A Novel Type of Calmodulin Interaction in the Inhibition of Basic Helix−Loop−Helix Transcription Factors†

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ABSTRACT: Calmodulin is the predominant intracellular receptor for Ca^{2+} signals, mediating the regulation of numerous cellular processes. Previous studies have shown that calcium-loaded calmodulin can bind to and inhibit the activity of certain basic helix−loop−helix (bHLH) transcription factors. The basic sequence within the bHLH domain is the primary target for calmodulin binding, and sequences modulating the calmodulin interaction reside directly N-terminal to the basic sequence. Here we show that the interaction of calmodulin with bHLH proteins is of a novel type, displaying characteristics very different from those of previously characterized calmodulin−target complexes. We show that calmodulin interacts much stronger with a dimeric basic sequence than with the monomeric form. The calmodulin−bHLH protein complex contains equimolar amounts of calmodulin and bHLH chains. The interaction is unusual in being to a large extent polar in nature, and it is highly resistant to tested calmodulin inhibitors. Both the N-terminal and C-terminal domains of calmodulin can independently bind to and inhibit the DNA binding of bHLH proteins. The C-terminal domain preferentially binds to the basic sequence, whereas the N-terminal domain is essential for the effect of the modulatory sequence. We propose a model for the calmodulin−bHLH complex where two calmodulin molecules interact with one bHLH dimer, with one domain of calmodulin preferentially binding to the basic sequence of bHLH proteins and the other domain interacting with the modulatory sequence.

The basic helix−loop−helix (bHLH)[1] family of proteins is a group of transcription factors that are important regulators in a variety of eukaryotic systems, many of which involve the control of cell growth and differentiation. Therefore, it is not surprising that inappropriate expression or aberrant bHLH proteins are associated with developmental dysfunctions and/or tumorigenesis (reviewed in *1*, *2*). The majority of bHLH proteins can be classified into two groups based upon their expression patterns. Class A bHLH proteins, also called E-proteins, are broadly or ubiquitously expressed, whereas class B proteins show tissue-specific expression (*1*). Dimerization of bHLH proteins, which is mediated by their helix−loop−helix (HLH) domains, is required for DNA binding and results in the juxtaposition of the DNA binding basic sequence to target DNA (*3*, *4*). E-proteins can form homodimers, whereas the functional activity of class B proteins in vivo requires heterodimerization with an E-protein (*5*). In mammals, three genes for E-proteins have been identified: E2A, encoding the proteins E12 and E47 (*6*); E2-2/SEF2-1 (*7*, *8*); and HEB (*9*, *10*). In contrast, numerous class B proteins have been described, including the myogenic family of proteins: MyoD (*11*), myogenin (*12*, *13*), MRF4 (*14*−*16*), and Myf5 (*17*); and the neurogenic proteins NeuroD (*18*), MASH1, and MASH2 (*19*).

To constantly adapt to their environment, eukaryotic cells express a multitude of receptors that transmit environmental signals. Stimulation of specific receptors leads to an uptake of extracellular Ca^{2+} ions and/or a release of Ca^{2+} from internal stores (*20*, *21*). This surge in intracellular Ca^{2+} concentration is sensed by a family of related Ca^{2+} binding proteins, of which calmodulin is the predominant one. Calmodulin is an evolutionary highly conserved protein that is ubiquitously expressed in eukaryotic cells and essential for cell growth. Calmodulin is important for numerous cellular processes including many nuclear functions (reviewed in *22*−*25*). Calmodulin possesses four high-affinity EF-hand Ca^{2+} binding sites, each composed of two α-helices with a calcium binding loop. Upon binding Ca^{2+}, calmodulin undergoes a conformational change exposing previously inaccessible hydrophobic patches, allowing interaction with numerous target proteins and activation of signaling pathways (reviewed in *25*). All calmodulin−target complexes structurally characterized at atomic resolution share common features: the target presents an amphipathic α-helix around which calmodulin collapses so that its two domains form a continuous surface that interacts with the hydrophobic face

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[1] Abbreviations: bHLH, basic helix−loop−helix; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFP, trifluoperazine; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-12, *N*-(4-aminobutyl)-2-naphthalenesulfonamide; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide.

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of the amphipathic α-helix. Most of the binding energy is derived from van der Waals forces associated with those hydrophobic interactions, with minor polar and ionic contributions (*25*). There are, however, other calmodulin targets where a different binding mode has been suggested (*26*−*28*).

We have previously shown that Ca^{2+}-loaded calmodulin can selectively interact with and inhibit the DNA binding of E-proteins in vitro, whereas other bHLH proteins such as the heterodimers E12/MyoD and E12/MASH2 were essentially unaffected. In cultured cells, transcriptional activation of a reporter gene by calmodulin-sensitive bHLH proteins was specifically inhibited when intracellular Ca^{2+} levels were raised with ionomycin (*29*). Subsequently, we mapped the calmodulin binding site to the basic DNA binding sequence of the bHLH domain and identified sequences directly N-terminal to the basic sequence that modulate the interaction (*30*, *31*).

In this study, we characterize the calmodulin−bHLH protein interaction further and show that this interaction is of a novel type displaying many features distinct from previously investigated calmodulin−target interactions. We present data favoring that the two calmodulin domains interact with different regions of a bipartite target within the bHLH protein.

EXPERIMENTAL PROCEDURES

General Chemicals and Radiochemicals. Unless otherwise noted, all general laboratory chemicals were obtained through KEBO Lab (Spånga, Sweden). Radiochemicals were supplied by Amersham Pharmacia Biotech (U.K.).

Plasmids, Proteins, and Peptides. Plasmids for overexpression of calmodulin and the His-tagged bHLH proteins in *E. coli* have been described previously (*30*). Deletion mutants of E12 were derived from the previously described construct (*29*) by polymerase chain reaction mutagenesis with *Pfu* polymerase and cloned into pET20b+His (*30*). Overexpressed calmodulin was purified by heat denaturation of *E. coli* proteins and subsequent purification of the soluble calmodulin by cation exchange chromatography and phenyl-Sepharose chromatography as described (*30*). Purified Ca^{2+}-free calmodulin was dialyzed exhaustively against water and lyophilized, and the dry powder was stored at −20 °C. Tryptic digestion of calmodulin was performed in the presence of Ca^{2+} as described (*32*). The tryptic fragments Tr1C (amino acids 1−78) and Tr2C (amino acids 79−149) were purified by ion exchange chromatography as described (*33*). Purified tryptic fragments were dialyzed against water, lyophilized, and stored as dry powder at −20 °C. Prior to use, lyophilized proteins were dissolved in water at a concentration of 1 mg/mL. Overexpressed His-tagged bHLH proteins were purified by metal chelation chromatography and heparin−Sepharose affinity chromatography as described (*30*). Aliquots were stored at −80 °C in 20 mM HEPES, pH 8.0, 100 mM NaCl, 1% glycerol, 0.01% Triton X-100, and 2 mM DTT. Purity of the proteins was analyzed by SDS−polyacrylamide gel electrophoresis, and protein concentrations were determined using BCA Protein Assay Reagent (Pierce). Radiolabeled E12 and derivatives and calmodulin were in vitro translated using the TnT-coupled reticulocyte lysate system (Promega) with T7 RNA polymerase, incorporating ^{35}S-labeled methionine. The proteins were purified by heparin−Sepharose (Pharmacia) (E12 and derivatives) or phenyl-Sepharose chromatography (Pharmacia) (calmodulin) as described (*30*). The SEF2-1 basic sequence peptide **KERRMANNARERLRVR**GGCGY (SEF2-1 sequence shown in boldface type) was custom-synthesized by the biotechnology facility at Uppsala University. To obtain monomeric peptide, 2 mM DTT was included in the reaction mixture, thus fully reducing the cysteines. Dimers of the peptide were obtained by instead adding 1 mM 1,1′-azobis-(*N*,*N*-dimethylformamide) (diamide), forcing the formation of cystine bridges. The redox state of the peptide was verified by SDS−PAGE in the absence of reducing agents in the loading buffer. The control peptide representing the calmodulin binding site of calmodulin-dependent protein kinase II (CaMK II, amino acids 290−309) was obtained from Sigma.

Calmodulin Binding Assay. Dansylated calmodulin was prepared using standard procedures (*34*). Binding of peptides and proteins to dansylated calmodulin was performed in binding buffer (20 mM HEPES, pH 8.0, 100 mM NaCl, and 3 μM $CaCl_2$) and monitored by spectrofluorometry, exciting at a wavelength of 340 nm and recording the emission at 490 nm. Data analysis was performed as previously described (*30*). Binding of calmodulin tryptic fragments was measured by competition. Here, sufficient target protein was added to reach half-saturation of dansylated calmodulin, and the ability of calmodulin or the tryptic fragment to compete for the target with the dansylated calmodulin was monitored.

Electrophoretic Mobility Shift Assay. EMSAs were performed as previously described (*35*) using an end-labeled DNA segment containing either the μE5 E-box sequence CAGAACACCTGCAGCAGCT (*8*, *36*) or the binding site of the muscle creatine kinase (MCK) enhancer (*29*). Where indicated, bHLH proteins were preincubated with calmodulin, tryptic fragments of calmodulin, and/or calmodulin inhibitors.

Native PAGE. Proteins were premixed in the presence of 1 mM $CaCl_2$ or EDTA as indicated for 1 h at room temperature. Then 1 volume of 2× sample buffer (100 mM Tris, pH 8.8, 20% glycerol, 0.2% Bromophenol Blue) was added, and the protein complexes were separated on a 10−15% Tris−glycine (pH 8.8) gradient gel, using 40 mM Tris−glycine running buffer (pH 8.8). Gels were electrophoresed at 30 mA at 4 °C and either Coomassie-stained or autoradiographed to visualize proteins.

RESULTS

Calmodulin Preferentially Targets Dimers of the Basic Sequence. One model for the calmodulin inhibition of bHLH proteins could be that calmodulin binds to and dissociates bHLH dimers into their monomeric form, thereby preventing DNA binding. To test this hypothesis, E12 homodimers and homodimers of the shorter E12bHLH derivative were preincubated with calmodulin and mixed together, and the calmodulin inhibition was subsequently relieved by brief chelation of the calcium. If calmodulin were to dissociate bHLH dimers into their monomeric form, one would expect to find not only the original homodimers but also the E12/E12bHLH heterodimer. However, only the DNA binding activity of the two original homodimers was found to reemerge in electrophoretic mobility shift analysis (EMSA)

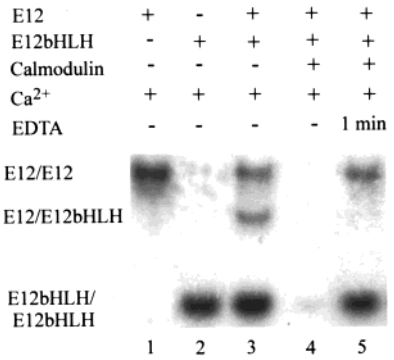

FIGURE 1: Calmodulin does not dissociate E12 into monomers. DNA complexes of homodimers of E12 and the shorter derivative E12bHLH (amino acids 271−340) are shown in lanes 1 and 2, respectively. Premixing E12 with E12bHLH produces in addition to the DNA binding homodimers also DNA complexes with E12/E12bHLH heterodimers (lane 3). Homodimers of E12 and E12bHLH were preincubated with 2.5 μM calmodulin in the presence of 0.1 mM $CaCl_2$ for 5 min to allow formation of the calmodulin−bHLH complex and subsequently mixed without (lane 4) or with (lane 5) addition of 1 mM EDTA for 1 min. The complexes were analyzed by electrophoretic mobility shift analysis (EMSA) using the μE5 binding site as ^{32}P-labeled probe as described under Experimental Procedures.

without any significant amount of E12/E12bHLH heterodimer detected (Figure 1, lane 5). Since mixing of E12 and E12bHLH leads to formation also of E12/E12bHLH heterodimers active in DNA binding (Figure 1, lane 3), this argues against a dissociation of bHLH dimers by calmodulin and instead favors that the dimer, or another oligomeric state, is the calmodulin target in the complex. Similar results were found with full-length and shortened derivatives of another bHLH protein, SEF2-1 (data not shown).

Previously, we have shown that a synthetic peptide representing the basic sequence of SEF2-1 could bind to dansylated calmodulin, thereby identifying this sequence as a calmodulin target. However, the affinity for the basic peptide was much lower than that determined for the intact bHLH domain (*30*). One possible reason for the weaker calmodulin affinity of the basic peptide compared to the bHLH domain could be its monomeric form. We therefore asked if artificial dimerization of the basic peptide could functionally replace the HLH domain and increase the affinity of the peptide for dansylated calmodulin. A cysteine was incorporated near the C-terminal end of the basic peptide, separated from the basic sequence by a flexible linker of two glycines. A peptide dimer could therefore be formed by the addition of diamide, forcing an intermolecular disulfide bond. The ability of this peptide dimer to bind dansylated calmodulin was then measured by fluorescence spectroscopy (Figure 2A). We found that the basic peptide bound dansylated calmodulin approximately 50−100-fold stronger in the presence of diamide ($K_D \approx 10$ nM) compared to the binding of the monomeric basic peptide in the presence of DTT ($K_D \approx 700$ nM). The binding curve of the monomeric basic peptide was biphasic and reached saturation at high concentrations (data not shown) as previously reported (*30*). In contrast, addition of diamide or DTT had little or no effect on binding of dansylated calmodulin to another target, the calmodulin-dependent protein kinase II (CaMK II) peptide (K_D approximately 70 nM in the presence of diamide and 40 nM in the presence of DTT). This was also the case when the dimerizable amino acid sequence corresponding to the

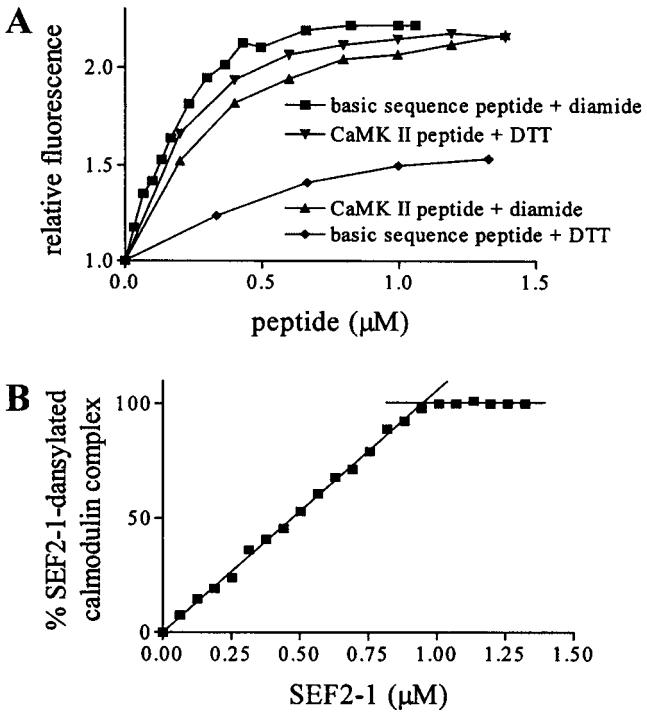

FIGURE 2: Preferential calmodulin binding to dimeric basic peptide and determination of the stoichiometry of calmodulin binding to SEF2-1. (A) The basic sequence peptide of SEF2-1 was pretreated with 2 mM DTT or 1 mM diamide for at least 10 min to achieve full reduction of the cysteines or cystine dimerization, respectively. Aliquots were added to 300 nM dansylated calmodulin, and after 5 min incubation to ensure equilibrium, the emission at 490 nm was recorded. A peptide representing the calmodulin binding domain of CaM kinase II (amino acids 290−309) was also added to dansylated calmodulin after the pretreatment with DTT or diamide. Plotted here is the emitted fluorescence light relative to the fluorescence of free dansylated calmodulin, taken as 1. (B) Dansylated calmodulin and untreated calmodulin were mixed to a total calmodulin concentration of 1 μM. SEF2-1 protein aliquots were added, and after 5 min incubation, the emission at 490 nm was recorded. Shown here is the fractional saturation of calmodulin, as determined by the relative fluorescence, versus the concentration of bHLH protein (in monomers). A straight line was fitted through the 12 initial data points. It reaches the saturation point at a bHLH concentration of approximately 940 nM.

one used in the basic peptide was introduced at the C-terminus of the control peptide (data not shown). The affinity of the dimerized basic sequence peptide for dansylated calmodulin was approximately equal to that determined for the complete bHLH domain of SEF2-1 (*30*). These results show that two basic sequences in close spatial proximity bind to dansylated calmodulin much better than an isolated basic peptide, arguing that a bHLH dimer (or another multimer) is the preferred calmodulin target. In addition, these results also strongly suggest that the HLH domain only contributes dimerization to the calmodulin−bHLH interaction, since the function of the HLH can be completely replaced by a heterologous dimerization mechanism.

Calmodulin Binds to bHLH Proteins with a 1:1 Overall Stoichiometry. Either one or multiple calmodulin molecules could interact with a dimer (or another multimer) of a bHLH protein. To determine the molar ratio between bHLH molecules and calmodulin in the CaM−bHLH complex, the binding of SEF2-1 to dansylated calmodulin was monitored at a calmodulin concentration of 1 μM. Under these conditions, the binding curve is almost linear until saturation,

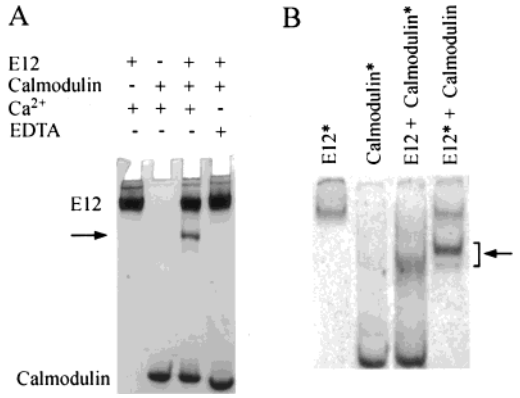

FIGURE 3: Formation of a calcium-dependent calmodulin−E12 complex. (A) Recombinant E12 and calmodulin were premixed in the presence or absence of calcium for 1 h at room temperature prior to electrophoresis on a native polyacrylamide gel as described under Experimental Procedures. Protein bands were visualized by Coomassie staining. The new band upon mixing is indicated by an arrow. (B) Recombinant and ^{35}S-labeled in vitro translated E12 and calmodulin were premixed in the presence of calcium. The resulting complexes were analyzed by native polyacrylamide gel electrophoresis and visualized by autoradiography. ^{35}S-labeled proteins are denoted by an asterisk, and the E12−calmodulin complexes are indicated by an arrow.

which is reached at 940 nM, thus approximating a ratio of one calmodulin per bHLH monomer (Figure 2B). Titration curves at lower protein concentrations showed the same calmodulin:bHLH ratio for E12 and also for SEF2-1 bHLH that lacks the modulatory sequence N-terminal to the basic sequence (data not shown). Binding curves for the dimerized basic peptide also showed this ratio (Figure 2A and data not shown). Thus, the E-proteins SEF2-1 and E12 bind calmodulin with a 1:1 stoichiometry, which is not affected by the N-terminal modulatory sequence.

Calmodulin Does Not Interact with E12 as a Large Complex. bHLH proteins exist in a variety of oligomeric states (*37*, *38*), with the DNA binding moiety being the bHLH dimer (*3*, *4*). Our findings that calmodulin interacts at a 1:1 molar ratio and preferentially with the bHLH dimer and not with monomers (Figures 1 and 2) do not rule out that calmodulin might induce the formation of very large non-DNA binding complexes with bHLH proteins. To investigate this alternative, calmodulin−E12 complexes were analyzed by native PAGE. The small, monomeric, and highly negatively charged calmodulin migrates very fast (Figure 3A). The recombinant E12 migrates slower and predominantly as a single band although other complexes with slower mobility are apparent. Upon preincubation of E12 with calmodulin in the presence of calcium, a new complex with an intermediate mobility appears. Formation of this new complex is calcium-dependent as the chelator EDTA prevents complex formation. This new complex could be composed of both E12 and calmodulin, or be a different oligomeric state of E12 with altered mobility. To differentiate between these possibilities, radio-labeled in vitro translated E12 and calmodulin were used in combination with *E. coli* produced unlabeled proteins. Mixing either labeled E12 with recombinant calmodulin or labeled calmodulin with recombinant E12 produced in both cases a new, labeled complex with an intermediate mobility (Figure 3B). The slightly faster migrating and less distinct band for the complex with in vitro translated calmodulin compared to nonlabeled *E. coli* produced calmodulin is likely to reflect calmodulin modifications affecting charge, and therefore mobility, that do not occur in *E. coli* (unpublished results and *39*−*41*). The results show that the complex contains both E12 and calmodulin. The much faster mobility of the E12−calmodulin complex compared to free E12 strongly argues against calmodulin "pushing" E12 to a very large non-DNA binding multimeric E12−calmodulin complex.

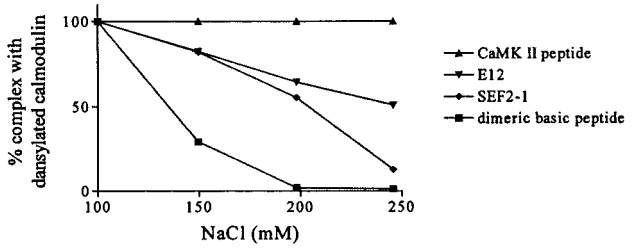

FIGURE 4: Salt sensitivity of the calmodulin interaction with the SEF2-1 basic sequence and with bHLH proteins. Dansylated calmodulin (300 nM) in binding buffer supplemented with 1 mM diamide was saturated with the dimeric basic peptide of SEF2-1 (squares), E12 (inverted triangles), SEF2-1 protein (amino acids 547−667) (diamonds), or CaM kinase II peptide (triangles). Through stepwise addition of NaCl, the salt concentration was increased, and after 5 min incubation, the emission at 490 nm was recorded. Plotted here is the fractional saturation of dansylated calmodulin as determined by the relative fluorescence of free and saturated dansylated calmodulin.

The Calmodulin−bHLH Interaction Is Polar in Nature. A common feature of calmodulin targets is that they form an amphipathic α-helix with a hydrophobic face and a polar face (*42*). While the basic sequence of bHLH proteins can form an α-helix, at least in the bHLH−DNA complex (*3*, *4*), it is not amphipathic in nature. Instead, 9 of the 16 amino acids are charged, and the 7 positively charged amino acids are distributed over the entire basic sequence. Calmodulin, on the other hand, is negatively charged. This suggests that the binding of calmodulin to the basic sequence may not be predominantly hydrophobic, but rather facilitated by ionic or polar interactions. To test this hypothesis, the basic peptide in its dimeric form, SEF2-1, or the CaMK II control peptide was bound to dansylated calmodulin. The resulting peptide or SEF2-1 complex with dansylated calmodulin was then challenged with increasing ionic strength (Figure 4). As expected, the largely hydrophobic nature of the CaMK II peptide−dansylated calmodulin complex made it inert to the increased salt concentration. In contrast, the dimeric basic peptide dissociated from dansylated calmodulin when the ionic strength was raised. This suggests that a significant portion of the binding energy for the dimeric basic peptide is derived from forces that can be shielded by salt ions. Sensitivity to increased ionic strength was also found for SEF2-1 protein (Figure 4). E12 was also sensitive to increased ionic strength, both when NaCl and KCl were used (Figure 4 and data not shown). Thus, this is not just a phenomenon restricted to peptides. The complex formed between a bHLH protein and calmodulin is also to a large extent through ionic interaction.

Calmodulin Inhibitors Have Little Effect on the Calmodulin−bHLH Interaction. Several small organic molecules are known to inhibit the interaction of calmodulin with targets, both in vitro and in vivo (*43*, *44*). These molecules are usually hydrophobic and are thought to bind to the

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Calmodulin Inhibitor	Phosphodiesterase inhibition, IC_{50} (μM)*	E12 DNA binding inhibition, IC_{50} (μM)	Relative IC_{50} for E12	E12bHLH DNA binding inhibition, IC_{50} (μM)	Relative IC_{50} for E12bHLH
W-5	240	1390 ± 38	5.8	798 ± 9	3.3
W-7	28	473 ± 38	16.9	282 ± 69	10
W-12	260	2063 ± 68	7.9	980 ± 190	3.8
W-13	68	522 ± 8	7.7	345 ± 25	5.1
Chlorpromazine	17	217 ± 39	12.7	301 ± 76	18
Trifluoperazine	5.8	148 ± 22	25.5	400 ± 22	69
Calmidazolium	0.04	5.8 ± 0.3	145	3.4 ± 0.4	85

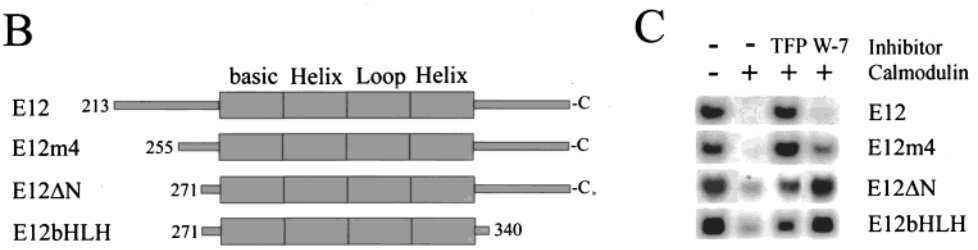

FIGURE 5: Differential effects of calmodulin inhibitors on the inhibition of E12 DNA binding and mapping of the E12 modulatory sequence. (A) Comparison of IC_{50} values for inhibition of the calmodulin inhibition of *E. coli* produced E12 and E12bHLH, and published IC_{50} values for inhibition of calmodulin activation of phosphodiesterase (asterisk, refs *62*−*65*). IC_{50} values were determined as the calmodulin inhibitor concentrations necessary to regain 50% of the DNA binding activity of E12 or E12bHLH. IC_{50} values are shown both as absolute values and relative to IC_{50} values for inhibition of phosphodiesterase activation, which were set to 1. (B) Schematic illustration of the E12 protein derivatives used. (C) The ability of intermediary concentrations of W-7 (350 μM) and TFP (275 μM) to relieve the calmodulin inhibition of DNA binding of the in vitro translated truncated E12 proteins was analyzed. EMSA conditions were as described (*35*) using the μE5 DNA binding site.

hydrophobic patches of calmodulin that become exposed upon Ca^{2+} binding, thereby blocking the interaction with target molecules. The salt sensitivity of the interaction between calmodulin and bHLH proteins suggested that the binding is not predominantly hydrophobic in nature. Therefore, some calmodulin inhibitors may not efficiently affect this interaction. To test this possibility, we analyzed several commonly used calmodulin inhibitors for their ability to block the calmodulin inhibition of bHLH proteins. E12 or E12bHLH, lacking the modulatory sequence, was added to calmodulin preincubated with various concentrations of calmodulin inhibitor, and the residual ability of E12 or E12bHLH to bind DNA was assayed by EMSA. IC_{50} values were estimated from the concentrations needed to relieve 50% of the calmodulin inhibition and compared to published IC_{50} values for another functional interaction of calmodulin, inhibition of phosphodiesterase (Figure 5A). None of the tested calmodulin inhibitors was able to significantly affect the calmodulin inhibition at a concentration sufficient to block the calmodulin interaction with other targets. The IC_{50} values for inhibition of E12 and E12bHLH were from 3.3- to 145-fold higher than for the inhibition of phosphodiesterase (Figure 5A). The modulatory sequence of E12 was found to influence the ability of certain calmodulin inhibitors to relieve the inhibition of DNA binding (Figure 5A,C). Most inhibitors showed a lower efficiency on the calmodulin inhibition of E12 than of E12bHLH. This, however, was not the case for chlorpromazine and trifluoperazine (TFP). This difference between E12 with and without the modulatory sequence in the relative efficiency of inhibition by W-7 and TFP was subsequently used to more finely map the modulatory sequence of E12. N-Terminal truncations of E12 were produced as illustrated in Figure 5B, and the ability of intermediary concentrations of W-7 and TFP to relieve calmodulin inhibition in EMSA was assayed (Figure 5C). TFP is more potent at relieving the calmodulin inhibition for E12 compared to W-7, whereas the opposite is true for E12bHLH (cf. upper and lower panels in Figure 5C). E12ΔN, which possesses the same N-terminus as E12bHLH but has a longer C-terminus, behaves as E12bHLH, showing that the difference observed resides in the N-terminal region of the protein. E12m4 behaves as E12, showing that this construct still possesses the modulatory sequence. Interestingly, E12m4 contains only 16 amino acids not present in E12ΔN, which is comparable to the 13 amino acid modulatory sequence identified for MyoD (*30*).

The N- and C-Terminal Domains of Calmodulin Can Bind Independently to bHLH Proteins. The bipartite calmodulin interaction with both a primary calmodulin binding site and a modulatory sequence (*30*) raised the possibility that calmodulin may bind to the basic sequence with only one of its domains, whereas the other domain would be free to interact with the modulatory sequence. To address this, we separated the N-terminal domain of calmodulin, Tr1C, and the C-terminal domain, Tr2C, by tryptic cleavage in the central α-helix (Figure 6A), isolated the domains as described under Experimental Procedures, and investigated whether the domains could compete with calmodulin for binding to a bHLH protein. Dansylated calmodulin was half-saturated with either N-terminally truncated E12 or E12ΔN (see Figure 5B). The ability of calmodulin, Tr1C, or Tr2C to compete with dansylated calmodulin for the binding of either E12 (Figure 6B) or E12ΔN (Figure 6C) was measured. Intact calmodulin could efficiently compete with dansylated calmodulin for target binding, both in the presence (Figure 6B) and in the absence (Figure 6C) of the modulatory sequence of E12. The C-terminal Tr2C domain could efficiently displace dansylated calmodulin from E12ΔN, whereas Tr1C

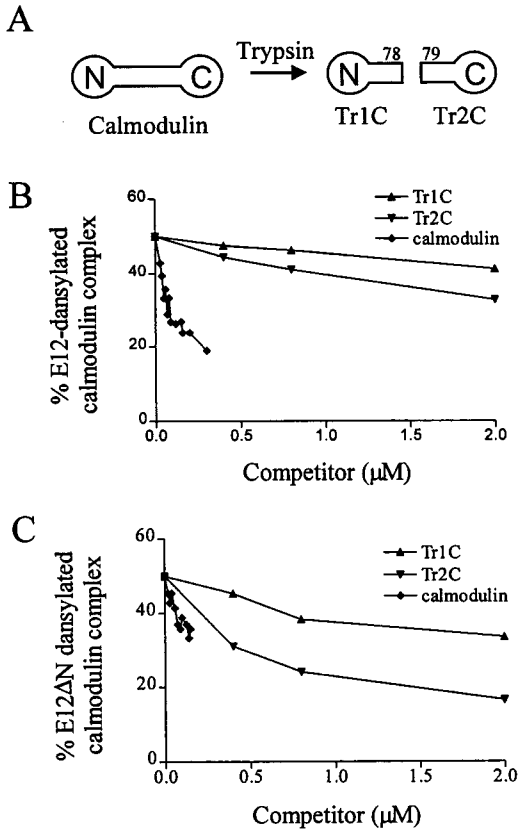

FIGURE 6: Independent binding of isolated calmodulin domains to E12 derivatives. (A) Schematic representation of calmodulin and its tryptic fragments Tr1C and Tr2C. (B and C) Dansylated calmodulin (150 nM) was half-saturated with homodimers of E12 (B) or E12ΔN (C) with and without the modulatory sequence, respectively. The preformed complexes were then challenged with increasing concentrations of either unlabeled calmodulin (diamonds), Tr1C (triangles), or Tr2C (inverted triangles), and the remaining emitted fluorescence at 490 nm was recorded.

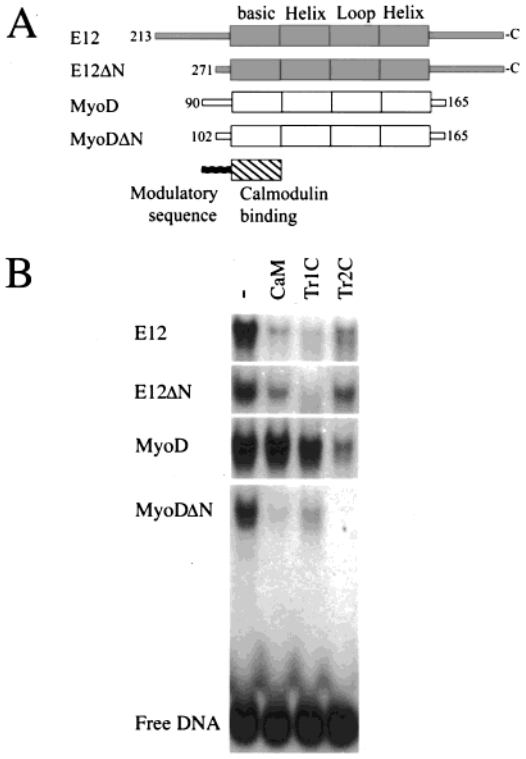

FIGURE 7: Inhibition of bHLH proteins by isolated calmodulin domains. (A) Schematic representation of the bHLH protein constructs used. bHLH domains and N- and C-terminal amino acids are indicated. The sequences identified as the calmodulin binding domain and the directly N-terminal modulatory sequence are shown. (B) Binding of bHLH proteins to a DNA probe containing an MCK site in EMSA. Binding reactions were in the absence or presence of 2.5 μM calmodulin, Tr1C, or Tr2C with binding conditions as described under Experimental Procedures.

was a much weaker competitor. A 1:1 mixture of the two domains behaved as the sum of the calmodulin halves, and no significant synergism or antagonism could be detected (data not shown). Since Tr2C but not Tr1C efficiently competes with dansylated calmodulin in the absence of modulating sequences, we conclude that it is preferentially the C-terminal domain of calmodulin that interacts with the basic sequence of E12, although the N-terminal domain is not completely inert. In the presence of the modulatory sequence (Figure 6B), neither domain was able to strongly compete with dansylated calmodulin, although Tr2C was still more efficient than Tr1C. We conclude that the interaction between the E12 modulatory sequence and calmodulin provides additional stabilization to the E12-dansylated calmodulin complex, making it more difficult for the individual domains, in particular Tr1C, to compete with intact calmodulin.

The N- and C-Terminal Domains of Calmodulin Can Independently Inhibit bHLH Proteins. Having shown that Tr2C, and to a lesser extent Tr1C, can bind to bHLH proteins, we wanted to investigate if the binding of one calmodulin domain to the basic sequence was sufficient to cause inhibition of DNA binding. To address this question, we analyzed the ability of Tr1C and Tr2C to inhibit the DNA binding of bHLH proteins in EMSA (Figure 7B). High concentrations of either Tr1C or Tr2C (2.5 μM) could inhibit the DNA binding of E12, both in the absence (E12ΔN) and in the presence (E12) of the modulatory sequence. As previously found for the inhibition by calmodulin, the inhibition mediated by Tr1C and Tr2C was calcium-dependent (data not shown). We wanted to further analyze if this functional binding was limited to one bHLH protein, or whether other bHLH proteins could also be inhibited. Therefore, we tested the ability of MyoDΔN, a derivative of MyoD lacking the negative modulatory sequence [Figure 7A and (*30*)], to be inhibited by the calmodulin fragments. Both Tr1C and Tr2C could inhibit the DNA binding of MyoDΔN (Figure 7B), showing that the ability of tryptic fragments of calmodulin to inhibit DNA binding is not restricted to one bHLH protein, but rather is a more common phenomenon. As previously reported (*30*), the MyoD modulatory sequence blocked the inhibition by calmodulin (cf. MyoDΔN and MyoD in Figure 7B). Surprisingly, in contrast to calmodulin, Tr2C could also inhibit MyoD. This suggests that the modulatory sequence of MyoD acts by a negative interference through the N-terminal domain of calmodulin.

DISCUSSION

Previously we have shown that calmodulin can inhibit the DNA binding of several class A bHLH proteins, whereas heterodimers between class A and class B proteins remain largely unaffected (*29*). The inhibition of DNA binding is dependent on a direct physical interaction between calmodulin and the DNA binding basic sequence of the bHLH

domain. We also identified sequences residing directly N-terminal to the basic sequence, which were capable of modulating the interaction with calmodulin (*30*). bHLH proteins exist in multiple oligomeric states in solution (*37*, *38*) of which the dimer is the DNA binding moiety (*3*, *4*). Previously, we identified a peptide representing the basic sequence of SEF2-1 as the calmodulin target within the bHLH domain (*30*). However, the oligomeric state of the target remained unknown. Here we show that calmodulin does not dissociate bHLH proteins into monomers upon binding (Figure 1) and that the binding of calmodulin to a dimeric target is much stronger compared to a monomeric target (Figure 2A). The finding that a simple disulfide bridge can mimic the effect of the HLH domain (Figure 2A) argues that the HLH domain is needed only for dimerization of the basic region and is not involved in any direct interaction with calmodulin. We have demonstrated that the calmodulin−bHLH complex has a 1:1 overall stoichiometry (Figure 2B). Taken together, these results would suggest that two calmodulin molecules would interact with one bHLH dimer. Although we cannot rule out higher stoichiometries, the much faster native PAGE mobility of the complex than the free E12 strongly argues against formation of a very large non-DNA binding multimeric E12−calmodulin complex.

The existence of a primary calmodulin interaction site and the presence of modulating sequences suggest that calmodulin interacts with bHLH proteins in a novel manner. Several calmodulin−target peptide complexes have been structurally characterized by NMR and/or X-ray crystallography (*45*−*47*). A common feature of these calmodulin targets is that they form an amphipathic α-helix with a polar face and a hydrophobic face with bulky hydrophobic amino acids at specific locations within the α-helix (*42*). Calmodulin collapses around the α-helical target (wrap-around model) (*48*), gaining most of its binding energy from van der Waals contacts between the hydrophobic face of the α-helix and hydrophobic patches on calcium-loaded calmodulin, with the methionines in the hydrophobic patches playing a key role in the interaction (*46*, *49*−*52*). Several lines of evidence presented in this study suggest that calmodulin uses a different binding mode to interact with bHLH proteins. We have shown that a complex between calmodulin and the dimeric basic sequence peptide or an E-protein dissociates at high salt, whereas a control calmodulin−target complex was completely unaffected by the concentrations used (Figure 4). This salt destabilization effect reflects a fundamental difference between a "classical" calmodulin complex and a bHLH−calmodulin complex. As the name implies, the basic sequence is rich in positively charged amino acids while calmodulin has an overall negative charge. It is likely that, to a large extent, calmodulin interacts with the basic sequence via salt bridges and other polar interactions, which are more sensitive to changes in ionic strength than the hydrophobic van der Waals contacts from which "classical" calmodulin complexes derive most of their binding energy. A further argument in favor of polar rather than hydrophobic interactions is the finding that hydrophobic calmodulin inhibitors poorly block the calmodulin−bHLH interaction (Figure 5). These inhibitors are believed to interact with the hydrophobic patches exposed upon calcium binding. Since the inhibitors cannot efficiently block the calmodulin−bHLH interaction, it would follow that the hydrophobic patches on calmodulin contribute very little to the binding of bHLH proteins. The poor ability of calmodulin inhibitors to block the calmodulin−bHLH protein interaction could have two alternative explanations. The calmodulin−bHLH protein binding could be very tight and able to displace the inhibitor from calmodulin, or the inhibitor binds to calmodulin sequences that are not essential for the bHLH protein interaction. Several calmodulin inhibitors have been shown to bind calmodulin with constants in the micromolar range (*53*, *54*). It is possible that the bHLH protein displaces the inhibitor from calmodulin since the binding constant for the calmodulin−bHLH interaction is higher than the calmodulin−inhibitor constants (*30*). An argument against this explanation is that calmodulin binds to many "classical" calmodulin targets with the same affinity or in some cases even higher affinity than to bHLH proteins (*42*). Thus, despite binding calmodulin with approximately similar affinities, "classical" targets and the novel bHLH target can be differentially inhibited by calmodulin inhibitors. The second alternative, that calmodulin inhibitors bind to calmodulin sequences not involved in this novel interaction, therefore appears more likely but still remains unresolved. Work is currently in progress to determine the calmodulin sequences involved in the interaction with bHLH proteins.

A further line of evidence suggests that the calmodulin−bHLH complex has features that are incompatible with typical wrap-around binding. Both halves of calmodulin, Tr1C and Tr2C, can independently bind to and inhibit the DNA binding of bHLH proteins (Figures 6 and 7). This suggests that only one calmodulin domain is needed to interact with the basic region, leaving the other domain free to interact with the modulatory sequence. This dual site interaction stabilizes the E12−calmodulin complex. In the case of MyoD, the modulatory sequence prevents the formation of a stable MyoD−calmodulin complex. This negative effect of the modulatory sequence of MyoD appears to be mediated by an interaction with the N-terminus of calmodulin, since Tr2C, lacking the N-terminal domain of calmodulin, can inhibit MyoD (Figure 7B). The simplest explanation is that a steric clash between the modulatory sequence of MyoD and the N-terminal domain of calmodulin prevents the correct placement of the C-terminal domain of calmodulin that would allow stable binding to the basic sequence.

This is not the first time that individual domains of calmodulin have been shown to mimic the actions of the intact protein in activating calmodulin targets (*55*−*57*). A common theme arising from studies of calmodulin halves is that there are three classes of targets in terms of their ability to be activated by individual calmodulin domains: those able to be activated by the C-terminal domain, those activated by a mixture of the two domains, and those unable to be activated by the individual domains (*57*). We show that the bHLH proteins are able to bind better to the C-terminal domain of calmodulin (Figure 6B,C). However, we also demonstrate that the isolated N-terminal domain is able to functionally replace intact calmodulin in inhibiting the DNA binding of E12 (Figure 7B). The fact that the C-terminal domain of calmodulin binds more efficiently to the basic sequence probably reflects differences between the two domains. Although the N- and C-terminal hydrophobic pockets share 71% sequence identity, the hydrophobic pocket

of the C-terminal domain has additional adjacent glutamate residues (*58*) that could provide additional ionic interactions to stabilize the bHLH−calmodulin complex. Mapping studies of the modulatory sequence in E12 and MyoD identified a region of 16 and 13 amino acids, respectively [Figure 5C and (*30*)], which probably represents the minimal recognition domain for a calmodulin domain. The differential effect of high concentrations of calmodulin inhibitors on the calmodulin inhibition of E12 with and without the modulatory sequence (Figure 5) indicates that the modulatory sequence has a very significant effect on how calmodulin interacts with the bHLH protein.

Collectively, the data presented here suggest a model where calmodulin binds to the basic sequence of bHLH proteins with only one of its domains. This binding is to a large extent facilitated by salt bridges and other polar interactions between the negatively charged calmodulin and the positively charged basic sequence. The other domain of calmodulin interacts with the modulating sequences, providing either additional stabilization, as is observed for E12, or destabilization, as is the case for MyoD.

Although other types of calmodulin−target interactions have been found (*28*, *57*, *59*) in addition to the "classical wrap-around model" interactions (*45*−*47*), this is the first reported case of an interaction with a bipartite target. None of the previously characterized calmodulin−target interactions was largely based on electrostatic forces with a basic target rather than interaction with a predominantly hydrophobic target, and none has been between multiple calmodulin and target molecules in equimolar amounts.

Why does calmodulin bind much stronger to a dimer compared to a monomer? Several explanations are possible: Two calmodulin molecules could interact with each other in the complex. However, the highly negative surface charge of calmodulin would strongly argue against a direct calmodulin−calmodulin interaction stabilizing the complex. On the other hand, it is possible that Ca^{2+} acts as "glue" between two calmodulin molecules positioned on the bHLH dimer. It has been previously shown that negative surface charges of the calmodulin-related calcium binding protein calbindin D_{9K} attract calcium ions (*60*, *61*). Since Ca^{2+} is divalent, it is feasible that the same Ca^{2+} ion could interact with the negative charges of two calmodulin molecules positioned in close spatial proximity due to their interactions with the two basic sequences of a bHLH dimer. Ca^{2+} ions positioned in that way could provide the additional binding energy needed to account for the observed increased binding. Alternatively, each of the calmodulin molecules in the complex could interact with both monomers of the bHLH dimer. We consider this to be a more likely explanation. When the modulatory sequence is present, one domain of calmodulin would contact the modulatory sequence of one bHLH protein while the other calmodulin domain interacts with the basic sequence of the other bHLH protein in the bHLH dimer. Thus, both calmodulins contact both bHLH monomers in the dimer. However, stronger binding is also observed for the dimer in the absence of the modulatory sequence, which then would imply that the domains of calmodulin can contact both monomers of a bHLH without the modulatory sequence. Studies are under way to further investigate the atomic structure of the calmodulin−bHLH protein complexes.

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