Mutation of a Protein Kinase C Phosphorylation Site in the *erbB* Protein of Avian Erythroblastosis Virus

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Tumor promoter-stimulated phosphorylation of threonine 98 of the *erbB* protein of avian erythroblastosis virus (AEV) correlates with inhibition of *erbB*-dependent mitogenesis. To more clearly define the role of phosphorylation of this residue in regulation of the activity of the *erbB* protein, we have constructed *erbB* mutations which encode alanine (Ala-98), tyrosine (Tyr-98), or serine (Ser-98) at position 98. The biosynthesis and stability of the three mutant proteins were similar to those of the wild-type *erbB* protein, and all three retained the ability to transform chicken embryo fibroblasts. Treatment of transformed CEF with 12-tetradecanoylphorbol-13-acetate (TPA) stimulated incorporation of $^{32}\text{P}_i$ into wild-type and mutant *erbB* proteins and resulted in a slight decrease in the electrophoretic mobilities of all the *erbB* proteins. Tryptic maps of *erbB* phosphopeptides showed no endogenous or TPA-stimulated phosphorylation of alanine 98 or tyrosine 98 in cells transformed by the Ala-98 and Tyr-98 mutants. Analysis of tryptic phosphopeptides by high-pressure liquid chromatography revealed that TPA treatment of cells stimulated phosphorylation of other sites of the *erbB* protein in addition to threonine 98. A high endogenous level of phosphorylation of serine 98 of the Ser-98 mutant protein was found, and TPA treatment of cells did not result in further phosphorylation of this residue. Cells transformed by wild-type and mutant AEV were equally sensitive to TPA-dependent inhibition of growth in soft agar and TPA-dependent inhibition of $[^3\text{H}]$ thymidine incorporation. TPA treatment inhibited tyrosine phosphorylation to a similar extent in cells transformed by wild-type or Ala-98 AEV. These data indicate that phosphorylation of threonine 98 of the *erbB* protein is not responsible for TPA-dependent inhibition of growth of AEV-transformed cells or TPA-induced inhibition of *erbB*-dependent tyrosine phosphorylation. TPA-stimulated phosphorylation of the *erbB* protein at other sites may mediate these effects. The data also show that subtle changes in a phosphorylation site (i.e., changing threonine to serine) can drastically alter recognition by protein kinases.

Avian erythroblastosis virus (AEV) causes erythroblastosis and sarcomas in chickens (13) and transforms erythroblasts and fibroblasts in culture (11, 13). The *erbB* gene of AEV has been shown to be responsible for cellular transformation by AEV (11, 28) and encodes a protein related to the *src* gene tyrosine kinase family (29). The *erbB* protein shares extensive homology with the cytoplasmic domain of the epidermal growth factor (EGF) receptor (8). This region includes the tyrosine-specific protein kinase domain which appears to be responsible for the transmission of mitogenic signals by these proteins. For the EGF receptor, activation of this protein kinase requires ligand binding; however, the *erbB* protein lacks the ligand-binding domain and may be constitutively active.

The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been shown to stimulate in vivo phosphorylation of threonine 654 of the EGF receptor (5, 17) and phosphorylation of an equivalent threonine residue of the *erbB* protein (7). TPA-induced phosphorylation of the EGF receptor correlates with inhibition of both EGF binding (21) and inhibition of EGF receptor tyrosine kinase activity (4, 6, 10). TPA also inhibits EGF-dependent mitogenesis in normal human fibroblasts (6), anchorage-independent growth of AEV-transformed chicken embryo fibroblasts (CEF) (7), EGF-stimulated $\text{Na}^+\text{-H}^+$ exchange (27), EGF-dependent increases in intracellular Ca^{2+} in A431 cells (24), and the effects of EGF on phosphatidylinositol metabolism (26). These responses to TPA are probably mediated through activation of protein kinase C (3).

Protein kinase C regulates the activity of a number of other receptor systems including those for β -adrenergic agonists (20), thrombin (30), α_1 -adrenergic agonists (22), and muscarinic agonists (19). For some of these systems, phorbol esters induce receptor phosphorylation (22), although it has not been shown conclusively that uncoupling of receptor function is a result of receptor phosphorylation.

To more clearly define the mechanisms involved in TPA-dependent inhibition of growth of AEV-transformed CEF, we have examined the effects of site-directed mutagenesis of threonine 98 of the *erbB* protein from AEV strain H on *erbB* protein metabolism and on cellular responses to TPA.

MATERIALS AND METHODS

Materials. TPA and 4- α phorbol were from LC Biochemicals. L-1-Tosylamido-2-phenylethylchloromethyl ketone-treated trypsin was from Sigma Chemical Co. $[^{35}\text{S}]$ methionine (1,000 Ci/mmol) was from Dupont, NEN Research Products, and carrier-free $^{32}\text{P}_i$ was from ICN Pharmaceuticals, Inc.

Cell culture. Normal and transformed CEF were grown in Dulbecco modified Eagle medium-Ham's F-12 medium (1:1) containing 5% calf serum and 1% heat-inactivated chicken serum. Primary cultures of CEF were transformed by wild-type and mutant AEV by incubation with culture supernatants from cells previously transfected with AEV DNA and UR2AV helper virus DNA. Transfections were performed by the method of Neckameyer and Wang (25).

In vitro mutagenesis. Plasmid pAE7.7 containing the complete genome of AEV-H (29) was kindly provided by K. Toyoshima, University of Tokyo. The 3.5-kilobase *Kpn*I-

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XhoI *erbB* gene-containing fragment from pAE7.7 was subcloned into plasmid pIBI30 (International Biotechnologies, Inc.) which had been cut with *KpnI* and *XhoI* (pIBIKX). Oligonucleotides were synthesized in which the codon for threonine 98 was changed from ACC to either GCC (coding for alanine), TAC (tyrosine), or TCC (serine). These oligonucleotides were used for in vitro mutagenesis of the *erbB* gene in pIBIKX by using the gapped-duplex procedure. Briefly, pIBIKX was cut with *XhoI* or with *ApaI* and *HincII*, and the linearized plasmid and plasmid containing the *ApaI*-*HincII* gap were isolated, mixed, and then melted and reannealed in the presence of one of the mutant oligonucleotides. This procedure generates a proportion of circular plasmids with single-stranded gaps between the *ApaI* and *HincII* sites of the *erbB* gene to which the mutant oligonucleotides have hybridized. Gaps were filled in with the Klenow fragment of DNA polymerase and T4 DNA ligase, and the preparations were used for transformation. Colonies were screened by hybridization with the appropriate mutant oligonucleotides, and the entire *erbB* gene was sequenced to confirm the mutations. The *KpnI*-*XhoI* *erbB* inserts from pIBIKX containing the various mutations were ligated back into pAE7.7, and this DNA, along with UR2AV helper virus DNA (provided by L. H. Wang, Rockefeller University), was used to transfect secondary cultures of CEF.

Radiolabeling and immunoprecipitation of the *erbB* protein. For pulse-labeling with [³⁵S]methionine, approximately 5×10^5 cells in 35-mm dishes were incubated for 30 min in methionine-free F-12 medium containing 5% dialyzed calf serum and 100 μ Ci of [³⁵S]methionine per ml prior to lysis and immunoprecipitation with rabbit antibody to the *v-erbB* protein as described (6, 7). For equilibrium labeling, cells were incubated for 36 h in growth medium containing 20% of the normal amount of methionine and 100 μ Ci of [³⁵S]methionine. Cells were labeled with ³²P_i as previously described (6).

Peptide synthesis and preparation of ³²P-labeled peptide. The pentapeptide Lys-Arg-Ser-Leu-Arg was synthesized by the Rockefeller University Sequencing Facility. The peptide was purified by high-pressure liquid chromatography (HPLC) and phosphorylated by the catalytic subunit of the cAMP-dependent kinase (Sigma). The kinase reaction mixture contained 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2), 10 mM MgCl₂, 1 μ M peptide, 1 μ M [α -³²P]ATP (10 μ Ci), 0.5 mM phenethylsulfonyl fluoride, and 1 U of kinase activity and was incubated at 24°C for 15 min. Phosphorylated peptide was isolated by thin-layer electrophoresis at pH 8.9 [1% (NH₄)₂CO₃]. To generate the phosphopeptides Arg-P-Ser-Leu-Arg and P-Ser-Leu-Arg, we incubated the pentapeptide with trypsin for various times and separated the two less-basic species by electrophoresis at pH 8.9.

Tryptic peptide analysis by HPLC. The *erbB* proteins from ³²P-labeled cells were immunoprecipitated, reduced, and carboxymethylated by the method of Cassel et al. (2). The proteins were run on sodium dodecyl sulfate-gels, *erbB* bands were cut from the gels, and the gel slices were trypsin treated by the method of Gullick et al. (14). The digests were loaded onto a C₁₈ reverse-phase HPLC column equilibrated with 0.05% trifluoroacetic acid. Peptides were eluted with a gradient of 0 to 60% acetonitrile at 1 ml/min, and 1-ml fractions were collected and counted for Cerenkov radiation.

Phosphoamino acid analysis was performed by the method of (6). Two-dimensional peptide analysis, assay of soft-agar growth, and measurement of [³H]thymidine incorporation were performed as previously described (6, 7).

FIG. 1. Biosynthesis of the *erbB* protein in CEF transformed by wild-type and mutant AEV. Cells were labeled with [³⁵S]methionine for 30 min (lanes A through D) or for 36 h (lanes E through H) prior to lysis and immunoprecipitation with anti-*erbB* serum as described in the text. The *erbB* proteins were immunoprecipitated from wild-type AEV-transformed cells (lanes A and E), Ala-98 AEV-transformed cells (lanes B and F), Tyr-98 AEV-transformed cells (lanes C and G), and Ser-98 AEV-transformed cells (lanes D and H). Molecular weights (in thousands) are indicated.

RESULTS

To investigate the relationship between TPA-dependent phosphorylation of threonine 98 of the *erbB* protein and TPA-dependent inhibition of growth of AEV-transformed cells, we made mutations in the *erbB* gene of AEV H which resulted in the replacement of threonine 98 of the *erbB* protein with either an alanine (Ala-98), a tyrosine (Tyr-98), or a serine (Ser-98) residue. The alanine substitution was chosen because it was relatively conservative, and the tyrosine and serine substitutions were made to evaluate the effects of placing other potentially phosphorylatable amino acids at a threonine-specific protein kinase C site. It was also hoped that analysis of three mutations would minimize possible spurious interpretation of results as a result of destabilization of the *erbB* protein by a particular mutation.

erbB precursor proteins of M_r 72,000 and 74,000 were synthesized in similar amounts in cells transformed by the wild-type virus and by the three mutant viruses (Fig. 1). Steady-state levels of the mature M_r 78,000 to 88,000 *erbB* protein were also unaffected by the mutations at position 98.

Since the biosynthesis of the *erbB* proteins appeared to be unaltered by the mutations, the effects of TPA on in vivo phosphorylation of the *erbB* proteins were examined (Fig. 2). Figure 2A shows wild-type and mutant *erbB* proteins immunoprecipitated from ³²P-labeled cells treated for 5 min with 100 ng of 4- α -phorbol per ml or with 25 ng of TPA per ml. TPA treatment caused an 80 to 120% increase in incorporation of ³²P into all the *erbB* proteins. In addition, TPA treatment caused a slight decrease in the electrophoretic mobility of the *erbB* proteins.

As shown previously for the *erbB* protein from CEF transformed by the ES4 strain of AEV (7), TPA treatment of wild-type AEV-H-transformed CEF stimulated the phosphorylation of a basic phosphothreonine containing peptide (Fig. 2, panel B2, peptide z). This phosphopeptide is identical to peptide z, which has been shown by Hunter et al. (17) to be phosphorylated at threonine 654 of the EGF receptor when A431 cells are treated with TPA (6; S. Decker, unpublished results). The sequence of this phosphopeptide is Lys-Arg-PThr-Leu-Arg, and it contains threonine 98 of the *erbB* protein.

FIG. 2. Immunoprecipitation and tryptic phosphopeptide analysis of in vivo ^{32}P -labeled *erbB* proteins from AEV-transformed CEF. (A) ^{32}P -labeled *erbB* proteins were immunoprecipitated from CEF transformed by wild-type AEV (lanes 1 and 2), Ala-98 AEV (lanes 3 and 4), Tyr-98 AEV (lanes 5 and 6), or Ser-98 AEV (lanes 7 and 8) and analyzed on a sodium dodecyl sulfate-7.5% polyacrylamide gel. Prior to lysis, cells were treated for 5 min with 100 ng 4- α -phorbol per ml (lanes 1, 3, 5, and 7) or 25 ng of TPA per ml (lanes 2, 4, 6, and 8). Cells were labeled for 12 h as described in the text. (B) Tryptic phosphopeptide analysis of *erbB* proteins from panel A. Tryptic patterns of wild-type *erbB* proteins from control and TPA-treated cells are shown in panels 1 and 2, respectively; patterns from Ala-98 *erbB* protein are shown in panels 3 and 4, patterns from Tyr-98 *erbB* protein are shown in panels 5 and 6, and patterns from Ser-98 *erbB* protein are shown in panels 7 and 8. Two-dimensional peptide analysis was performed as described in the text. Electrophoresis was in the horizontal dimension with the cathode to the left.

As expected for the Ala-98 and Tyr-98 mutations, TPA treatment does not stimulate phosphorylation of a peptide with mobility similar to that of peptide z, although overall phosphopeptide patterns are similar to that of the wild-type *erbB* protein. The two-dimensional map of Ser-98 *erbB* protein from control cells contained three phosphopeptides (Fig. 2, panels B7 and B8, peptides x, y, and z) not found in *erbB* protein from wild-type, Ala-98, or Tyr-98 control cells. TPA treatment did not appear to affect the phosphorylation of these peptides. All three peptides contained phosphoserine exclusively (data not shown), and the mobility of the

FIG. 3. Constitutive phosphorylation of serine 98 in the Ser-98 *erbB* protein. The peptide Lys-Arg-Ser-Leu-Arg was synthesized, ^{32}P labeled, and trypsin treated as described in the text. (A) This phosphorylated pentapeptide (designated z') and the peptides Arg-P-Ser-Leu-Arg (y') and P-Ser-Leu-Arg (x') derived from it were subjected to two-dimensional thin-layer analysis as described in the text. (B) The respective synthetic phosphopeptides were mixed with the in vivo-phosphorylated peptides x, y, and z from the Ser-98 *erbB* protein as shown in Fig. 2B, panels 7 and 8. Equal counts of peptide x and peptide x' were mixed and run in two dimensions in panel 1, equal counts of peptide y and y' were used in panel 2, and equal counts of peptides z and z' were used in panel 3. About 300 cpm (Cerenkov radiation) was applied for each peptide. Electrophoresis was in the horizontal dimension (cathode to the left). The origins are marked with dots.

Ser-98 z peptide was almost identical to the mobility of peptide z from TPA-treated cells transformed with wild-type AEV. This suggested that replacement of threonine 98 with serine may have altered the accessibility of trypsin to basic residues in this region and resulted in the generation of three phosphopeptides containing serine at position 98 (P-Ser-Leu-Arg, Arg-P-Ser-Leu-Arg, and Lys-Arg-Ser-Leu-Arg). To examine this possibility, we eluted these three peptides from thin-layer plates and exhaustively retrypsinized them. This treatment had no effect on the mobility of peptide Ser-98 x but resulted in the loss of peptides Ser-98 y and Ser-98 z and the generation of phosphopeptides which comigrated with peptide Ser-98 x from these peptides (data not shown). To further substantiate the identity of these peptides, the peptide Lys-Arg-Ser-Leu-Arg was synthesized, ^{32}P -labeled with cyclic AMP kinase catalytic subunit, and trypsinized. As expected, the phosphorylated peptide contained only phosphoserine, and trypsin treatment resulted in the appearance of two less-basic phosphorylated peptides presumed to be Arg-P-Ser-Leu-Arg and P-Ser-Leu-Arg. The positions of the tripeptide (labeled x'), the tetrapeptide (y'), and the pentapeptide (z'), in two-dimensional analysis are shown in Fig. 3. Peptides x', y', and z' were mixed with peptides x, y, and z, respectively, from in vivo-labeled Ser-98-*erbB* protein (as in Fig. 2, panels B7 and B8) and run in two dimensions. Peptides x and x' comigrated, as did peptides y and y' and peptides z and z' (Fig. 3B). These data support the premise that Ser-98 x, Ser-98 y, and Ser-98 z form a set of related peptides containing phosphorylated serine 98.

FIG. 4. Tryptic phosphopeptide analysis of *erbB* proteins from cells transformed by wild-type (A) or Ala-98 (B) AEV by reverse-phase HPLC. Cells were treated for 5 min with 100 ng of 4- α -phorbol per ml (●) or 25 ng of TPA per ml (○) prior to lysis. Cells were labeled with 32 P, *erbB* proteins were immunoprecipitated and trypsin treated, and phosphopeptides were analyzed on a C_{18} column as described in the text.

Since TPA stimulated the phosphorylation of the *erbB* proteins from the Ala-98 and Tyr-98 mutants, sites in the *erbB* protein other than threonine 98 must be phosphorylated. To more closely examine this possibility, *erbB* proteins from control and TPA-treated cells transformed by wild-type or Ala-98 AEV were trypsin treated, and resultant peptides were analyzed by reverse-phase HPLC with a C_{18} column (Fig. 4). TPA treatment led to increased phosphorylation of several peptides. A phosphopeptide which eluted after about 4 min was found only in TPA-treated cells transformed by wild-type AEV. When this peptide was run in two-dimensional thin-layer analysis, it was found to comigrate with peptide z (Fig. 2, panel B2), indicating that it is the pentapeptide Lys-Arg-PThr-Leu-Arg, containing threonine 98 (data not shown). TPA treatment stimulated phosphorylation of other peptides from wild-type and Ala-98 *erbB* proteins, which eluted between 18 and 28 min, and phosphorylation of these peptides was stimulated to a similar extent in *erbB* proteins from wild-type and Ala-98 AEV.

The effects of TPA on the growth of wild-type AEV- and mutant AEV-transformed CEF were compared. All three mutant AEVs were judged to be comparable to wild-type AEV in terms of their ability to alter the cellular morphology of infected cells, to cause loss of contact inhibition in monolayer culture, and to induce formation of large colonies in soft agar. As previously shown for AEV-ES4-transformed CEF (7), relatively low concentrations of TPA caused marked inhibition of soft-agar growth of AEV-H-transformed CEF (Table 1). The mutations at position 98 of the *erbB* protein had no discernable effect on TPA sensitivity of AEV-transformed CEF. The effects of TPA on incorporation of [3 H]thymidine in normal and transformed CEF were also

TABLE 1. Effects of TPA on anchorage-independent growth of CEF transformed by wild-type and mutant AEV

Type of AEV-H	No. of colonies per dish ^a		
	Control	Plus TPA (5 ng/ml)	Plus TPA (25 ng/ml)
Wild-type	1,060 $\pm$ 126	174 $\pm$ 28	22 $\pm$ 6
Ala-98	1,275 $\pm$ 176	182 $\pm$ 14	18 $\pm$ 4
Tyr-98	956 $\pm$ 86	158 $\pm$ 6	24 $\pm$ 8
Ser-98	1,254 $\pm$ 192	156 $\pm$ 22	24 $\pm$ 6

^a Single-cell suspensions of cells (2×10^3 cells per 35-mm dish) were plated in 0.3% agar in normal growth medium containing either 100 ng of 4- α -phorbol per ml (control) or TPA as indicated. Cells were incubated for 10 to 14 days, at which time colonies were counted. Colonies larger than 0.1 mm in diameter were scored as positive. Assays were performed in duplicate, and the mean and range are given.

compared (Table 2). In normal CEF, serum starvation rapidly reduced [3 H]thymidine incorporation, and addition of TPA or serum resulted in stimulation of [3 H]thymidine incorporation. However, for CEF transformed by wild-type or mutant AEV, serum starvation had little effect on [3 H]thymidine incorporation, and TPA addition caused an approximate 50% inhibition of [3 H]thymidine incorporation.

Since phosphorylation of threonine 654 of the EGF receptor correlates with inhibition of receptor tyrosine kinase activity, we examined the effects of TPA treatment on phosphotyrosine levels in wild-type and Ala-98 AEV transformed CEF. Transformation by AEV-H resulted in an approximate twofold increase in whole-cell levels of phosphotyrosine (Table 3). TPA treatment of cells for 2 h led to

TABLE 2. Effects of TPA and serum on [3 H]thymidine incorporation by serum-starved cultures of normal and AEV-transformed CEF^a

Cell type and addition ^b	[3 H]thymidine incorporation (cpm/mg of protein) ^c
CEF	
4- α -Phorbol	2,254 $\pm$ 234
TPA	7,874 $\pm$ 1,280
Calf serum	17,934 $\pm$ 3,486
Thr-98 AEV CEF	
4- α -Phorbol	18,567 $\pm$ 2,162
TPA	8,646 $\pm$ 856
Calf serum	22,834 $\pm$ 3,398
Ala-98 AEV CEF	
4- α -Phorbol	26,865 $\pm$ 2,904
TPA	14,736 $\pm$ 2,476
Calf serum	25,626 $\pm$ 3,654
Tyr-98 AEV CEF	
4- α -Phorbol	19,550 $\pm$ 1,250
TPA	8,174 $\pm$ 1,079
Calf serum	25,712 $\pm$ 3,870
Ser-98 AEV CEF	
4- α -Phorbol	21,734 $\pm$ 1,794
TPA	12,022 $\pm$ 2,087
Calf serum	22,947 $\pm$ 1,956

^a Cells were incubated for 12 h in serum free F-12 medium before the additions were made. [3 H]thymidine incorporation was assayed 20 h after the additions.

^b 4- α -Phorbol was added at 100 ng/ml; TPA was added at 25 ng/ml; calf serum was added at 5%.

^c Mean and range of duplicate determinations.

TABLE 3. Effects of TPA on whole-cell levels of acid-stable phosphotyrosine in normal and AEV-transformed CEF

Cell type and addition ^a	% Phosphotyrosine ^b
Normal CEF	
4- α -Phorbol	0.084 $\pm$ 0.010
TPA	0.087 $\pm$ 0.006
AEV-H CEF	
4- α -Phorbol	0.194 $\pm$ 0.012
TPA	0.151 $\pm$ 0.014
AEV-H Ala-98 CEF	
4- α -Phorbol	0.201 $\pm$ 0.017
TPA	0.157 $\pm$ 0.009

^a 100 ng of 4- α -phorbol per ml or 25 ng of TPA per ml was included during the last 2 h of the 12-h labeling period.

^b Percent phosphotyrosine is expressed as the percentage of total acid-stable phosphoamino acids. In these experiments, percentages of phosphothreonine and phosphoserine were constant at about 8% and 92%, respectively. Procedures were performed as described in the text. The mean and range of duplicate samples are shown.

a partial (30 to 40%) inhibition of this increase in cells transformed by wild-type or Ala-98 AEV. Longer (or shorter) incubation with TPA did not lead to greater inhibition. As judged by two-dimensional thin-layer analysis, threonine 98 remained phosphorylated following TPA treatment of cells for at least 2 h (results not shown).

DISCUSSION

To investigate the functional significance of phosphorylation of threonine 98 of the *erbB* protein, we have introduced point mutations in the *erbB* gene at this position. Cells transformed by the mutant AEV were equally sensitive to TPA-dependent inhibition of growth, even though position 98 was not phosphorylated in TPA-treated Ala-98 AEV or Tyr-98 AEV-transformed cells. In addition, control Ser-98 AEV-transformed cells, in which considerable endogenous phosphorylation of serine 98 was evident, still exhibited the transformed phenotype. These data indicate that TPA-stimulated phosphorylation of threonine 98 is not responsible for the growth-inhibitory effects of TPA on AEV-transformed cells.

Phosphorylation at threonine 98 also does not appear to be responsible for TPA-induced inhibition of tyrosine phosphorylation in AEV-H-transformed cells. TPA inhibited tyrosine phosphorylation in Ala-98 AEV-transformed cells to the same extent as in wild-type AEV-transformed cells. Phosphorylation of the equivalent residue of the EGF receptor (threonine 654) may be involved in modulation of high-affinity binding of ligand by the EGF receptor as suggested by Lin et al. (23) or may serve some as yet unknown function.

TPA treatment of wild-type AEV- and mutant AEV-transformed CEF stimulated phosphorylation of all the *erbB* proteins, indicating that TPA induces phosphorylation of the *erbB* proteins at sites other than threonine 98. Analysis of tryptic peptides by HPLC confirmed that TPA treatment resulted in increased phosphorylation of several other sites of the *erbB* protein. Earlier studies of EGF receptor phosphorylation suggest that TPA treatment leads to increased phosphorylation of serine residues as well as threonine (5, 16, 18). The growth-inhibitory effects of TPA on AEV-transformed cells may be mediated through phosphorylation of these additional sites or through some unknown mechanism not involving *erbB* phosphorylation. Use of the *erbB*

mutants described in this study should facilitate identification of the additional TPA-induced phosphorylation sites in the *erbB* protein.

Surprisingly, considerable phosphorylation of serine 98 of the Ser-98 mutant *erbB* protein was found in normally growing cells. Protein kinase C is able to phosphorylate serine or threonine residues; however, there is no reason to believe that the threonine-to-serine mutation in the Ser-98 mutant would constitutively activate protein kinase C. The fact that the TPA still stimulates phosphorylation of the *erbB* protein in Ser-98-transformed cells supports this contention. Since many protein kinases recognize basic residues on the amino- and carboxy-terminal sides of phosphorylation sites, some other kinase may be responsible for serine 98 phosphorylation. Both the cyclic AMP- and cyclic GMP-dependent kinases require multiple basic residues on the amino-terminal side of a phosphorylation site and prefer serine to threonine (9). On the basis of work with peptide substrates, a general cyclic AMP kinase recognition sequence of Arg-Arg-X-Ser-Y- has been determined (1).

Serine 32 of histone H2B is preferentially phosphorylated by cyclic GMP-dependent kinase and is contained within the sequence Arg-Lys-Arg-Ser-Arg-Lys (15), a region similar to the sequence surrounding serine 98 in the Ser-98 *erbB* protein. Calmodulin-dependent kinase and phosphorylase kinase (9), among others, also recognize basic residues in the vicinity of the phosphorylation site. On the basis of present knowledge of protein kinase sequence requirements and the data presented in this study, it is not possible to determine which protein kinase is responsible for constitutive phosphorylation of serine 98. It is interesting, however, that such a mutation can radically alter the phosphorylation state of a protein, and this suggests an approach for engineering specific changes in protein function. It would be of interest to examine the effects of substitution of serine at position 654 on the function of the EGF receptor.

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