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Phosphorylation of E47 as a Potential Determinant of B-Cell-Specific Activity

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The E2A gene encodes two basic helix-loop-helix proteins designated E12 and E47. Although these proteins are widely expressed, they are required only for the B-lymphocyte lineage where DNA binding is mediated distinctively by E47 homodimers. By studying the properties of ΔE47, an N-terminal truncation of E47, we provide evidence that phosphorylation may contribute to B-cell-specific DNA binding by E47. Two serines N terminal to the ΔE47 basic helix-loop-helix domain were found to be phosphorylated in a variety of cell types but were hypophosphorylated in B cells. Phosphorylating these serines in vitro inhibited DNA binding by ΔE47 homodimers but not by ΔE47-containing heterodimers, such as ΔE47:MyoD. These results argue that hypophosphorylation may be a prerequisite for activity of E47 homodimers in B cells, suggesting the use of an inductive (nonstochastic) step in early B-cell development.

Basic helix-loop-helix (bHLH) proteins have been implicated in a variety of developmental processes. Certain cell-restricted members of this protein family are necessary and, in some cases, sufficient for generating particular cell lineages. For example, all four of the myogenic bHLH proteins, MyoD, myf-5, myogenin, and MRF4, can program muscle gene expression when introduced into a variety of nonmuscle cell lines in culture (reviewed in references 29, 41, and 51). Gene-targeting experiments indicate that either MyoD or myf-5 is sufficient for the generation of myoblasts while myogenin is required for later stages of differentiation (20, 37, 43). Mash-1, a neuron-restricted bHLH protein, is required for the early development of olfactory and autonomic neurons (18), and ectopic expression of NeuroD/BETA2 can direct *Xenopus* epidermal precursor cells towards the neuronal lineage (30, 38). All of these cell-restricted bHLH proteins bind DNA as heterodimers with members of a more widely expressed subfamily of bHLH proteins, known as the E proteins (25, 29, 33, 35). In vertebrates, E proteins include those encoded by the E2A gene (E12 and E47/E2-5), E2-2, and Heb.

E47 was first identified as a protein that binds immunoglobulin and insulin gene regulatory elements (21, 34, 39). Despite the protein's wide tissue distribution, DNA binding by E47 homodimers is B cell restricted (45). In other cell types, E47 either does not bind DNA or binds as a heterodimer with cell-restricted bHLH proteins such as MyoD or NeuroD/BETA2 (28, 35, 36, 38). E47 homodimers efficiently activate immunoglobulin transcription in vivo, and this is related to their unique ability to displace an enhancer-bound repressor (12, 44, 52). Recently, we have shown that overexpressed E47 is sufficient to activate the chromosomal immunoglobulin heavy chain and terminal deoxynucleotidyltransferase loci in non-B cells, suggesting that E47 may have properties expected of a master regulatory protein (8). The importance of E47 in B-cell transcription is underscored by gene-targeting experiments that showed that the principal defect in E2A-deficient mice was a lack of B cells (2, 53).

Given that E47 is widely expressed, it is reasonable to expect that B-cell-restricted DNA binding by E47 homodimers is regulated by protein modification. Understanding the nature of such modifications would be important in its own right but may also have broader implications related to how cell-specific transcription is established in general. Most studies of lineage-restricted transcription have implicated regulatory proteins that are also transcribed in a limited number of cell types. This applies to all of the aforementioned cell-specific bHLH proteins, including MyoD and myogenin, whose mRNAs are restricted to cells of the muscle lineage. Accordingly, none of these bHLH proteins are lineage determining in a true inaugural sense; they must be activated by transcription factors that work further upstream. Two general models, centered on either stochastic or inductive events, have been proposed to account for the inauguration of cell type-specific transcription (40). Stochastic models argue that expression of a lineage-determining transcription factor is activated within a precursor cell by chance but that the consequences of such activation may be irreversible and thus the progenitor cell becomes committed (49). Inductive models argue that cell type-specific transcription is ultimately governed by defined events, most likely involving extracellularly derived signals, that lead to transcription factor activation and lineage determination (10, 50). Posttranslational regulation of E47 may well fall into this latter category, suggesting that at least one early step of B-cell development is governed by an inductive event.

In order to ultimately define the unique properties of B cells that permit DNA binding by E47 homodimers, we have undertaken a study of E47 phosphorylation. Using an N-terminally truncated E47, we show here that phosphorylation of two serine residues inhibits DNA binding by E47 homodimers in vitro. Our results indicate that constitutive activity of E47 homodimers in B cells may require a low steady-state level of phosphorylation at these sites.

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MATERIALS AND METHODS

Protein expression. The eukaryotic expression plasmid pCHNE2-5C (45) was generated from the E2-5 cDNA and used to express ΔE47. In addition to being

shorter, this ΔE47 protein differs from that described by Baltimore and coworkers (26) in that it contains one less serine in its acidic region. The originally published E47 contains an additional serine immediately N terminal to Ser-529 as a consequence of alternative 3′-splice site selection. Despite this difference, we have employed the name ΔE47 (as opposed to ΔE2-5) as a manner of convenience. Moreover, other commonly used E47 cDNAs express proteins that do not contain the additional serine (32a). Point mutations were introduced into pCHNE2-5C with a Transformer site-directed mutagenesis kit (Clontech Laboratories, Inc., Palo Alto, Calif.). ΔE47 and ΔE47(AAS), complete with hemagglutinin tags and nuclear localization signals, were expressed in bacteria as oligonucleotide-histidine fusion proteins by using the bacterial expression plasmid pET-15b (Novagen, Madison, Wis.). ΔE47 and ΔE47(AAS) were purified by nickel-chelate chromatography (Novagen) as per the manufacturer's instructions. Bacterially expressed MyoD protein was generously provided by Hal Weintraub.

Metabolic labeling. NIH 3T3 cells, P2 cells, and HeLa cells were transfected by calcium phosphate essentially as described previously (16) by using 20 μg of plasmid per 100-mm plate (approximately 40% confluent). Ag8 cells (10^6) were transfected with 10 μl of Lipofectin reagent (Gibco BRL) and 3.5 μg of plasmid DNA. Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum was added to a final volume of 5 ml 5 h later. S194 cells (2×10^7 in 20 ml) were transfected with 6.5 μg of DNA with DEAE dextran as previously described (17, 42). Jurkat cells (10^7 in 10 ml) were transfected by electroporation with 40 μg of plasmid DNA (11).

Following the addition of DNA, cells were incubated for the following times prior to the addition of label: NIH 3T3 cells, 2 days; P2 cells, 2 days; Jurkat cells, 1 day; HeLa cells, 1 day; S194 cells, 6 h; and Ag8 cells, 18 h. Adherent cells (NIH 3T3, P2, and HeLa) were each transfected, incubated, and pulse labeled in a single 100-mm dish. $^{32}P_i$ and [^{35}S]Met labelings were carried out in cultures transfected in parallel. Transfected suspension cells (20-ml total volume; Ag8 [4 × 5 ml], S194 [20 ml], and Jurkat [2 × 10 ml]) were split into two 10-ml samples for parallel labeling with [^{35}S]Met and $^{32}P_i$.

For labeling with [^{35}S]Met, cells were washed twice with 10 ml of warm DMEM lacking cysteine and methionine and then incubated for 1 h at 37°C in 5 ml of DMEM (minus Met and Cys) containing 10% dialyzed fetal bovine serum. Cells were then incubated for 1 h at 37°C in the presence of 5% CO_2. A total of 1 mCi of [^{35}S]Met (EXPRE^{35}S^{35}S protein labeling mix containing [^{35}S]Cys; NEN) was added, and cells were incubated for an additional 2 h at 37°C in the presence of 5% CO_2.

For labeling with $^{32}P_i$, cells were treated as for labeling with [^{35}S]Met, except that the DMEM lacked phosphate instead of methionine and cysteine. The phosphate-free medium was also buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Following the addition of 2 mCi of $^{32}P_i$ (>8,000 Ci/mmol; NEN), cells were incubated for 2 h at 37°C (in the absence of CO_2).

Preparation of nuclear extracts. Labeled cells as described above (5 ml) were rinsed twice in ice-cold Tris-buffered saline, resuspended in 900 μl of ice-cold phosphate-buffered saline (PBS), and centrifuged at 4°C in a microcentrifuge. Cell pellets were suspended in 600 μl of hypotonic buffer (25 mM Tris-Cl [pH 7.4], 1 mM $MgCl_2$, 5 mM KCl), and incubated for 5 min on ice. A total of 600 μl of hypotonic buffer containing 1% Nonidet P-40 (NP-40) was then added, and incubation was continued for 5 min on ice. After centrifugation in a microcentrifuge for 3 min at 4°C, nuclear pellets were suspended in 500 μl of radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 8.0]). Nuclei were then sonicated at full power for 30 s in a cup horn (model W-375 sonicator; Heat Systems Ultrasonics, Inc.), rocked at 4°C for 15 min, and centrifuged (100,000 × *g*, 30 min). Supernatants (nuclear extracts) were collected for immunoprecipitations.

Immunoprecipitations. Total trichloroacetic acid (TCA)-precipitable counts (5) in 5-μl nuclear extracts were determined in order to make direct comparisons of ^{32}P and ^{35}S labeling of ΔE47. For each cell type, immunoprecipitations were carried out with 30 times more ^{35}S counts per minute than $^{32}P_i$ counts per minute. Volumes varied between 80 and 400 μl. Immunoprecipitations employed the 12CA5 anti-hemagglutinin monoclonal antibody (Boehringer Mannheim) as described by Bain et al. (1), except that protein A-agarose was used instead of protein A-Sepharose. Samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to autoradiography by using standard techniques.

Phosphoamino acid analysis and V8 peptide maps. Labeled, immunoprecipitated ΔE47 proteins were purified on SDS–10% polyacrylamide gels. Samples (approximately 400 Cerenkov cpm) were eluted from gel slices and analyzed as described previously (6), using endoproteinase Glu-C (V8 protease). Peptides were resolved by using pH 1.9 buffer for electrophoresis (1 kV, 25 min) and phosphochromatography buffer for chromatography.

Kinase reactions and EMSAs. All electrophoretic mobility shift assays (EMSAs) and kinase and phosphatase reactions were performed in silanized tubes. Proteins purified from bacteria (40 μg) were treated simultaneously with cyclic AMP-dependent protein kinase (PKA) and casein kinase II (CKII) (3.15 U each; New England Biolabs) in 150 μl of 0.75× CKII buffer–0.25× PKA buffer for 90 min at 30°C. A total of 6 μl of the kinase reaction was then removed for treatment with λ-phosphatase (200 U; New England Biolabs) for 1 h at 30°C.

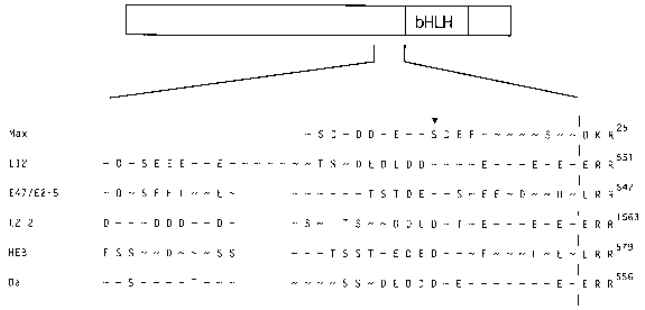

FIG. 1. An acidic domain is conserved in several widely expressed bHLH proteins. Negatively charged residues, aspartic acids (D) and glutamic acids (E), and potential phosphorylation sites, serines (S) and threonines (T), in the region immediately N terminal of the basic region of the indicated bHLH proteins are shown. Numbers on the right indicate the positions of the arginine (R) residues for each protein. No tyrosines are present in this region in any of the proteins. The "∼" symbol indicates any other amino acid and does not signify homology between proteins. The "▼" symbol identifies the serine in Max that, when phosphorylated, inhibits DNA binding by homodimers. E47 proteins have been described that possess an additional serine adjacent to Ser-529 (see Materials and Methods). K, lysine.

DNA binding of bacterially expressed ΔE47 proteins (1.6 μg) to a labeled μE5 E-box was carried out as previously described (45) except that the binding buffer contained 10 mM Tris-HCl (pH 7.5), 40 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 10% glycerol, and 100 μg of bovine serum albumin per ml. One microgram of poly(dI-dC) was included in each reaction mixture. For binding reaction mixtures containing both MyoD (5 μg) and ΔE47 (0.8 μg), proteins were mixed in binding buffer with 0.1% NP-40. The reaction mixtures were heated to 42°C for 15 min prior to adding the DNA probe, and then the reaction mixtures were incubated for an additional 15 min at room temperature.

For DNA binding assays using labeled protein from transfected NIH 3T3 cells, cells (one 100-mm plate) were transfected and labeled and protein was immunoprecipitated as described above except that after 3 washes with radioimmunoprecipitation assay buffer, E47-bound protein A-agarose beads were washed one time in elution buffer (20 mM HEPES [pH 7.9], 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). Beads were then incubated with 40 μl of elution buffer with 0.1% NP-40 and 1 μg of hemagglutinin peptide per ml for 1 h at room temperature. Aliquots were then incubated with cold μE5 oligonucleotide (30 ng) without or with anti-E47 antibody (generously provided by Cornelis Murre) as indicated elsewhere (1).

RESULTS

A conserved acidic region N terminal to the E47 bHLH domain identifies a potential target for phosphorylation. To narrow the field of potential modifications that could influence DNA binding by E47, we focussed on the properties of two other HLH proteins, E12 and Max. E12 dimerizes through its HLH domain while Max dimerizes through a more extended HLHZip domain. Both proteins contact DNA though their respective basic regions; these comprise clusters of negatively charged amino acids just N terminal to the HLH domains. Sun and Baltimore (48) showed that DNA binding by E12 homodimers could be inhibited by an acidic domain immediately N terminal to the basic region. Berberich and Cole (4) showed that the corresponding region of Max could similarly inhibit DNA binding by homodimers but only after phosphorylation of a particular serine within the region. Inhibition of DNA binding was specific for homodimers because DNA binding by E12:MyoD heterodimers and by phosphorylated Max:Myc heterodimers was readily detected.

When we aligned the amino acids N terminal to the basic regions of several bHLH proteins, including E47, E2-2, Heb, and *Drosophila* daughterless (da), we noted a striking conservation of acidic amino acids, e.g., aspartates and glutamates, mixed with serines and threonines (Fig. 1). We speculated that the overall net negative charge may weaken DNA binding by

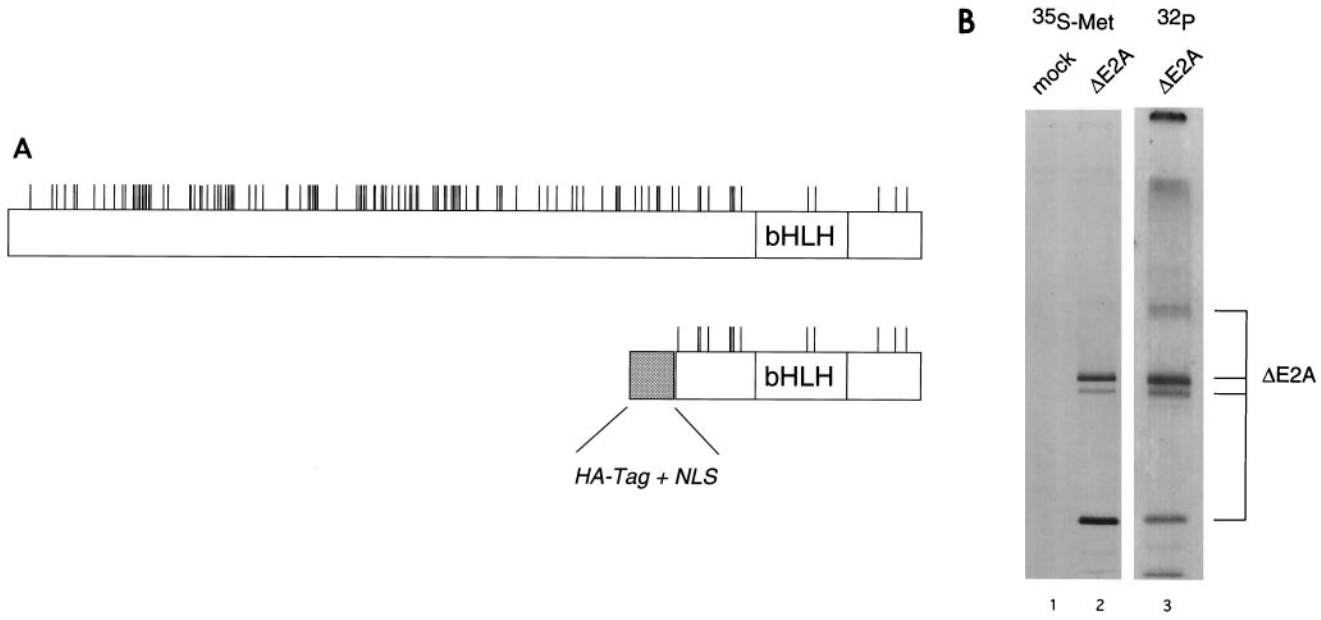

FIG. 2. ΔE47 is expressed and phosphorylated in NIH 3T3 cells. (A) Schematic representation of ΔE47 which corresponds to the carboxy third of E47 (amino acids 492 to 650). Potential phosphorylation sites (serines, threonines, and tyrosines) are depicted as vertical lines. A hemagglutinin tag (HA-Tag) and a nuclear localization signal (NLS) were added at the N terminus. (B) Analysis of ΔE47 phosphorylation. NIH 3T3 cells were transfected with a plasmid expressing ΔE47 (pCHNE2-5C) or with vector alone. Two days later the cells were pulsed with [^{35}S]Met or $^{32}P_i$, immunoprecipitated with antihemagglutinin antibody, and the precipitates were analyzed by SDS-PAGE and autoradiography. Lane 1, cells transfected with vector alone and labeled with [^{35}S]Met; lane 2, cells transfected with pCHNE2-5C and labeled with [^{35}S]Met; lane 3, cells transfected with PCHNE2-5C and labeled with $^{32}P_i$. The lower bands in lanes 2 and 3 represent ΔE47 monomers.

homodimers, as seen acutely with E12, and that phosphorylation of serines and threonines may augment the effect, as seen with Max (the phosphorylated serine residue of Max is indicated in Fig. 1). We wanted to know if B-cell-specific DNA binding by E47 homodimers might follow the example of Max, namely, that phosphorylation of serines and threonines within the acidic region would inhibit DNA binding by E47 homodimers and that the extent of such phosphorylation would be low in B cells.

A truncated E47 protein is phosphorylated on two serines within the acidic region. In order to examine phosphorylation within the acidic region of E47, we employed an amino-truncated version of the protein, denoted ΔE47 (Fig. 2A). This truncated protein spans the acidic region and the entire bHLH domain and extends to the C-terminal end of E47. We engineered an expression vector so that the N terminus of the protein would also contain a hemagglutinin epitope for immunoprecipitations and a nuclear localization signal to ensure nuclear targeting (although we have not verified its necessity). We reasoned that the truncated protein would provide a simpler pattern of phosphorylation, given that full-length E47 contains over 15% serine plus threonine plus tyrosine (over 100 potential sites) and is known to be phosphorylated in both B cells and non-B cells (45). ΔE47 contains only 13 potential phosphorylation sites (9 serines and 4 threonines).

Transfection and labeling experiments revealed that ΔE47 could be expressed and phosphorylated in NIH 3T3 fibroblasts. Cells were transfected with a vector expressing ΔE47 and then labeled for 2 h with either [^{35}S]Met or $^{32}P_i$. ΔE47 protein was then immunoprecipitated from nuclear extracts by using an antihemagglutinin monoclonal antibody and analyzed by autoradiography of SDS-polyacrylamide gels. Metabolic labeling with [^{35}S]Met revealed that ΔE47 protein was expressed and that both monomeric (fast-migrating) and presumably dimeric (slower-migrating) forms were generated (Fig. 2B). Metabolic labeling with $^{32}P_i$ revealed that both forms of ΔE47 were phosphorylated in NIH 3T3 cells.

Gel-purified protein was used to determine the sites of phosphorylation. Partial amino acid hydrolysis followed by two-dimensional electrophoresis revealed that phosphorylation occurred exclusively on serines (Fig. 3A). A two-dimensional peptide map of V8 protease-digested protein revealed one discrete spot and an additional cluster of spots (Fig. 3B, upper left panel). This same pattern was obtained for both the monomeric and apparently dimeric forms of ΔE47 (not shown). The mobilities of the labeled peptides were consistent with phosphorylation occurring within the acidic region of ΔE47, with the cluster of spots generated by partial V8 digestion of a single peptide fragment.

We then analyzed phosphorylation of ΔE47 proteins carrying alanine substitutions in the three serines immediately N terminal of the basic region, Ser-514, Ser-529, and Ser-535 (numbering derived from reference 26; see Materials and Methods for additional details). Proteins with single missense mutations in Ser-514, Ser-529, and Ser-535 are referred to as ASS, SAS, and SSA, respectively, with the wild-type proteins being designated SSS. ΔE47 expression vectors were transfected into NIH 3T3 cells, and V8 protease maps of $^{32}P_i$-labeled proteins were generated (Fig. 3B). The ASS mutant protein failed to generate the lower cluster of spots present in the wild-type protein, SSS, while the SAS mutant protein failed to generate the upper discrete spot. The relative mobilities of the lower cluster of spots and the upper spot were also consistent with those predicted for the V8 fragments which include Ser-514 and Ser-529, respectively. The labeled peptides generated by the SSA mutant were not qualitatively different from those of the wild-type protein. A protein carrying two changes, Ser-514 to Ala and Ser-529 to Ala (AAS) incorporated no detectable $^{32}P_i$ in labeled cells (data not shown). We conclude that Ser-514 and Ser-529 are necessary for phosphorylation of

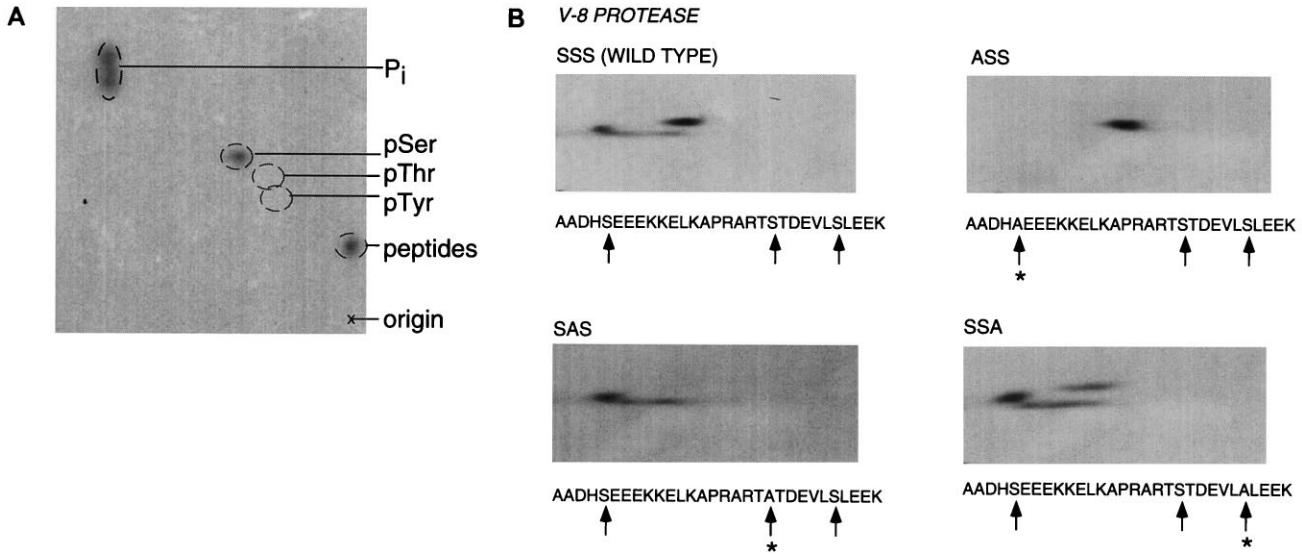

FIG. 3. ΔE47 is phosphorylated at two serines within the acidic region. (A) Phosphoamino acid analysis. $^{32}P_i$-labeled ΔE47 was generated as described in the legend for Fig. 2, eluted from the gel, and subjected to partial amino acid hydrolysis and two-dimensional electrophoresis; this was followed by autoradiography. The positions of phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) markers are indicated. (B) Two-dimensional peptide maps of $^{32}P_i$-labeled ΔE47 proteins. Plasmids expressing wild-type ΔE47 (SSS) or single Ser-to-Ala mutations (ASS, SAS, and SSA) were transfected into NIH 3T3 cells, and gel-purified protein was treated with V8 protease. Peptide fragments were separated by electrophoresis in the horizontal dimension and chromatography in the vertical dimension. Autoradiographs of the resulting labeled peptides are shown.

ΔE47 and are likely to be direct sites of phosphorylation of E47 in fibroblasts.

We have not been able to confirm that Ser-514 and Ser-529 are phosphorylated on the endogenous full-length E47 protein. Although we have shown previously that the endogenous protein is phosphorylated (45), our analyses currently lack the sensitivity and resolution required to unambiguously assign identities to the various phosphopeptides.

In vitro phosphorylation of Ser-514 and Ser-529 abolishes DNA binding by ΔE47 homodimers. We introduced the double Ser-to-Ala mutations (AAS) into the full-length E47 protein to see if an inability to phosphorylate the sites would affect activity in vivo. Transfections of E47 expression plasmids with E47-responsive reporters confirmed that the mutant AAS E47 protein was active in both fibroblasts and B cells (data not shown). However, the mutations also had the effect of severely destabilizing the full-length protein (but not ΔE47), and we therefore could not determine its specific activity relative to wild-type E47.

As an alternative to in vivo analyses, we determined the effects of phosphorylation on DNA binding by ΔE47 in vitro. In separate studies, we determined that Ser-514 could be phosphorylated efficiently in vitro by purified CKII while Ser-529 could be phosphorylated by PKA. As shown in Fig. 4A, treatment of bacterially expressed ΔE47 with the two kinases in the presence of ATP completely destroyed DNA binding. Further treatment with phosphatase restored binding, confirming that inhibition was due to phosphorylation. Although Konieczny and coworkers (24) have shown that phosphorylation by CKII alone can inhibit DNA binding by E47 homodimers, we required PKA in addition to CKII. When we carried out the same analysis on the double serine-to-alanine mutant protein (AAS), binding was largely resistant to kinase treatment. This confirms that phosphorylation specifically at Ser-514 and Ser-529 inhibits DNA binding by ΔE47 homodimers in vitro. We assume that the residual inhibition of the mutant protein AAS upon kinase treatment is due to phosphorylation of a subset of the remaining 11 potential serines and threonines (these include one additional consensus PKA site and three additional consensus CKII sites). Our in vitro mapping studies indicated that Ser-514 and Ser-529 were the primary targets of the two kinases (e.g., Ser-535, despite being a consensus CKII site, was not efficiently phosphorylated in vitro; data not shown). Although phosphorylation at these unidentified sites may affect DNA binding, it is not observed in vivo and thus probably reflects an artifact of the in vitro reaction.

ΔE47 can bind DNA if only one of the two partners of the homodimer is phosphorylated. Our results show that ΔE47 is phosphorylated in NIH 3T3 cells and that such phosphorylated proteins do not bind DNA in vitro. How, then, can we explain the ability of transfected E47 to activate transcription in 3T3 cells (44, 52)? A possible explanation would be that transfected E47 is highly expressed and outruns kinase activity within the cell. E47 activity would then be a reflection of the E47 proteins that escape phosphorylation. Indeed, under the particular conditions used for the transfections shown in Fig. 3 (high input), only a small fraction of the ΔE47 protein becomes phosphorylated. This conclusion is based on our observation that phosphorylation reduces the mobility of ΔE47, yet [^{35}S]Met-labeled protein appears as only a single faster-migrating band (data not shown).

Activity of transfected E47 might result from dimers that escape phosphorylation completely and perhaps also from those that contain only one phosphorylated partner. To address the latter possibility, we assessed DNA binding by phosphorylated ΔE47 in the presence of full-length, unphosphorylated E47 (also produced in bacteria). As shown in Fig. 4B, mixing ΔE47 with the full-length E47 generates DNA-bound heterodimers of intermediate mobility. When phosphorylated ΔE47 is mixed with full-length E47, the heterodimers are still apparent, despite the complete lack of DNA binding by the phosphorylated ΔE47 homodimers. We conclude that DNA binding is not abolished when only one of the two partners is phosphorylated. A more detailed analysis will be required to

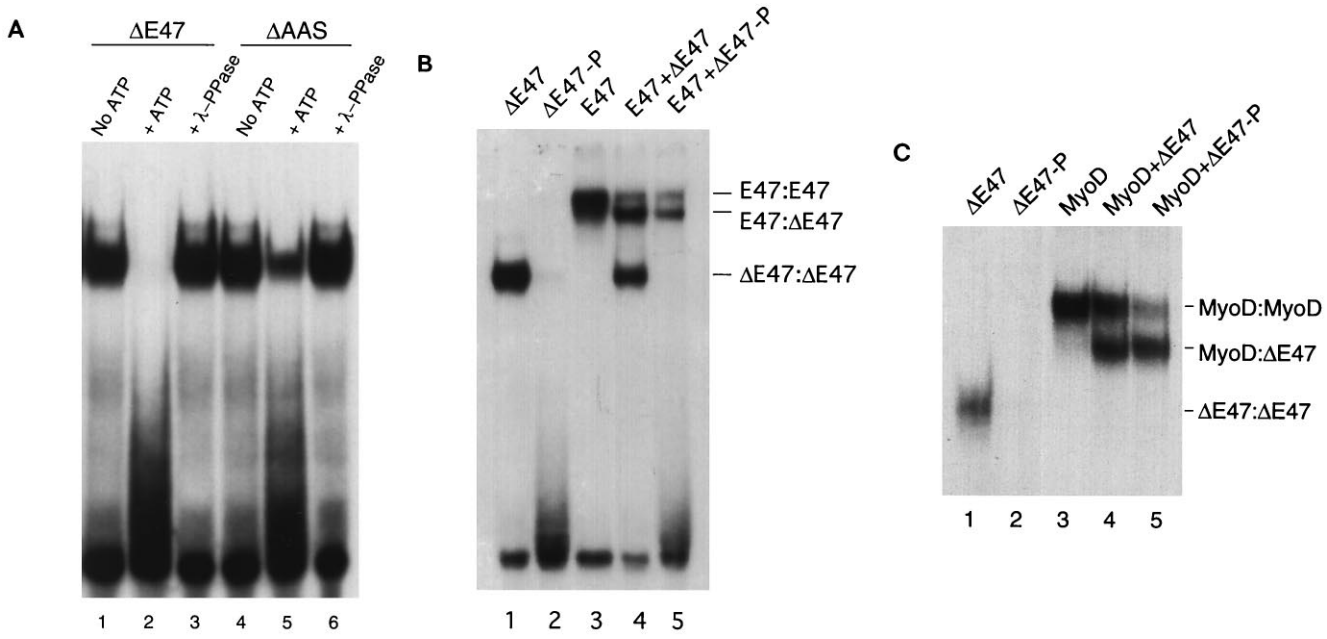

FIG. 4. Effects of phosphorylation on DNA binding by dimers containing ΔE47. (A) Phosphorylation inhibits DNA binding by ΔE47 homodimers. Wild-type ΔE47 (SSS) and double Ser-to-Ala mutant ΔE47 (AAS) proteins were expressed in bacteria and purified by nickel-chelate chromatography and treated with purified CKII and PKA either in the presence or absence of ATP and either with or without subsequent treatment with λ-phosphatase (λ-PPase) as indicated. The ability of each protein to bind a μE5-containing oligonucleotide was determined by EMSA. (B) Phosphorylation of ΔE47 does not inhibit DNA binding by ΔE47:E47 mixed dimers. EMSAs of bacterially expressed ΔE47 with (lanes 4 and 5) or without (lanes 1 and 2) bacterially expressed full-length E47 (lane 3) are indicated. Mobilities of DNA-bound ΔE47 homodimers, E47 homodimers, and ΔE47:E47 mixed dimers are also indicated. Free DNA probe is visible at the bottom of the gel. ΔE47-P, ΔE47 treated with kinases in the presence of ATP. (C) Phosphorylation of ΔE47 does not inhibit DNA binding by ΔE47:MyoD heterodimers. EMSAs of bacterially expressed ΔE47 with (lanes 4 and 5) or without (lanes 1 and 2) bacterially expressed MyoD (lane 3) are indicated. Mobilities of DNA-bound MyoD homodimers, MyoD:ΔE47 heterodimers, and ΔE47 homodimers are also indicated. To increase resolution of the protein-DNA complexes free probe was electrophoresed off the gel. ΔE47-P, ΔE47 treated with kinases in the presence of ATP.

determine if phosphorylating one of the two partners modestly affects DNA binding. The results of Fig. 4B also support the observation of Konieczny et al. (24) that phosphorylation by CKII does not affect protein homodimerization. If homodimerization were inhibited under our conditions of phosphorylation, we may have expected to see a corresponding increase in the number of bound heterodimers. Instead, the intensities of the heterodimer bands were roughly the same whether or not ΔE47 was phosphorylated.

We also assessed DNA binding by $^{32}P_i$-labeled ΔE47 from transfected cells. Since our transfected cells produced mostly unphosphorylated ΔE47, we reasoned that most of the phosphorylated ΔE47 would exist as a dimer with an unphosphorylated partner. Accordingly, any DNA binding by $^{32}P_i$-labeled protein would reflect the binding of such mixed dimers. DNA binding by labeled protein can be assessed by examining changes in protein mobility in the presence of unlabeled DNA. In a native gel, protein-DNA complexes often run faster than protein alone because of the increased net negative charge imparted by the DNA.

We found that both [^{35}S]Met-labeled and $^{32}P_i$-labeled ΔE47 bound DNA. We examined DNA binding of labeled ΔE47 by first labeling transfected cells, immunoprecipitating protein from nuclear extracts with antihemagglutinin antibody, and dissociating the protein from the antibody with excess hemagglutinin peptide. We then resolved the protein on a native polyacrylamide gel either alone or in the presence of a cold oligonucleotide bearing an E47 binding site. As shown in Fig. 5, both [^{35}S]Met- and $^{32}P_i$-labeled protein behaved similarly. In the absence of DNA, protein ran as a diffuse smear in the gel (Fig. 5, lanes 1 and 5). However, in the presence of the cold oligonucleotide, a rapidly migrating band was readily apparent (lanes 2 and 6). We confirmed that this band contained ΔE47 because (i) it was present only with cells transfected with ΔE47 (compare lanes 2 and 6 with lanes 4 and 8), and (ii) it was shifted in the gel by the addition of anti-E2A antibody (lanes 3 and 7). We conclude that $^{32}P_i$-labeled ΔE47 can bind DNA but that the observed binding is due to homodimers in which only one of the two partners is phosphorylated.

Phosphorylated ΔE47 can bind DNA as a heterodimer with MyoD. The preceding experiments demonstrate that ΔE47 fails to bind DNA as a homodimer when both partners in the homodimer are fully phosphorylated but that ΔE47 can bind DNA if only one of the two partners is phosphorylated. In some non-B cells E47 is known to bind DNA in heterodimeric form with tissue-specific bHLH proteins such as MyoD. It was therefore of interest to determine if phosphorylated ΔE47 could bind DNA as a heterodimer with MyoD. As shown in Fig. 4C, under conditions of in vitro phosphorylation where DNA binding by ΔE47 homodimers was completely blocked, we saw no inhibition of DNA binding by ΔE47:MyoD heterodimers.

Our results thus far have addressed only the behavior of a truncated E47 protein. In an attempt to confirm that phosphorylation also inhibits binding of full-length E47 homodimers, we purified full-length E47 and full-length E47 carrying the double serine-to-alanine mutation (AAS) from bacteria and treated them with PKA and CKII. Our results varied from experiment to experiment (data not shown). DNA binding by the mutant protein was never inhibited by kinase treatment, while DNA binding by the wild-type protein was sometimes inhibited and sometimes not. We attribute this to

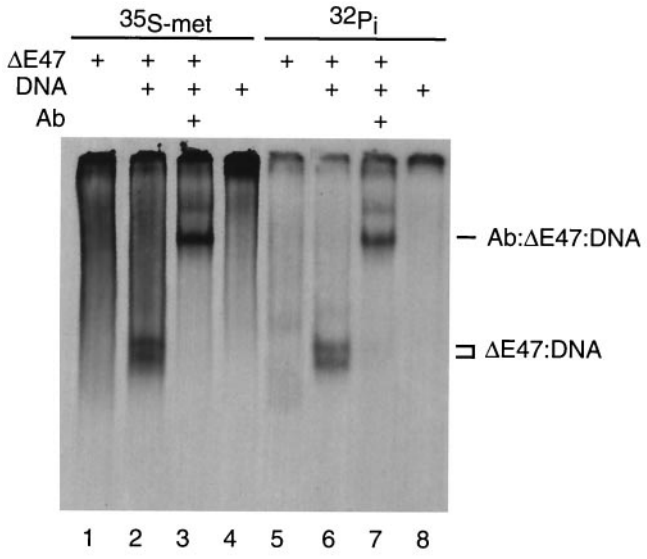

FIG. 5. $^{32}P_i$-labeled ΔE47 from transfected cells can bind DNA. ΔE47 was immunoprecipitated from nuclear extracts of transfected, labeled NIH 3T3 cells by using an antihemagglutinin antibody. The protein was then eluted from the protein A-agarose beads by adding an excess of hemagglutinin peptide and resolved on a native gel. Lanes 1 to 4 contain protein from [^{35}S]Met-labeled cells, and lanes 5 to 8 contain protein from $^{32}P_i$-labeled cells. Lanes 1 and 5, eluted protein alone; lanes 2 and 6, protein plus cold μE5-containing oligonucleotide; lanes 3 and 7, protein plus cold μE5-containing oligonucleotide plus anti-E2A antibody (AB); lanes 4 and 8, protein prepared from cells transfected with vector alone plus cold μE5-containing oligonucleotide.

inefficient and variable phosphorylation. According to the behavior of ΔE47 we reasoned that we would need at least 40% of the E47 protein phosphorylated to see a clear decrease (16%) in DNA binding activity.

The acidic region of ΔE47 is hypophosphorylated in B lymphocytes. If phosphorylation of the E47 acidic region influences binding of the homodimer, we might expect to see cell type-specific differences in phosphorylation. We therefore examined phosphorylation of transfected ΔE47 in a variety of cell lines. In addition to NIH 3T3 fibroblasts, we analyzed T cells (Jurkat), epithelial cells (HeLa), myoblasts (10T1/2-derived P2 cells) and two B-cell lines (Ag8 and S194). To determine ΔE47 phosphorylation levels in the cell lines independent of differences in their transfection efficiencies, we compared the amount of $^{32}P_i$ incorporated into the protein (phosphorylation) with the amount of [^{35}S]Met incorporated (total ΔE47 synthesized). To adjust for slight differences in metabolic rates among the cell lines, we immunoprecipitated ΔE47 from an amount of $^{32}P_i$-labeled extract that was adjusted to the total amount of [^{35}S]Met incorporated into TCA-precipitable counts.

All of the non-B-cell lines gave rise to comparable ratios of $^{32}P_i$-labeled and [^{35}S]Met-labeled ΔE47 (Fig. 6, compare the intensities of the ΔE47 bands in the lower panels with those in the upper panels). However, the two B-cell lines gave either very low (Ag8) or undetectable (S194) $^{32}P_i$-labeled ΔE47 compared with [^{35}S]Met-labeled protein. When our data were quantitated by using a PhosphorImager, we found that the steady-state level of ΔE47 phosphorylation in these B-cell lines is less than 10% that obtained with any of the other cell types. A representative comparison between Jurkat (T) and Ag8 (B) cells is shown in Fig. 7. Despite comparable levels of protein synthesis as measured by [^{35}S]Met incorporation (Fig. 7, left panels), the level of phosphate incorporated into ΔE47 was vastly reduced in the B cells (compare right two panels).

DISCUSSION

The E47 protein is present in many different cell types yet forms homodimers that bind DNA exclusively in B cells. We have studied the properties of a truncated version of the protein, ΔE47, to provide a possible explanation. Our results argue that cell type-specific binding is not due to B cells specifically activating the E47 protein but, rather, may be a consequence of E47 being inactivated in other cell types. Inactivation results, at least in part, from the phosphorylation of two serines that map to an acidic region just N terminal to the E47 bHLH. Phosphorylation inhibits DNA binding by homodimers, and since the sites are hypophosphorylated in B cells, DNA binding by the homodimers is permitted in B cells. Although we have been unable to confirm B-cell-specific hypophosphorylation of endogenous E47, because of uncertainties in assigning identities to the various phosphopeptides, we think it is likely that the full-length protein and ΔE47 behave similarly. We acknowledge this as a weakness in our model.

Phosphorylation is known to affect directly the activity of many transcription factors (for a review, see reference 27).

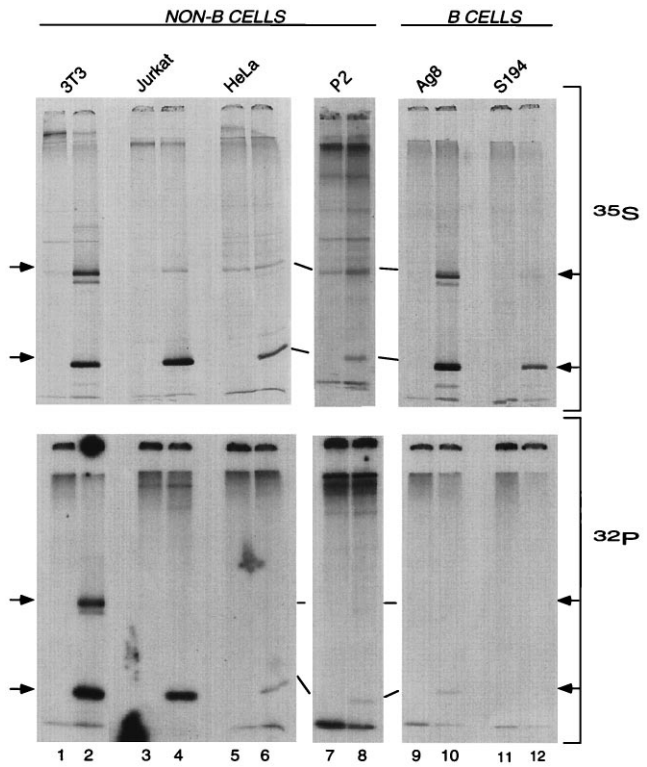

FIG. 6. ΔE47 is hypophosphorylated in B-cell lines. The indicated cell lines were transfected and labeled ΔE47 was analyzed as described in the legend for Fig. 2B. Upper panels show autoradiographs generated by [^{35}S]Met-labeled cells. Lower panels indicate results from $^{32}P_i$-labeled cells. Lanes 1, 3, 5, 7, 9, and 11 represent extracts prepared from cells transfected with vector alone. Lanes 2, 4, 6, 8, 10, and 12 represent cells transfected with pCHNE2-5C. Lanes 1 and 2, NIH 3T3 (fibroblast) cells; lanes 3 and 4, Jurkat (T) cells; lanes 5 and 6, HeLa (cervical carcinoma) cells; lanes 7 and 8, P2 (myoblast) cells; lanes 9 and 10, Ag8 (B) cells; lanes 11 and 12, S194 (B) cells. Arrows indicate electrophoretic positions of ΔE47. The upper arrows in both the upper and lower panels (identifying the ΔE47 dimer) sometimes correspond to a contaminating band seen in untransfected cells. For each cell type, the same ratio of total TCA-precipitable [^{35}S]Met-labeled protein to TCA-precipitable $^{32}P_i$-labeled protein was analyzed.

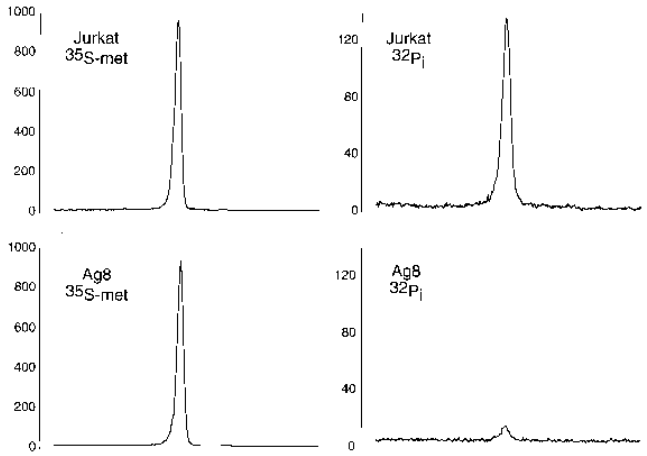

FIG. 7. ΔE47 is hypophosphorylated in B-cell lines. Jurkat (T) cells (top panels) and Ag8 (B) cells (bottom panels) were transfected and labeled as described in the legend for Fig. 5. The intensities of the [^{35}S]Met-labeled ΔE47 (left panels) and the $^{32}P_i$-labeled ΔE47 (right panels) were graphed using a PhosphorImager. The scales are arbitrary but consistent for a given isotope.

Examples include the STAT proteins, which are activated by tyrosine phosphorylation (46), and CREB and TCF/Elk-1, which are activated by serine phosphorylation (13, 14, 32, 54). Likewise, c-Jun is stimulated by serine phosphorylation within its amino-terminal activation domain, but DNA binding by c-Jun homodimers is inhibited by serine/threonine phosphorylation at its C-terminal DNA-binding domain (6, 9, 31). In these cases, phosphorylation is affected by extracellular signals that regulate transcription factor activity in multiple cell types and this does not involve cell type-specific transcription per se. E47 may represent the first example of differential phosphorylation participating in the generation of cell type-specific activity of a transcription factor.

Results obtained by Benezra (3) suggest an alternative, or perhaps additional, mode of B-cell-specific activation of E47. Those studies demonstrated a requirement for an intermolecular disulfide bond for DNA binding by E47 homodimers at 37°C. Indeed, covalently linked, oxidized E2A homodimers were detected in B cells and were postulated to result from a relative lack of E2A reducing activity in B cells. However, such oxidized homodimers represent only a small fraction of the total E2A in B cells. For example, Western analysis indicates that oxidized homodimers represent less than 1% of the total E47 in B-cell nuclear extracts (data not shown). Also, Benezra estimated that at 25°C, conditions that do not require the disulfide bond for DNA binding, only 10% of the DNA binding activity from B cells was due to oxidized E2A. Given that DNA binding by E47 homodimers is B cell specific at 25°C and that this largely represents binding by reduced homodimers (see, for examples, references 1, 23, 36, and 45), the B-cell-specific formation of oxidized homodimers cannot account for B-cell-specific DNA binding overall. E47 can also form oxidized homodimers in non-B-cell types. Transfected E47 proteins readily activate transcription in fibroblasts, and even in yeast cells, suggesting that covalent dimerization is permitted in these cells (21, 44). Moreover, we detected presumably dimeric forms of ΔE47 in additional non-B-cell lines in the present studies (Fig. 4). These dimeric forms of ΔE47 were apparent despite the 100 mM DTT used in our gel-loading buffer, consistent with the relative resistance of the E47 disulfide bond to reducing agents (3). Taken together these observations indicate that, while covalent dimerization of E47 may be important for the protein's activity in vivo, it is not restricted to B cells.

Experiments from our own laboratory lead us to the conclusion that phosphorylation of Ser-514 and Ser-529 cannot account totally for the inactivity of E47 homodimers in non-B cells. For example, we have been unable to activate E47 by phosphatase treating non-B-cell extracts. Moreover, if phosphorylation were the only modification preventing binding, then we would expect to generate a novel DNA binding activity after mixing bacterial ΔE47 with non-B-cell extracts. The binding activity would represent dimers of phosphorylated E47 and unphosphorylated ΔE47, analogous to the DNA binding complexes observed in Fig. 4B and 5. Despite repeated attempts, such binding activity has not been detected. While hypophosphorylation may not be the only modification affecting DNA binding by E47, our results argue that it is a necessary prerequisite.

We have only limited information regarding the kinases and phosphatases that affect phosphorylation at Ser-514 and Ser-529. Although our results show that CKII and PKA can phosphorylate these sites and inactivate ΔE47 in vitro, we cannot conclude that these proteins affect E47 under normal physiologic conditions. Overexpression of CKII in vivo can, indeed, inactivate E47, this has also been shown by Johnson et al. (24), but this also occurs with the AAS version of the protein (data not shown). Similar results were obtained with a PKA expression vector, while forskolin-treated cells showed no effect (data not shown). Thus, CKII and PKA may not be involved in the regulatory scheme we have postulated here. Alternatively, they may also target an additional site on the E47 protein that affects its activity or inhibit an unknown E47 accessory protein such as a coactivator.

E2A proteins are required only for the B-lymphocyte lineage (2, 53). This is surprising considering their broad tissue distribution and their proposed role as partners for several cell-restricted bHLH proteins. However, it is not surprising given the distinctive role of E47 homodimers in activating B-cell-specific transcription. Our results argue that, in addition to simply the presence of E47 protein, B cells require that E47 be hypophosphorylated (Fig. 8). A decrease in E47 phosphorylation at a critical point in development might be initiated by an extracellular ligand and be mediated through a signal pathway.

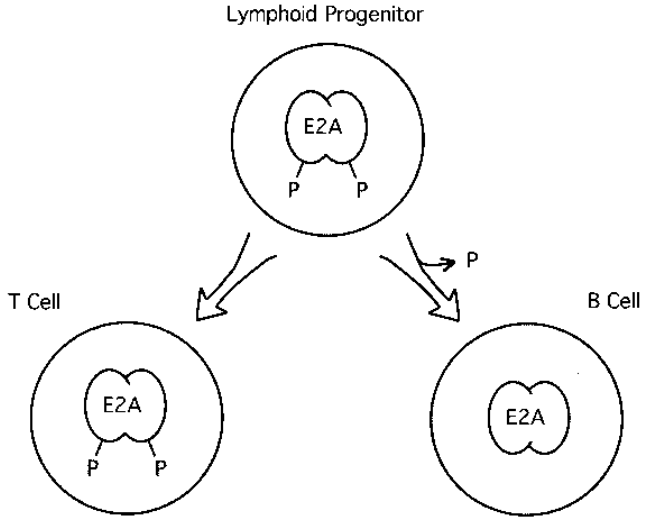

FIG. 8. A model depicting hypophosphorylation of E47 as a prerequisite for B-cell development.

Although we have no information about the ligand, its receptor, or any of the intracellular signal transducers, our results provide the first evidence that such a pathway may be important for early B-cell development. Stochastic models have been proposed to explain certain features of hematopoietic development (47, 49). From the perspective of transcriptional regulation, stochastic mechanisms would imply that genes encoding cell type-specific transcription factors are activated at random. Our results suggest a nonrandom means for activating a B-cell-specific transcription factor, namely, the E47 homodimer. Indeed, if E47 plays a role in B-cell determination, as opposed to B-cell maturation, then the decision that takes a lymphoid progenitor into the B-cell lineage may have an inductive component.

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