The Ca^{2+} sensor protein calmodulin can interact with the DNA binding basic helix-loop-helix (bHLH) domain of E12, E47, and SEF2-1 (E2-2), which belong to the E-protein subclass of bHLH transcription factors. This interaction inhibits the DNA binding of these bHLH proteins in vitro, and an ionophore that increases intracellular Ca^{2+} can inhibit transcriptional activation by the E-proteins. Here we have attempted to determine if these phenomena reflect a direct calmodulin-dependent inhibition of DNA binding by E-proteins in vivo. We have compared calmodulin effects on DNA binding in vitro and on activation of transcription in vivo using a series of E12 mutants harboring defined alterations within the basic sequence of the bHLH domain that reduce their ability to bind calmodulin to varying degrees. We find a striking direct correlation between the ability of calmodulin to inhibit their DNA binding in vitro and the ability of overexpressed calmodulin or cellular Ca^{2+} mobilization to inhibit their transcriptional activity in vivo. Furthermore, E12 and overexpressed calmodulin were co-localized in the nucleus, and calmodulin pull-down experiments with cell extracts showed a Ca^{2+}-dependent interaction between calmodulin and E12 but not with a calmodulin inhibition-deficient E12 mutant. Chromatin immunoprecipitation showed that calmodulin overexpression leads to decreased binding of E12 and E47, but not a calmodulin inhibition-deficient E12 mutant, to the DNA recognition sequence in vivo. The data suggest that Ca^{2+} signaling can inhibit the transcriptional activities of E-proteins through direct binding of Ca^{2+}/calmodulin to the basic sequence of E-proteins, resulting in inhibition of their DNA binding.

Calmodulin is a ubiquitous regulator of numerous cellular processes including cell cycle progression, cell mobility and contraction, ion homeostasis, and transcription. Most target proteins bind to the Ca^{2+}-loaded form of calmodulin. Therefore, upon signals leading to increased intracellular Ca^{2+} concentration, calmodulin is able to bind to a new set of target proteins and modulate their activity (1). Several transcriptional regulators are direct targets for calmodulin or are regulated by calmodulin-dependent kinases or the calmodulin-dependent phosphatase calcineurin (2, 3).

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‡ This paper is available on line at http://www.jbc.org.
Calmodulin Inhibition of E-protein Activity

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—The cDNAs coding for murine E12 and E47 (6) were inserted into pcDNA3/amp (Invitrogen) by excision with XhoI and BamH1 and subsequent blunt end ligation to the EcoRI site of pcDNA3/amp. The MyoD cDNA was inserted as an EcoRI fragment from pMSV-MyoD (6) into the EcoRI site of pcDNA3/amp. The cDNA coding for human SEF2-1 (E2-2) was isolated from an human AZF library containing genomic DNA inserts from human lymphoma cell line BL-29 (8). For expression vector construction, a SEF2-1 DNA of the SEF2-1B type (9) was isolated from this cDNA library using a SEF2-1B probe (9), and it was subcloned as a blunt-ended EcoRI fragment into blunt-ended XhoI-BalI-digested pKG346 (10), thereby replacing the probe (9), and it was subcloned as a blunt-ended EcoRV site of pcDNA3/amp. For construction of C-terminal FLAG derivatives of E12 and the E12 mutant m8-4-7, BamHI, BglII, and XhoI sites were introduced at the stop codon of E12 and m8-4-7. Double-stranded oligonucleotides coding for two tandem copies of the FLAG epitope sequence were inserted between the BamHI and BglII sites.

Cell Culture and Transfections—DG75 B-cells were maintained in RPMI medium supplemented with 5% fetal calf serum and antibiotics. DG75 cells were transiently transfected by electroporation as described (12). The IMR-32 neuroblastoma cell line was maintained in Hepes-buffered minimum essential medium supplemented with 10% fetal calf serum, nonessential amino acids, and antibiotics. IMR-32 cells were transfected using polyethylenimine (11). 10% of the beads were seeded per 5-cm plate 1 day before transfection. DNA-polyethylenimine complexes were formed in 1 ml of serum-free minimum essential medium for 10 min and added on cells with the addition of 1 ml of serum-free minimum essential medium. Cells were exposed to DNA-polyethylenimine complexes for 2 h, after which the medium was replaced with serum-containing minimum essential medium with or without 5 mM caffeine. Cells were harvested 20 h after the start of transfection and analyzed for luciferase and β-galactosidase activity as described (12).

Western Blot with Nuclear and Cytoplasmic Extracts—Cells were pelleted and washed once with cold phosphate-buffered saline. The cells were then resuspended in 10 volumes of ice-cold lysis buffer (10 mM Hepes, pH 8, 10 mM KCl, 0.1% Nonidet P-40, 1 mM EDTA) supplemented with protease inhibitor mixture (Roche Applied Science) and kept on ice for 10 min. The cells were then vortexed for 10 s, the lysate was pelleted by centrifugation at 10,000 × g for 1 min at +4°C, and the supernatant containing the cytoplasmic fraction was collected. The pellet was washed once with 1 ml of the hypotonic buffer, and nuclear proteins were extracted with one pellet volume of high salt buffer (20 mM Hepes, pH 8, 400 mM NaCl, 10% glycerol, 1 mM EDTA) supplemented with protease inhibitor mixture on ice for 30 min with occasional flicking of the tubes. The insoluble nuclei were pelleted by centrifugation at 20,000 × g for 3 min at +4°C, and the supernatant with the nuclear extract was collected. Western blot analysis was performed using SuperSignal (Pierce) according to the manufacturer's instructions using anti-E12 antibody (H-208, Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences).

Analysis of DNA Binding—E12 wild type and basic sequence-mutated E12 proteins containing the amino acid sequence from 483 to 652 were produced for DNA binding analyses as previously described (7). The wild type and most mutants were produced in E. coli, whereas mutants 1-m7 were produced in vitro translation. Electrophoretic mobility shift assays (EMSAs) of the E12 proteins were performed as previously described using an end-labeled DNA sequence containing the μE5 E-box as probe (7). DNA binding was quantified with a PhosphorImager and AIDA image analysis software (Amersham Biosciences).

Immunocytochemistry—Immunostaining and confocal microscopy of transfected DG75 cells was performed essentially as described (14) with the exception that cells were fixed with 2% paraformaldehyde in phosphate-buffered saline for 15 min. Primary antibodies used were rabbit anti-E12 (H-208) and goat anti-calmodulin (N-19) (both from Santa Cruz Biotechnology). The secondary antibodies were fluorescent isothiocyanate-conjugated donkey anti-rabbit IgG and rhodamine red X-conjugated donkey anti-goat IgG (both from Jackson Immunoresearch Laboratories). All antibodies were used at 1:50 dilutions. Higher laser power and detectivity was used to detect endogenous proteins compared with overexpressed proteins in the confocal microscopy.

Calmodulin-Sepharose Pull-down—DG75 cells were transiently transfected with the expression plasmid for E12, the m8-4-7 mutant, or the empty vector. Whole cell lysates were prepared with Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) and diluted 5-fold in EMSA binding buffer with either 0.5 mM CaCl2 or 1 mM EDTA. Diluted extracts were incubated with calmodulin-Sepharose and the beads were washed three times with the Ca2+- or EDTA-containing buffer. Calmodulin-Sepharose-bound proteins were separated by SDS-PAGE, blotted, and detected with the anti-E12 (H-208) antibody, or, in the case of the FLAG tagged protein, with the anti-FLAG antibody M2 (Sigma).

Chromatin Immunoprecipitations—DG75 cells were transiently transfected with the E-box luciferase reporter and with expression vector for either E47, E12, or the E12 mutant m8-4-7 and for calmodulin where indicated. 24 h after transfection, cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Cross-linking was quenched with a 20 mM volume of 2.5 mM glycine, and cells were washed with Tris-buffered saline. Cells were lysed on ice with 0.5 ml of radioimmunoprecipitation assay lysis buffer for 30 min with occasional flicking of the tubes. DNA was phenol-chloroform-extracted, ethanol-precipitated, and PCR-amplified for 28 cycles with the primer pair 5'-TATGTTTTTGGCGTCTTCCA-3' that flanks the promoter sequence of the E-box luciferase reporter plasmid.

RESULTS

Transcriptional Activity of E12, E47, and SEF2-1 Is Inhibited by Calmodulin Co-expression—Binding of calmodulin has been shown to inhibit DNA binding of E-proteins in vitro (6, 7, 15), and a Ca2+ ionophore that increases the intracellular Ca2+ concentration has been shown to inhibit activation of transcription by E47 (6). On the way to determining if these phenomena are linked, we analyzed whether overexpression of calmodulin can also inhibit the transcriptional activity of E-proteins. The B-cell line DG75 was co-transfected with an E-box luciferase reporter and an expression vector for either E12, E47, SEF2-1, or the empty vector together with either the calmodulin expression vector or the corresponding empty expression vector. Calmodulin overexpression was found to inhibit E12, E47, and SEF2-1-induced E-box reporter activity in a dose-dependent manner (Fig. 1A). Co-transfection of 10 μg of calmodulin expression plasmid decreased transcriptional activation by E12 more than 15-fold, and E47- and SEF2-1-induced E-box reporter activity was also strongly inhibited by calmodulin overexpression. In contrast, transcriptional activation by MyoD, a bHLH protein whose DNA binding shows little calmodulin sensitivity in vitro (6, 7, 15), was not significantly affected by calmodulin overexpression (Fig. 1A). Western blot analysis of cytoplasmic and nuclear cell extracts revealed that calmodulin overexpression did not affect the level of expressed E12 protein or its nuclear/cytoplasmic distribution (Fig. 1B). Thus, calmodulin overexpression can selectively inhibit the transcriptional activity of an E-protein without affecting the nuclear level of the protein.
Calmodulin inhibition of the transcriptional activity of E12, E47, and SEF2-1. A, expression vectors for bHLH proteins (2.5 μg) were transiently expressed in the B-cell line DG75 together with 2.5 μg of a luciferase reporter plasmid for E-proteins, 6×(μE5 + μE2)-luciferase, or for MyoD, 4×(MEF1)-luciferase. In addition, the hCMV-gal plasmid (2.5 μg) was included to normalize each assay. Where indicated, expression vectors for bHLH proteins were co-transfected with either 2.5 or 10 μg of the CaM expression plasmid pcDNAI/amp mCaM. Where 2.5 μg or no pcDNAI/amp mCaM was used, total transfected DNA was equalized with 7.5 or 10 μg of pcDNAI/amp vector DNA, respectively. As a control, the empty expression vector was used in place of the bHLH protein expression vectors (co). The luciferase reporter activities of harvested cells were measured and then normalized to the β-galactosidase activity from the co-transfected hCMV-gal plasmid. Transcriptional activities of bHLH proteins in the absence of calmodulin overexpression were set as 100%. The bars show the mean values ± S.E. of three transfections. B, Western blot analysis of E12 protein levels in cytoplasmic and nuclear extracts of DG75 cells transfected as indicated. Equal amounts of protein from the cytoplasmic and nuclear extracts were separated by SDS-PAGE, and E12 was detected by Western blot with anti-E12 antibody (H-208, Santa Cruz).

E12 Mutants with Decreased Sensitivity to Calmodulin—To analyze if calmodulin binding to the basic sequence of the bHLH domain of E12 causes the inhibition of the transcriptional activation by cellular Ca2⁺ mobilization and calmodulin overexpression, we generated E12 mutants with amino acid substitutions in the basic sequence that bind both DNA and calmodulin. With the aim of generating E12 mutants with disrupted binding to calmodulin but not to DNA, we used the known structure of the E47-DNA complex (16) to predict those amino acids likely not to be critical for the DNA binding by E12. These residues were preferentially mutated to alanine or, when necessary, to glutamine to produce the E12 mutants m1 to m8 (Fig. 2A).

Three of the eight point mutants, m1, m2, and m3 (I562A, R560A, and V559A, respectively), showed no detectable DNA binding in EMSA (Fig. 2B) and were subsequently excluded from further study. However, the remaining five point mutants all retained the DNA binding (Fig. 2B). Therefore, the effect of calmodulin on their DNA binding was analyzed in detail (Fig. 3). None of the five single amino acid substitutions led to a calmodulin-resistant E12 mutant protein. Compared with wild type E12, four of these mutants, m4, m5, m6, and m7, showed a slight decrease in sensitivity to calmodulin that was more evident at the higher calmodulin concentration (1 μM). Moreover, even though the fifth mutant, m8 (D561A), was less sensitive to calmodulin than the other four mutants, much of the sensitivity still remained (Fig. 3). In view of our recent NMR structural study, highlighting that the binding of the E12 basic sequence to calmodulin involves multiple alternative amino acid contacts, we proposed that combinations of amino acid substitutions would lead to less calmodulin-sensitive mutants of E12. Thus, the amino acid substitution of the m8 mutant (D561A) was combined with m5 or m7 to produce the double mutants m8-5 and m8-7 or with those of both m4 and m7 to produce the triple mutant m8-4-7 (Fig. 2A). As expected, the DNA binding of the double mutants m8-5 and m8-7 was more sensitive to calmodulin than the other three double mutants (Fig. 3) where 200 nM calmodulin (gray bars) and 1 μM calmodulin (white bars) on DNA binding are shown. Error bars represent the S.D. of three experiments.

Fig. 1. Calmodulin inhibition of the transcriptional activity of E12, E47, and SEF2-1. A, expression vectors for bHLH proteins (2.5 μg) were transiently expressed in the B-cell line DG75 together with 2.5 μg of a luciferase reporter plasmid for E-proteins, 6×(μE5 + μE2)-luciferase, or for MyoD, 4×(MEF1)-luciferase. In addition, the hCMV-gal plasmid (2.5 μg) was included to normalize each assay. Where indicated, expression vectors for bHLH proteins were co-transfected with either 2.5 or 10 μg of the CaM expression plasmid pcDNAI/amp mCaM. Where 2.5 μg or no pcDNAI/amp mCaM was used, total transfected DNA was equalized with 7.5 or 10 μg of pcDNAI/amp vector DNA, respectively. As a control, the empty expression vector was used in place of the bHLH protein expression vectors (co). The luciferase reporter activities of harvested cells were measured and then normalized to the β-galactosidase activity from the co-transfected hCMV-gal plasmid. Transcriptional activities of bHLH proteins in the absence of calmodulin overexpression were set as 100%. The bars show the mean values ± S.E. of three transfections. B, Western blot analysis of E12 protein levels in cytoplasmic and nuclear extracts of DG75 cells transfected as indicated. Equal amounts of protein from the cytoplasmic and nuclear extracts were separated by SDS-PAGE, and E12 was detected by Western blot with anti-E12 antibody (H-208, Santa Cruz).

Fig. 2. E12 basic sequence site-directed mutants used in the study. A, amino acids in the basic sequence of mouse E12 were mutated to decrease inhibition of the DNA binding by calmodulin. The amino acid substitutions are in bold. For DNA binding analyses, E12 wild type (wt) and basic sequence-mutated E12 proteins containing the amino acid sequence from 483 to 652 were used. For transient transfections, the mutations were incorporated into the full-length E12 expression vector (“Experimental Procedures”). The asterisks indicate the three mutants that showed no detectable DNA binding in EMSA. B, DNA binding of wild type E12 and E12 basic sequence mutants to μE5 E-box probe analyzed by EMSA.

Fig. 3. Effects of calmodulin on DNA binding of E12 and E12 mutants analyzed by EMSA. bHLH proteins were preincubated with Ca²⁺–loaded calmodulin and allowed to bind to a μE5 E-box probe. Complexes were resolved by EMSA, and the degree of E12 binding to DNA in the presence of calmodulin was quantified. The mean values of the effects of 200 nM calmodulin (gray bars) and 1 μM calmodulin (white bars) on DNA binding are shown. Error bars represent the S.D. of three experiments.

1 Larsson, G., Saariketta, J., Sveshnikova, N., Zdunek, J., Schleucher, J., Grundström T., and Wijmenga, S. S., submitted for publication.
slightly less sensitive to calmodulin than that of the single mutant m8 (Fig. 3). The triple mutant m8-4-7 was the least calmodulin-sensitive mutant (Fig. 3). We also created mutants m10, m11, and m12 by combining glutamine substitutions in the amino acids substituted in the four single point mutants m4-m7 (Fig. 2A). Although these new mutants were all more resistant to calmodulin than wild type E12, they were not more resistant than the single mutant m8. Therefore, we generated mutant m13, which combined the D561A mutation of mutant m8 with glutamine substitutions at all four amino acid positions mutated in m4-m7. The m13 mutant and mutant m8-4-7 were the most resistant mutants in the series, being ∼20-fold less sensitive than wild type E12 to 1 μM calmodulin (Fig. 3). The set of mutants provides ideal molecular tools to probe the connection between Ca2+/calmodulin inhibition of E-protein DNA binding in vitro and activation of transcription in vivo.

Decreased Inhibition of Transcriptional Activities of E12 Mutants by Ca2+/Calmodulin—We compared the effects of calmodulin overexpression on activation of transcription by the series of E12 mutants that represent a gradient of sensitivity to calmodulin inhibition of the DNA binding in vitro. DG75 cells were co-transfected with an expression vector for each E12 mutant in the presence of the calmodulin or empty expression vector. The relative effects of calmodulin overexpression on transcriptional activation by the various E12 mutants correlated to their ability to bind DNA in vitro in the presence of calmodulin (cf. Figs. 3 and 4A). Transcriptional activation by the single amino acid substitution mutants m4, m6, and m7, but not mutant m5, were slightly less inhibited by calmodulin overexpression than that of wild type E12. Moreover, among the single amino acid mutants tested, transcriptional activity by mutant m8 was the least inhibited by calmodulin overexpression (Fig. 4A). The transcriptional activity of the multiple amino acid substitution mutant m13 was inhibited by calmodulin overexpression to a similar extent as m8, whereas the activities of mutants m8-7 and m8-4-7 were nearly unaffected by calmodulin overexpression (Fig. 4A).

We next analyzed the effect of increased intracellular Ca2+ on transcriptional activation in transfected DG75 B-cells by E12 and the most calmodulin-resistant mutant of E12, m8-4-7, by treatment with the Ca2+ pump blocker thapsigargin that leads to Ca2+ signaling in lymphocytes. The activity of the E-box reporter without overexpression of E12 was inhibited by thapsigargin treatment (Fig. 4B). This thapsigargin-sensitive activity is mainly due to E12/E47 since this is the predominant E-protein in DG75 cells (data not shown). Transcriptional activation by overexpressed E12 was as thapsigargin-sensitive as the endogenous E-protein activity (Fig. 4B). In contrast, the increase in transcriptional activation by overexpression of the mutant m8-4-7 was not affected by the Ca2+ inducer. We conclude that the transcriptional activity of E12 can be inhibited by Ca2+ stimulation in the presence of endogenous levels of calmodulin in DG75 cells, whereas transcriptional activation by a calmodulin-resistant mutant of E12 is not inhibited by Ca2+ stimulation in the B-cell line.

Treatment of cells with caffeine has been shown to increase the intranuclear Ca2+ concentration in some cell types (17, 18). Therefore, to further study the effects of increased intracellular Ca2+ on transcriptional activity of E12 and E12 mutants, transfected IMR-32 neuroblastoma cells were stimulated with caffeine. The Ca2+ inducer caffeine decreased the transcriptional activity of wild type E12 to 40% of that of the activity in non-stimulated cells (Fig. 4C). Moreover, the effects of caffeine on the transcriptional activities were not significantly different from the wild type E12 for most mutants. However, the transcriptional activities of the multiple amino acid substitution mutants m8-7, m8-4-7, and m13 were all clearly less affected by caffeine than wild type E12. Thus, the three multiple amino acid substitution mutants whose DNA binding showed low...
Calmodulin Overexpression Sensitivity in Vivo and the Effects of Calmodulin Overexpression

To analyze the correlation between the Ca\(^{2+}\)/calmodulin sensitivities of E12 mutants in vitro and in vivo, the transcription levels of each E12 variant in the presence of overexpressed calmodulin and the Ca\(^{2+}\)/inducer caffeine were expressed as a function of the DNA binding in vitro in the presence of 200 nM CaM (Fig. 5; regression lines are in gray and black, respectively). Linear regression analysis showed a striking direct correlation between calmodulin inhibition of DNA binding by E12 variants in vitro and the effects of calmodulin overexpression (r\(^2\) = 0.97, p < 0.0001) and caffeine stimulation (r\(^2\) = 0.96, p < 0.0001) on their transcriptional activities in vivo. Importantly, no individual mutant displayed any significant lack of correlation between Ca\(^{2+}\)/calmodulin sensitivity in vitro and in vivo. Furthermore, the activities of the mutants upon calmodulin overexpression correlated well with their activities upon caffeine stimulation (r\(^2\) = 0.97, p < 0.0001). Thus, Ca\(^{2+}\)/calmodulin sensitivity of E12 in vitro and in vivo shows indistinguishable amino acid sequence specificity.

Nuclear Co-localization of Overexpressed E12 and Calmodulin—Because overexpression of calmodulin could inhibit the transcriptional activity of E12, we wondered whether these proteins were co-localized in cells. We immunostained overexpressed wild type E12 transcription factor and the E12 mutant m8-4-7 were all exclusively nuclear (Fig. 6). The calmodulin localization in this cell line was primarily cytoplasmic, although some nuclear calmodulin could also be detected. However, overexpressed calmodulin was mainly localized in the nucleus. Co-expression of E12 and calmodulin resulted in a striking overlap in the subnuclear localization of these two proteins (observed as yellow color in Fig. 6, A and B). In contrast, the calmodulin binding-deficient E12 mutant m8-4-7 co-localized poorly with calmodulin in the nucleus on the basis of limited overlap between areas of red fluorescence and green fluorescence (Fig. 6, A and B).

Binding of E12 to Calmodulin-Sepharose—To study if wild type E12, but not a calmodulin binding deficient mutant, interacts with calmodulin in a cell extract, DG75 cells were transfected with an expression vector for either E12 or the calmodulin-insensitive mutant m8-4-7. Pull-down analyses of the resulting cell extracts were subsequently performed with calmodulin-Sepharose. Both endogenous E12 and overexpressed E12 bound to calmodulin-Sepharose in the presence of...
**Calmodulin Inhibition of E-protein Activity**

**Figure 7. Binding of wild type E12 but not the m8-4-7 mutant to calmodulin-Sepharose.**

A. Whole cell extracts from DG75 cells transfected with 5 μg of pcDNA/amp vector control or a pcDNA/amp derivative expressing wild type (wt) E12 or the m8-4-7 mutant were incubated with calmodulin-Sepharose (CaM Seph.) in the presence of Ca²⁺ or the chelator EDTA. Bound proteins were eluted by boiling in sample buffer, separated by SDS-PAGE, and detected with anti-E12 antibody. The top panels show typical pull-down experiments, and the lower panels show the average results ± S.D. of three experiments. The relative amounts of input E12 are expressed as percent of the input in cells transfected with wild type E12 plasmid in A and wild type E12-FLAG plasmid in B, and the relative amounts of eluted E12 are expressed as percent of the eluted amounts in cells transfected with wild type E12 plasmid in A and wild type E12-FLAG plasmid in B.

**Figure 8. Inhibition of DNA binding of E47 and wild type E12 in vivo by calmodulin.**

A. Chromatin immunoprecipitation performed with DG75 cells transiently transfected with 5 μg of expression vector for E47 in the presence of 5 μg of expression vector for calmodulin (CaM) or the empty CaM expression vector and 5 μg of E-box luciferase reporter. B. Chromatin immunoprecipitation (IP) performed with DG75 cells co-transfected with 5 μg of expression vector for either FLAG-tagged E12, the E12 mutant m8-4-7, or vector alone in the presence of 5 μg of expression vector for CaM or the empty CaM expression vector and 5 μg of E-box luciferase reporter. Cells were formaldehyde-cross-linked, and protein-DNA complexes were precipitated by chromatin immunoprecipitation (“Experimental Procedures”). Immunoprecipitated DNA and input DNA were amplified by PCR and resolved on an agarose gel.

**Discussion**

Calmodulin binding to the basic sequence of the bHLH transcription factors E12, E47, and SEF2-1 and how this inhibits their DNA binding in vivo has been extensively characterized (6, 7, 15, 19). Furthermore, increasing intracellular Ca²⁺ by ionomycin treatment inhibits the transcriptional activity of E47 and SEF2-1 (6). Before this study, however, a correlation between these results through the inhibition of the DNA binding of the E-protein in vivo by calmodulin had not yet been established. In this report, we provide evidence for the hypothesis that Ca²⁺ signaling through calmodulin can inhibit the transcriptional activity of E-proteins in vivo through direct binding of calmodulin to the basic DNA binding sequence.

Overexpression of calmodulin in DG75 B-cells could inhibit the transcriptional activities of E12, E47, and SEF2-1 (Fig. 1A).

Notably, an increase in intracellular Ca²⁺ was not needed for the inhibitory effect of overexpressed calmodulin on the transcriptional activity of the E-proteins. A likely explanation is that the non-induced Ca²⁺ level is sufficient for a proportion of cellular calmodulin to be Ca²⁺-bound. This is supported by the fact that calmodulin overexpression in absence of Ca²⁺ stimulation can inhibit the transcriptional activity of a Smad-responsive promoter, an effect that was proposed to occur through Ca²⁺-dependent interaction of calmodulin with Smad proteins (20, 21). Furthermore, we have shown that the intracellular Ca²⁺ level of DG75 cells in the absence of ionomycin stimulation is sufficient for potentiation of phorbol ester-activated...
transcription from a granulocyte-macrophage colony-stimulating factor promoter by the Ca\(^{2+}\)/calmodulin-dependent phosphatase calcineurin (22). Thus, higher calmodulin levels would likely lead to an increase in the level of Ca\(^{2+}\)-loaded calmodulin in the cell.

Calmodulin can accumulate rapidly in the nucleus upon an increase in intracellular Ca\(^{2+}\) (23, 24), presumably through the action of nuclear calmodulin-binding proteins that preferentially sequester the Ca\(^{2+}\)-loaded form (24, 25). In some neurons calmodulin is primarily nuclear. In these cells, CREB (cAMP-response element-binding protein) phosphorylation by Ca\(^{2+}\)/calmodulin-dependent kinase IV is not dependent on the nuclear import of any protein component but only on increased cellular Ca\(^{2+}\) (26). Therefore, we assume that an increased Ca\(^{2+}\)/calmodulin effect on transcriptional activity of E-proteins can occur either through nuclear accumulation of calmodulin that is dependent on an elevated Ca\(^{2+}\) concentration or through activation by Ca\(^{2+}\) binding of pre-existing nuclear calmodulin. Although Ca\(^{2+}\) stimulation by ionomycin (6) and thapsigargin (Fig. 4B) inhibited the luciferase activity from the E-box reporter in the B-cell line DG75, we did not detect any increase in nuclear calmodulin in the cell line upon either treatment (data not shown). This argues that Ca\(^{2+}\) loading of the pre-existing amount of nuclear calmodulin upon increase in intracellular Ca\(^{2+}\) concentration is sufficient to inhibit E-protein transcriptional activity.

Endogenous calmodulin was mainly localized in the cytoplasm in DG75 cells, whereas overexpressed calmodulin showed preferential nuclear localization (Fig. 6). The change in calmodulin distribution upon overexpression could be dependent on the high amount of calmodulin in the transfected cells. Because calmodulin is considered to be a limiting factor in cells, overexpression could lead to the binding of new nuclear targets with low affinity but high abundance.

E12 and overexpressed CaM were co-localized in the nucleus in DG75 cells (Fig. 6). In contrast to E12, the mutant m8-4-7 showed only partial sub-nuclear co-localization with overexpressed calmodulin. These data together with the differential effects of overexpressed calmodulin on transcriptional activity of wild type E12 and some of the mutants most likely suggests that nuclear calmodulin binds wild type E12 through basic domain interactions and thereby forms complexes that are unable to bind DNA and, therefore, are transcriptionally inactive. Further evidence supporting this interpretation is demonstrated in the chromatin immunoprecipitation experiment, where calmodulin overexpression resulted in decreased binding of E12 and E47, but not the calmodulin binding-deficient mutant m8-4-7, to the E-box recognition sequences in vitro (Fig. 8).

An alternative explanation for calmodulin inhibition of E12 activity could be that calmodulin expression leads to a modification or protection from modification of E12 or another protein(s) involved in the E12 transcriptional complex. Many transcriptional activators are targets for calmodulin-dependent kinases or the calmodulin-dependent phosphatase calcineurin (2, 3). The transcriptional co-activator CBP is also regulated by calmodulin-dependent kinase IV (27). However, overexpression of constitutively active variants of calmodulin-dependent kinase II or IV or calcineurin did not have any significant effect on the transcriptional activity of E12 or E47 (data not shown). Furthermore, specific inhibitors of calmodulin-dependent kinases II and IV and calcineurin did not affect ionomycin-induced inhibition of the transcriptional activity of E47 (28). Collectively, these data suggest that no calmodulin-dependent change in the phosphorylation status of E12/E47 or any other component in the E12/E47 transcription complex contributes to the Ca\(^{2+}\) regulation of the transcriptional activity of E12/E47. Furthermore, analysis of partially purified E47 from the nuclear extracts of transfected cells revealed that overexpression of calmodulin did not affect DNA binding of E47 to an E-box probe in the absence of Ca\(^{2+}\)/calmodulin (data not shown). Therefore, we believe that calmodulin-dependent inhibition of the DNA binding activity of bHLH proteins is not mediated by protein modification. Another alternative mechanism for calmodulin inhibition of E-protein activity would be a potential activation of Id expression by calmodulin. This we find unlikely because calmodulin overexpression in DG75 cells did not lead to any increase in Id1, Id2, or Id3 transcripts in reverse transcription-PCR analysis (data not shown). Moreover, because calmodulin overexpression did not lead to a decrease in nuclear level of E12 (Fig. 1B), inhibition of E12 transcriptional activity by calmodulin occurs in the nucleus through the formation of an E12/calmodulin complex that cannot bind DNA. In addition, bound calmodulin may also prevent further protein interactions with the basic sequence region potentially important for the formation of a functional bHLH transcriptional protein complex. In this context it is notable that the positive effect of the transcriptional co-activator P300 on the transcriptional activity of E47 is mediated through the bHLH domain (29). However, the Ca\(^{2+}\)/calmodulin sensitivity of DNA binding of E12 in vitro and transcriptional activation by E12 in vitro were affected in an indistinguishable way by amino acid substitutions (Figs. 3–5). Thus, the results strongly favor inhibition of the DNA binding as the dominant if not the only mechanism of calmodulin inhibition of E-proteins.

T-cell receptor stimulation could potentially inhibit E-proteins through calmodulin, since T-cell receptor activation is well known to increase intracellular Ca\(^{2+}\). Bain et al. (30) have shown that T-cell receptor activation leads to a decreased DNA binding activity of E47, mediated by an increased transcription of the inhibitory Id3 HLH-protein that forms a non-DNA binding complex with E47. It is nevertheless still possible that a direct Ca\(^{2+}\)/calmodulin interaction may partially contribute to the E47 inhibition after T-cell receptor activation.

Various bHLH proteins show differential sensitivity to calmodulin (6, 7). Therefore, Ca\(^{2+}\) signaling through calmodulin could selectively lead to exclusion of certain bHLH protein dimers from E-boxes of target genes. This regulatory mechanism may have positive effects in differentiation through inhibiting E-protein dimers and, thereby, favor dimer formation of differentiation specific Class II/E-protein heterodimers. In line with this hypothesis, it was recently shown that decreased intracellular Ca\(^{2+}\) inhibits maturation of a myoblast cell line to myotubes without affecting the level of the myogenic myf5 bHLH transcription factor (31). This indicates that elevated Ca\(^{2+}\) could be a determinant for myogenesis through calmodulin inhibition of E-protein dimers and thereby favoring of DNA binding of myogenic bHLH protein/E-protein heterodimers. Ca\(^{2+}\) signaling has already been linked to the positive regulation of myogenesis, since the myogenic transcription factor MEF2 is activated by Ca\(^{2+}\)/calmodulin-dependent kinases (32). In this context it is also interesting that a domain in E-proteins, designated the RBP domain, was recently identified to play a role in keeping E-protein homodimers inactive on myogenic enhancers (33).

The calmodulin binding basic sequence shows high homology between the three E-proteins. In fact, this sequence is identical in SEP2-1 and in the third E-protein HEB. Therefore, we suggest that the transcriptional activities of all E-proteins and heterodimers within the E-protein subfamily can potentially be inhibited directly by Ca\(^{2+}\) signaling through calmodulin binding. There are a high number of potential bHLH protein dimers
with different sensitivities to calmodulin that could potentially regulate differentiation and/or other cellular functions in various tissues. In this report, identification of combinations of amino acid substitutions that block calmodulin regulation of E-proteins enables future mutant analyses to determine the role of calmodulin/bHLH-protein interactions in regulation of various target genes and cellular functions.

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