal of ATP concentration in μM. Linear regression was performed to calculate the K_m values for ATP.

***In Vivo* Autokinase Assay of IA c-*erbB* and V157I Mutant by Western Analysis.** The *in vitro* immune complex assay detected no increase in the V157I mutant's kinase activity. This result was not totally unexpected in view of the subtle nature of the point mutation. The effect of this change may very easily be obscured by the artificial environment created by the immune complex assay. We noted that the *erbB* product is a membrane protein whose conformation may be very different when in a soluble form. An additional limitation of this assay is that the antibodies capable of immunoprecipitating the *erbB* product are directed either against the kinase domain or against the carboxyl-terminal domain (28). Both are likely to affect the efficiency of the autophosphorylation reaction and thereby mask the effect of the Val → Ile change.

We therefore decided to investigate the *in vivo* kinase activity of the *erbB* proteins. To do this, we analyzed the *erbB* proteins isolated from the transfected/infected CEFs by Western analysis. Duplicate blots were probed with either a polyclonal antibody specific for the carboxyl-terminal domain of the *erbB* product (28) or an anti-phosphotyrosine monoclonal antibody. The anti-*erbB* blot shows that the *erbB* protein products were expressed comparably in cells transfected/infected by the different *erbB* virus constructs (Fig. 4*A*, lanes 2 and 3). The presence of multiple bands and size heterogeneity were due to the two products of alternatively spliced *erbB* transcripts as well as different states of glycosylation (11, 28, 31). Interestingly, the anti-phosphotyrosine blot showed that the level of autophosphorylation of the V157I mutant (Fig. 4*B*, lane 3) was 5- to 6-fold greater (based on densitometric scans from three independent experiments) than its Val^{157} counterpart, the IA c-*erbB* gene product (Fig. 4*B*, lane 2). This suggests that changing the ATP-binding site can modulate the autokinase activity of the *erbB* product.

Alternative Amino Acid Substitutions at Residue 157. Val^{157} lies within the invariant motif Gly-X-Gly-X-X-Gly-X-Val, which comprises a portion of the ATP-binding pocket in nearly all kinases (Fig. 1*B* and ref. 21). Since this site is so highly conserved, one intuitively, would think that mutations there would severely compromise the kinase activity, possibly rendering it inactive altogether. Instead, the isoleucine mutation at this site activates fibroblast-transforming potential and apparently enhances the *in vivo* kinase activity. To test the specificity of this mutation, we substituted additional amino acids for Val^{157} and analyzed their *in vitro* transform-

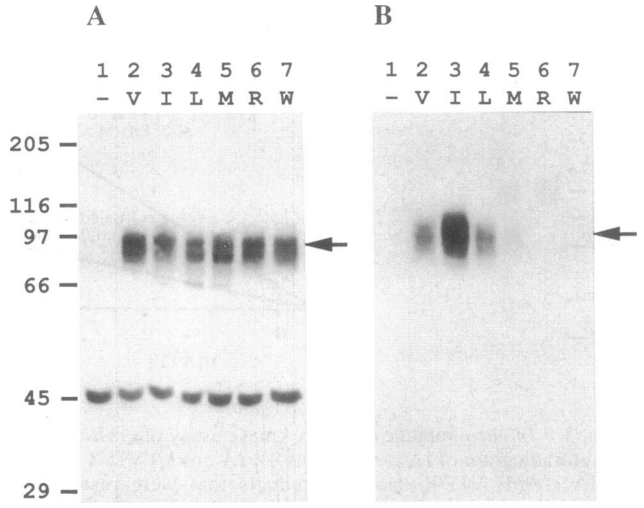

FIG. 4. Western blots showing the level of *erbB* expression and tyrosine phosphorylation in CEFs transfected with RCAN containing no insert (lanes 1), IA c-*erbB* (lanes 2), IA c-*erbB* V157I (lanes 3), IA c-*erbB* V157L (lanes 4), IA c-*erbB* V157M (lanes 5), IA c-*erbB* V157R (lanes 6), and IA c-*erbB* V157W (lanes 7). Total cellular lysate from 5×10^5 to 1×10^6 cells was loaded in each lane. The blots were probed with a rabbit polyclonal antibody specific for the carboxyl-terminal domain of the *erbB* product (*A*) and a mouse anti-phosphotyrosine monoclonal antibody (*B*). ^{125}I-labeled protein A was used to detect the antibody probes. Arrow indicates the *erbB* band. Size markers are in kilodaltons.

ing potential as well as their ability to autophosphorylate *in vivo*. When either arginine or tryptophan was substituted for valine, CEFs transfected/infected with these mutants were unable to form even small baseline clusters (Table 1; Fig. 2 *E* and *F*). In contrast, methionine substitution resulted in growth patterns approximately the same as for the IA c-*erbB* construct (Table 1; Fig. 2*D*). Finally, leucine substitution showed a somewhat increased transforming activity, although its level was not comparable to the activity displayed by the isoleucine mutant (Table 1; Fig. 2*C*). Of these new substitutions, the leucine mutant displayed the highest transforming potential, which is consistent with leucine's structural similarity to isoleucine. These new mutants' *in vivo* autophosphorylation activities appeared to correlate with their *in vitro* transforming ability, with the arginine and tryptophan mutants displaying no autophosphorylation activity, the methionine mutant displaying some autophosphorylation activity, and the leucine mutant displaying the highest autophosphorylation activity of these four mutants (Fig. 4, lanes 4–7). The arginine mutant, which has a completely inactivating alteration according to the Western blot data, was also inactive in the *in vitro* immune complex kinase assay (Fig. 3*A*, lane 3). Thus, the phosphorylation state of the *erbB* product *in vivo* as measured by anti-phosphotyrosine immunoblot correlated well with the ability to induce anchorage-independent growth in CEFs. The *in vitro* immune complex assay did not show quantitative differences between transforming and partially transforming mutants but did confirm totally inactive ("knock-out") mutations.

DISCUSSION

We have identified a point mutation in the ATP-binding pocket of the *erbB*/EGFR kinase domain that increases the sarcomagenic potential of this oncogene and the *in vivo* autophosphorylation activity of the enzyme. Previously engineered mutations in the kinase domain of the human EGFR, especially in the ATP-binding pocket, most frequently resulted in loss of kinase activity (32–35). This indicates that spacing and conformation of the highly conserved amino acids in the ATP pocket are crucial for the kinase activity. The $Val^{157} \rightarrow$ Ile mutation, a conservative substitution, can activate the IA c-*erbB* product by introducing only a slight modification in the amino acid side chain. Our attempts to change this moiety to other residues with a much greater difference in amino acid size and/or charge led to inactivation of the kinase, whereas changes to other residues with similar chemical characteristics led to only slight changes in the kinase activity.

Since residue 157 lies within the ATP-binding pocket, it seemed plausible that the enzymatic properties relative to ATP might be altered. One possibility was that affinity for ATP might be increased, lowering the K_m for ATP and resulting in a higher kinase activity. Another possibility is that the rate of phosphate transfer might be increased, which would raise the V_{max} of the enzyme and hence its kinase activity. The K_m values of the IA c-*erbB* and V157I products appeared to be identical (Fig. 3*B*), although this was determined with the caveat of the *in vitro* kinase assay described above. Nevertheless, a shift in the K_m for ATP is not likely to effect an increase in kinase activity, since the cellular levels of ATP in fibroblasts are generally in the millimolar range (36), far above our observed K_m. We therefore favor a model in which this mutation leads to a subtle shift in the conformation of the ATP-binding pocket that affects the efficiency of the phosphotransfer reaction. However, our observations do not exclude the formal possibility that the increased phosphorylation of the *erbB* product is due to other tyrosine kinases.

One fascinating feature of the avian *erbB* system is the display of tissue-specific transformation. IA c-*erbB*, although a very potent leukemia oncogene, does not have significant fibroblast-transforming activity. The $Val^{157} \rightarrow$ Ile change engendered an *erbB* product with appreciable sarcomagenic activity, in addition to its leukmogenic activity. At least three explanations can account for the increased fibroblast-transforming activity displayed by this mutant. (*i*) This mutation may alter the substrate binding specificity, permitting the protein to recognize a fibroblast-specific substrate that is critical for its transformation, whereas, in erythroblasts, a different substrate is present and the mutation is not necessary. This model awaits a better definition of the substrates phosphorylated by the *erbB*-encoded kinase in these different tissues. Recently, a number of substrates (e.g., phospholipase C-γ, phosphatidylinositol 3-kinase, Raf-1) have been implicated in growth signaling for human EGFR (37–42). (*ii*) It is possible that similar substrates are present in both erythroblasts and fibroblasts but their phosphorylation thresholds for transformation are different. A 5-fold increase in kinase activity might attain the threshold of fibroblast transformation. (*iii*) Bertics, Gill, and coworkers (43, 44) have proposed that autophosphorylated tyrosine residues in the carboxyl-terminal domain competitively inhibit phosphorylation of exogenous substrates by EGFR. The V157I mutant possesses a much higher autokinase activity, which removes the hindrance and enables the receptor to interact with critical substrates more effectively in fibroblasts. In erythroblasts, it is possible that a cellular kinase (similar to the c-*src* kinase) exists that will leave the receptor already phosphorylated and in an "open" conformation. These models are not mutually exclusive and are testable.

Since the valine residue is so highly conserved among kinases, it would be interesting to see whether this activation is specific for the *erbB* product or whether it is generally applicable to other kinases. We do not know whether the $Val^{157} \rightarrow$ Ile mutation will activate the intact *erbB*-encoded growth factor receptor in a ligand-dependent manner. It is possible that this activating event is specific to the truncated receptor, since ligand binding may effect such a gross con-

formational change of the receptor that it obscures the subtle perturbation of the local ATP environment by the mutation at the conserved valine.

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