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Basic Helix-Loop-Helix Protein Sequences Determining Differential Inhibition by Calmodulin and S-100 Proteins*

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Basic helix-loop-helix (bHLH) proteins are a group of transcription factors that are involved in differentiation and numerous other cellular processes. The proteins include the widely expressed class A bHLH proteins (E proteins) and the tissue-specific class B proteins. Previous studies have shown that calmodulin can inhibit the DNA binding activity of certain E proteins but not their heterodimers with class B proteins. Here we show that calmodulin binds to the DNA-interacting basic sequence within the bHLH domain of E proteins. The strength of the binding of bHLH proteins to calmodulin correlates directly with the calmodulin sensitivity of their DNA binding. The basic sequence of MyoD, a class B protein, can also interact with calmodulin. This interaction, however, is blocked by MyoD sequences directly N-terminal of the basic sequence. We further demonstrate that S-100 proteins can interact with and differentially inhibit the DNA binding of bHLH proteins through interaction with the basic sequence. Both the binding to the basic sequence and the effect of the directly N-terminal sequence vary for different S-100 proteins and bHLH proteins. The results suggest the involvement of both calmodulin and S-100 proteins in the differential regulation of bHLH proteins.

Basic helix-loop-helix (bHLH)[1] proteins are a class of transcription factors that are important regulators in numerous systems, often involving the control of cell growth and differentiation (1). Most bHLH proteins (with some exceptions) can be broadly classified into two groups based on their patterns of expression. Class A bHLH transcription factors, also called E proteins, are broadly expressed and include the *E2A* gene products E12/E47 (2) and products of the *E2–2/SEF2–1* gene (3, 4). E proteins are capable of forming both homodimers with themselves and heterodimers with cell type-specific class B proteins. This large group of tissue-restricted proteins includes the myogenic proteins (myogenin (5, 6), MyoD (7), MRF4 (8–10), and Myf5 (11)) and proteins involved in neurogenesis, including MASH2 (12) and NeuroD (13). Functional activity of a class B protein *in vivo* requires heterodimerization with an E protein, resulting in the commitment of cells to differentiation pathways (14).

Calcium plays a crucial role in many cellular processes (15, 16). Its actions are largely mediated through a family of calcium-binding proteins, of which calmodulin is the major calcium sensor. Calmodulin is a highly conserved, ubiquitously expressed protein that is essential for cell growth (17, 18). Calmodulin has four high affinity calcium binding sites called EF-hands, each composed of two α-helices connected by a calcium binding loop (19). Upon calcium binding, calmodulin undergoes a conformational change to expose hydrophobic patches (Ref. 20 and references therein), which allows interaction with numerous target proteins and the subsequent activation of signaling pathways.

S-100 proteins are other members of the EF-hand protein family that also modulate the activities of various proteins. At least 17 members of the S-100 family have been identified. They vary in their target specificity, cell type distribution, and cell cycle regulation (21). The best studied members of the S-100 family, S-100α and S-100β, have been shown to exist as both homodimers with themselves and heterodimers with each other. Their expression patterns differ; S-100α is predominantly found in muscle, whereas S-100β is highly expressed in cells within the nervous system (22–26). S-100 proteins are believed to interact with many proteins, and the identification of several common targets with calmodulin suggests that a common structural domain mediates these interactions (27–30).

We have previously shown that calcium-loaded calmodulin can selectively inhibit the DNA binding of E protein homodimers *in vitro* and that the calcium ionophore ionomycin inhibits their activity *in vivo*. In contrast, the heterodimers E12/MASH2 and E12/MyoD were either less sensitive or completely resistant (31).

Here we identify the protein sequences within the bHLH proteins that determine the differential inhibition by calmodulin. We show that this inhibition is the result of a physical interaction between the DNA binding basic sequence and calmodulin. Both E12 and MyoD basic sequences are capable of binding calmodulin. However, sequences directly N-terminal to the basic sequence inhibit this interaction in the case of MyoD. We show that S-100 proteins can also inhibit bHLH proteins through interaction with the basic sequence. S-100$\alpha\alpha$, S-100$\beta\beta$, and calmodulin show distinct bHLH protein preferences, and these differential interactions are determined by the basic sequences and the directly N-terminal sequences. These results suggest that both calmodulin and S-100 proteins play roles in the differential regulation of bHLH proteins.

MATERIALS AND METHODS

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

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[1] The abbreviations used are: bHLH, basic helix-loop-helix; EMSA, electrophoretic mobility shift assay.

Plasmid Constructs—Constructs for *in vitro* translation and overexpression in *Escherichia coli* of chimeric E12/MyoD proteins were based on the previously described E12 and MyoD constructs (31). Domain swapping was carried out by oligonucleotide-directed polymerase chain reaction mutagenesis using *Pfu* polymerase (Stratagene). The first amino acids defining each region within the bHLH domain are as follows: for MyoD, basic sequence, 108; helix 1, 123; loop, 138; helix 2, 146; for E12, basic sequence, 277; helix 1, 292; loop, 307; helix 2, 317. Proteins were deleted at the N termini up to amino acids 102 (MyoD) and 272 (E12) by polymerase chain reaction mutagenesis. For *in vitro* translation, constructs were cloned into pSP64-poly(A)$^+$ (Promega). For overexpression in *E. coli*, constructs were cloned into a pET-20b+ derivative (Novagen) modified to include an N-terminal His_6 tag (pET-20b+His). To maintain the frame, a glutamine was added between the tag and the N termini of E12ΔN and MyoD, and a leucine was added for MyoDΔN.

The deleted SEF2–1 (amino acids 547–667), SEF2–1ΔN (amino acids 558–667), SEF2–1-bHLH (amino acids 563–622), and MASH2ΔN (amino acids 98–174) expression constructs were derived from previously described constructs (31) by polymerase chain reaction mutagenesis with *Pfu* polymerase and were cloned into pET-20b+His.

The nucleotide sequences of all polymerase chain reaction-amplified DNA segments were confirmed by DNA sequencing. pETCaM, which contains the calmodulin-encoding sequences from pCaMRW (32) in a modified pET-3 vector (Novagen), was a kind gift from Dr. Peter Brodin.

Proteins—pSP64-poly(A)$^+$-based constructs were *in vitro* translated using the TnT coupled reticulocyte lysate system (Promega) with SP6 RNA polymerase. For reactions involving heterodimers, both partners were co-translated. The proteins were purified by heparin-Sepharose (Pharmacia) chromatography (31).

Recombinant bHLH proteins were expressed in the *E. coli* strain BL21(DE3) pLysS according to the manufacturer's instructions (Qiagen). 400 ml of cells were harvested after a 3-h induction and resuspended in 10 ml of buffer containing 10 mM Tris, pH 8.0, and 0.5 mM of the protease inhibitor phenylmethylsulfonyl fluoride (Sigma). Cells were lysed by freeze-thawing followed by sonication, and soluble His-tagged proteins were purified by metal chelation chromatography on a Ni-nitrolotriacetic acid column according to the manufacturer's instructions (Qiagen). The manufacturer's suggested buffer, 20 mM HEPES, pH 8.0, 500 mM NaCl, and variable imidazole, was supplemented with 1% glycerol and 0.01% Triton X-100 (Boehringer Mannheim). SEF2–1 proteins were further purified by affinity chromatography on heparin-Sepharose (31). Aliquots of the proteins 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 dithiothreitol. Purity of the proteins was determined by SDS-polyacrylamide gel electrophoresis, and protein concentrations were calculated using BCA protein assay reagent (Pierce).

Calmodulin was overexpressed in *E. coli* in a corresponding manner. Cells were lysed with the aid of a French pressure cell (Aminco, Silver Springs, MD) at 30 megapascal pressure, and initial purification was achieved by heat denaturation and subsequent removal of *E. coli* proteins as described (33). Soluble calmodulin was further purified to apparent homogeneity on DEAE-cellulose (DE-32, Whatman) and phenyl-Sepharose (Pharmacia) as described (32). Purified calmodulin was dialyzed exhaustively against water and lyophilized, and the dry powder was stored at −20 °C.

DNA Binding and Calmodulin Sensitivity Assay—EMSAs were performed as described previously (34) using an end-labeled DNA segment containing the binding site of the muscle creatine kinase enhancer (31). All calmodulin binding reactions were carried out in the presence of 0.1 mM $CaCl_2$. Recombinant proteins were diluted in 2 × calmodulin binding buffer (31) with the addition of 1 mg/ml bovine serum albumin (Sigma) prior to the reaction and premixed at room temperature for 10 min to allow heterodimer formation. Reactions were performed in the presence of S-100 proteins (Sigma), recombinant calmodulin, or calbindin D_{9K} (35) where indicated.

Synthetic Peptides—Synthetic peptides were custom synthesized by the biotechnology facility at Uppsala University (SEF2–1 peptide b), by AMS Biotechnology Ltd. (Täby, Sweden) (MyoD peptide b), or by Research Genetics (Huntsville, AL). The peptide sequences were as follows: SEF2–1 peptide b, KERRMANNARERLRVRGGCGY; peptide bH, NARERLRVRDINEAFKGY; peptide H1, RDINEAFKELGRMVQLGY; peptide H2, KLLILHQAVAVILSLEGY; and MyoD peptide b, ADRRKAATMRERRRLGGCGY.

Calmodulin Binding Assay—Lyophilized recombinant calmodulin was resuspended at a concentration of 1 mg/ml in 10 mM $NaHCO_3$, pH 10, and dansylated calmodulin (5-(dimethylamino)naphtalene-1-sulfonylcalmodulin) was prepared using standard procedures (36).

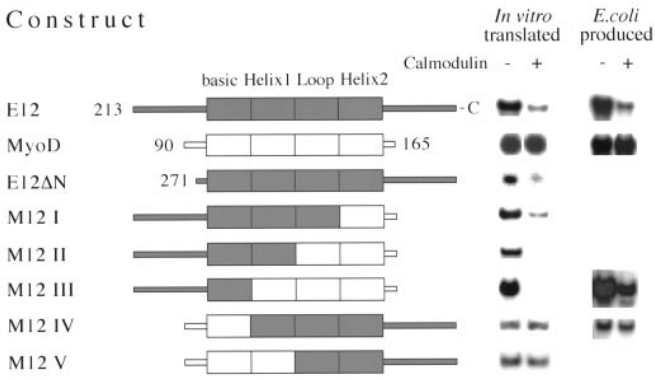

FIG. 1. **Localization of E12 and MyoD sequences determining differential sensitivity to calmodulin.** The chimeric proteins used are schematically illustrated. E12 sequences are represented by *shaded areas*, and bHLH domains and N- and C-terminal amino acid residues are indicated. Binding of these chimeric proteins to DNA containing a binding site from the muscle creatine kinase enhancer in EMSA are shown. Binding reactions were either in the absence or presence of 2.5 μM calmodulin for each bHLH protein, using binding conditions as described under "Materials and Methods." The DNA binding of *in vitro* translated proteins was analyzed for the homodimers, except for MyoD, M12 IV, and M12 V. Due to the inefficient DNA binding of these homodimers, they were analyzed as co-translated heterodimers with the E12 protein. Results of EMSA using *E. coli* produced homodimers are also shown for selected chimeras.

Binding of proteins to dansylated calmodulin was monitored by spectrofluorimetry using a Shimadzu RF5000 spectrofluorimeter. Dansylcalmodulin was excited at 340 nm, and the emission at 490 nm was recorded. Unless otherwise indicated, all reactions were performed in 3 ml of buffer containing 20 mM HEPES, pH 8.0, 100 mM NaCl, 3 μM $CaCl_2$ and 75 nM dansylated calmodulin. Proteins were incubated for 5 min at room temperature with dansylated calmodulin prior to each measurement. Binding constants, K_D, were estimated from the titration curves using a simple binding model and the equation,

$$\theta = \frac{(C_t + P_t + K_D) - \sqrt{(C_t + P_t + K_D)^2 - 4P_tC_t}}{2C_t} \qquad \text{(Eq. 1)}$$

where P_t is total added bHLH protein, C_t is total calmodulin, and θ is the fractional saturation $(F - F_0)/(F_{max} - F_0)$.

RESULTS

Differential Calmodulin Sensitivity of E12, Compared with MyoD, Lies N-terminal of Helix 1—We have previously shown that E12 and SEF2–1 homodimers display a calcium/calmodulin-dependent inhibition of DNA binding in an EMSA, whereas E12/MyoD heterodimers are completely resistant. For SEF2–1, we localized the calcium/calmodulin sensitivity to the bHLH domain (31). To further characterize this differential calmodulin sensitivity, the DNA binding of *in vitro* translated derivatives of E12 and MyoD proteins was assayed in the presence and absence of calmodulin as shown in Fig. 1. As expected, DNA binding of an N-terminally deleted derivative of E12 homodimers was inhibited upon preincubation with calmodulin, whereas heterodimers of a deletion derivative of MyoD with E12 remained unaffected by 2.5 μM calmodulin. The calmodulin sensitivity of E12 was further localized to a shortened fragment of E12, E12ΔN, that includes the bHLH domain but lacks all except five amino acids N-terminal to it (Fig. 1).

To determine the regions of E12 and MyoD responsible for sensitivity/resistance to calmodulin, E12/MyoD chimeric proteins were constructed. The chimeras (Fig. 1) were designed so that regions within the bHLH domain of the E12 derivative were replaced with analogous regions from the deletion derivative of MyoD, producing chimeras M12 I to M12 V. Analysis of the chimeric proteins showed that only sequences N-terminal of helix 1 were important for determining the sensitivity of E12 to calmodulin; homodimers of M12 III, where the HLH region

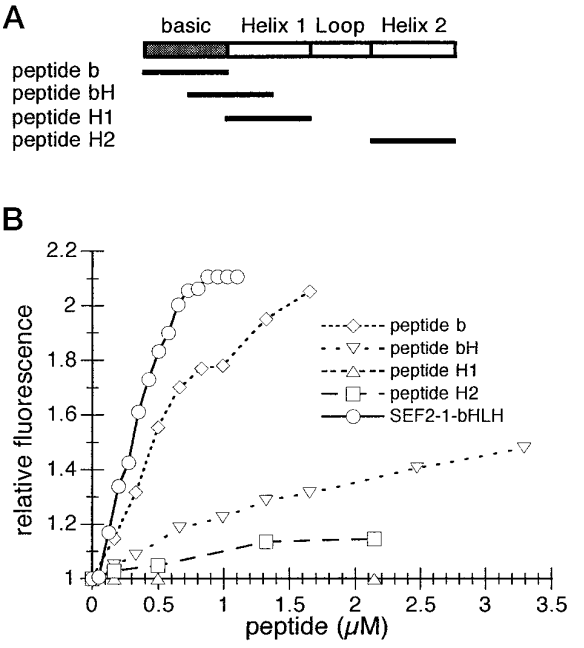

FIG. 2. **Mapping of the calmodulin binding site of SEF2–1 to the basic sequence.** *A*, schematic representation of the basic helix-loop-helix domain of SEF2–1 (SEF2–1-bHLH, amino acids 563–622) and the employed four synthetic peptides spanning the basic sequence, helix 1, and helix 2. *B*, spectrofluorimetric analysis of calmodulin binding of SEF2–1-bHLH and each of the four peptides. Representative recordings of emitted fluorescent light at 490 nm using 560 nM dansylated calmodulin are shown.

of E12 was replaced by that of MyoD, showed inhibition of DNA binding upon preincubation with calcium-loaded calmodulin (Fig. 1). The reciprocal chimera, M12 IV, which has a MyoD basic sequence followed by the E12 HLH, resulted in a protein that, when heterodimerized with the E12, showed no inhibition of DNA binding (Fig. 1). One interpretation of these results is that sequences N-terminal to helix 1 are responsible for calmodulin sensitivity. Alternatively, homodimers could be sensitive, whereas heterodimers could be resistant. To distinguish between these two possibilities, we repeated selected experiments with *E. coli*-produced proteins. Due to the higher protein concentration that could be achieved, analysis of DNA binding of homodimers was possible with recombinant MyoD and M12 IV. Both recombinant MyoD and M12 IV homodimers were resistant to calmodulin inhibition, in contrast to E12 and M12 III homodimers (Fig. 1), showing that it is not homo- or heterodimerization that determines calmodulin sensitivity. From these results, we conclude that sequences N-terminal of helix 1 are responsible for determining resistance or sensitivity to calmodulin.

The Basic Sequence of SEF2–1 Is a Calmodulin Target—Dansylated calmodulin undergoes a change in emitted fluorescence upon binding to a target (36). Monitoring this fluorescence change can be used to determine the affinity of calmodulin for the target. Dansylated calmodulin was titrated with the bHLH domain of SEF2–1 (amino acids 563–622; Ref. 31), and the emitted fluorescent light was recorded (Fig. 2). SEF2–1-bHLH bound to dansylated calmodulin with high affinity, and saturation was reached (Fig. 2*B*). The binding characteristics were similar to those of longer versions of SEF2–1 and E12 (data not shown), indicating that all sequences required for efficient calmodulin binding are contained within the bHLH domain. There is no change in fluorescence of dansylated calmodulin upon the addition of bHLH protein in the absence of calcium. To further determine sequences responsible for calmodulin binding, synthetic peptides derived from bHLH protein sequences were analyzed by the same assay. Since it has previously been shown for SEF2–1 that its bHLH domain (SEF2–1-bHLH) retains its sensitivity to calmodulin (31) and its ability to bind dansylated calmodulin (Fig. 2*B*), only sequences within the bHLH domain were considered. Well characterized calmodulin targets interact with calmodulin via a short basic and amphipathic α-helix (37). The basic sequence of the bHLH proteins is an α-helix, at least in the DNA complex (38, 39). Therefore, with the exception of the central loop, bHLH domains are composed of sequences that fulfill at least some of the criteria of a calmodulin target. We therefore designed peptides covering those sequences. Fig. 2*A* shows SEF2–1-bHLH and illustrates the sequences spanned by the synthetic peptides. Of these peptides, only peptide b, representing the basic sequence, bound with high affinity to dansylated calmodulin. Peptide bH bound to calmodulin to a much lower extent, and peptides H1 and H2 did not bind at the concentrations used (Fig. 2*B*). This strongly suggests that the sequence corresponding to peptide b, the basic sequence of SEF2–1, is the primary target for calmodulin binding.

The Calmodulin Affinity of bHLH Proteins Correlates with the Degree of DNA Binding Inhibition—The basic sequence of a bHLH protein can bind calmodulin, and sequences N-terminal to helix 1, including the basic sequence, determine calmodulin sensitivity in a DNA binding assay. Taken together, this suggests that the binding of calmodulin to the DNA-interacting basic sequence of a bHLH protein inhibits its binding to DNA. To further investigate this hypothesis, we tested whether there is a correlation between the degree of calmodulin inhibition of the DNA binding of a bHLH protein and its affinity for calmodulin. To that effect, we took a semiquantitative approach to analysis of DNA binding inhibition using homodimers of *E. coli*-produced bHLH proteins. A series of chimeric E12/MyoD bHLH proteins (Fig. 3*A*) were preincubated with various concentrations of calcium-loaded calmodulin, and inhibition constants (K_i) were estimated from the concentration of calmodulin necessary to achieve 50% inhibition of DNA binding. Typical results are shown in Fig. 3*B*, and the average results are summarized in Fig. 3*C*.

In a separate set of experiments, dansylated calmodulin was titrated with the above analyzed bHLH proteins, and the affinity constants (K_D) were determined using the simple binding model described under "Materials and Methods." The *numbers* shown in Fig. 3*C* are the averages of three separate titrations, with the values agreeing to within 25%. Titration curves of three representative proteins, E12, MyoDΔN, and MyoD, with strong, intermediate, and no calmodulin binding, respectively, are shown in Fig. 3*D*. All binding curves showed systematic deviations from the theoretical titration curve for simple binding, indicating that the binding process is more complex. This will be discussed elsewhere.[2] The bHLH proteins that were resistant to calmodulin inhibition did not bind dansylated calmodulin at the concentrations used (Fig. 3*C*). All bHLH proteins that were inhibited by calmodulin could also be shown to directly bind to calmodulin. Within the resolutions of these methods, the estimated K_i values correlated directly with the measured affinity constants, K_D. This is consistent with the notion that it is the physical interaction with calmodulin that renders a bHLH protein incapable of DNA binding.

Sequences N-terminal to the Basic Sequence Inhibit Calmodulin Binding and Sensitivity of MyoD—MyoD-E12basic and E12-MyoDbasic, with the basic sequences interchanged, behaved as MyoD and E12, respectively, both in the calmodulin

[2] S. Hermann, J. Onions, and T. Grundström, manuscript in preparation.

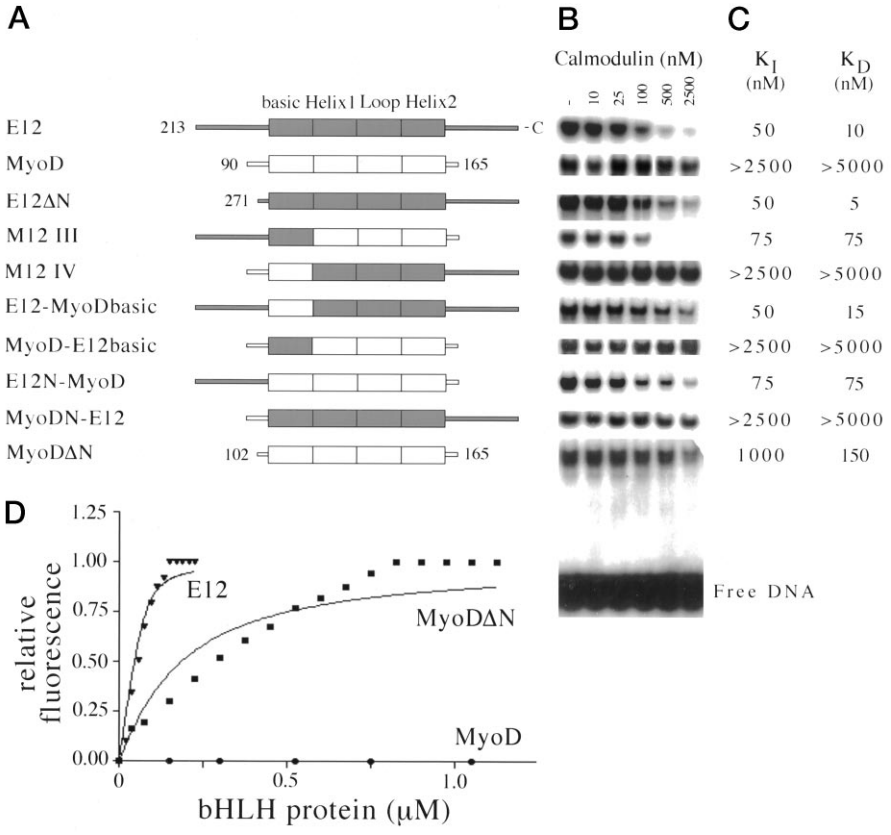

FIG. 3. **Correlation between calmodulin sensitivity and calmodulin binding strength of constructed bHLH proteins and localization of sequences within the bHLH domain that modulate calmodulin sensitivity.** *A*, schematic representation of the E12/MyoD chimeras analyzed. *Shaded areas* represent E12 sequences, and bHLH domains and N- and C-terminal amino acids are indicated. *B*, representative EMSAs of the *E. coli*-produced protein homodimers incubated with increasing concentrations of calmodulin. *C*, summary of calmodulin inhibition data and calmodulin binding data for the bHLH proteins. Inhibition constants (K_i) were estimated from the calmodulin concentration necessary to achieve 50% inhibition of the DNA binding, based on at least three EMSAs. The bHLH protein concentrations were as follows: E12 (3 nM), MyoD (200–2000 nM), E12ΔN (50–250 nM), M12 III (35 nM), M12 IV (350 nM) E12-MyoDbasic (4 nM), MyoD-E12basic (200–2000 nM), E12N-MyoD (28 nM), MyoDN-E12 (150–1500 nM), MyoDΔN (50–250 nM). To determine binding constants (K_D), calmodulin binding was analyzed spectrofluorimetrically. Increasing concentrations of the bHLH proteins were added to 75 nM dansylated calmodulin, and the emitted fluorescence was monitored at 490 nm. Binding constants were calculated from the best fitting titration curves as described under "Materials and Methods." The *numbers* represent the averages of at least three separate experiments. Individual experiments usually agreed within 25%. *D*, representative titrations of fluorescence emission of dansylated calmodulin with E12 (*triangles*), MyoD (*circles*), and MyoDΔN (*squares*). Theoretical titration curves for simple binding are superimposed onto the data as described under "Materials and Methods."

sensitivity and the calmodulin binding assay (Fig. 3). This suggested that the MyoD basic sequence is as good a calmodulin binding sequence as that of E12 but that additional MyoD sequences inhibit this calmodulin binding ability. To directly test whether the basic sequence of MyoD can bind calmodulin but is prevented from doing so by inhibitory N-terminal sequences, an additional construct was made, MyoDΔN, deleting most of the N-terminal sequences of MyoD. This variant was able to bind calmodulin, and its DNA binding could be inhibited by calmodulin (Fig. 3). Inhibition of MyoDΔN as well as all other bHLH proteins tested was dependent on calcium. Replacing calcium with the chelator EGTA completely abolished the calmodulin inhibition of bHLH proteins (data not shown).

To investigate the role of the N-terminal sequences more closely, we replaced the sequences directly N-terminal to the basic sequence of E12 with those of MyoD in MyoDN-E12 and *vice versa* in E12N-MyoD. The origin of the directly N-terminal sequences was found to determine calmodulin binding and sensitivity. MyoDN-E12, the chimeric protein with the N-terminal sequences derived from MyoD, did not bind calmodulin and was calmodulin-resistant, whereas E12N-MyoD, the chimeric protein with the N-terminal sequences derived from E12, bound to calmodulin and was calmodulin-sensitive (Fig. 3). Taken together, these results show that the bHLH sequences of both the calmodulin-sensitive bHLH proteins and the calmodulin-resistant MyoD are targets for calmodulin binding and that MyoD contains sequences directly N-terminal to the basic sequence that inhibit interaction with calmodulin.

Differential Sensitivity of bHLH Sequences to Calmodulin, S-100αα, S-100αβ, S-100ββ, and Calbindin D_{9k}—It was previously shown that the inhibition of DNA binding of bHLH proteins was not a general property of all calcium-binding proteins, since calbindin D_{9K} was not inhibitory (31). This, however, does not exclude the possibility that there are calcium-binding proteins in addition to calmodulin that also interact with bHLH proteins. S-100 proteins have several targets in common with calmodulin (27–30). We therefore preincubated various bHLH homodimers and heterodimers with calmodulin, S-100αα, S-100αβ, S-100ββ, or calbindin D_{9k} and analyzed their effects on bHLH DNA binding activity. Representative results are shown in Fig. 4. The calmodulin-sensitive E12ΔN and SEF2–1ΔN homodimers showed high sensitivity also to S-100 proteins but were unaffected by calbindin D_{9k}. Interestingly, a differential sensitivity was observed. SEF2–1ΔN showed the highest sensitivity toward the S-100 proteins, whereas E12ΔN was more sensitive to calmodulin and S-100αα than to S-100αβ and S-100ββ. E12ΔN/MyoDΔN heterodimers were resistant to calbindin D_{9k} and slightly sensitive to calmodulin, S-100αβ, and S-100ββ but surprisingly showed greater sensitivity to S-100αα. Homodimers of MyoDΔN dis-

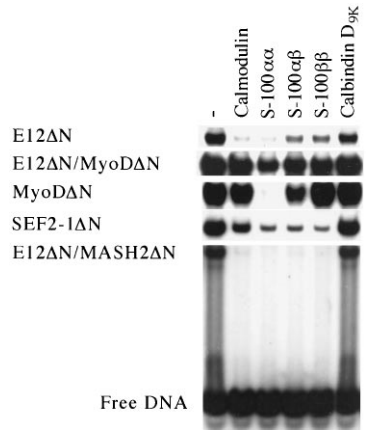

FIG. 4. **Differential inhibition of DNA binding of bHLH transcription factors by calmodulin, S-100 proteins, and calbindin D_{9k}.** EMSAs of recombinant bHLH proteins in the absence and presence of 2.5 μM calmodulin, 2.5 μM S-100 proteins, or 1.25 μM calbindin D_{9k} are shown. The binding conditions were as in Fig. 3*B*.

played properties similar to those shown by the heterodimer with E12ΔN except that they displayed extremely efficient inhibition by S-100$\alpha\alpha$. The differential sensitivity seen for the E12ΔN/MyoDΔN heterodimer did not appear to be a property of all heterodimers between class A and class B proteins, since E12ΔN/MASH2ΔN was equally sensitive to calmodulin and the S-100 proteins (Fig. 4).

S-100 Proteins Inhibit bHLH Proteins through Binding to the Basic Sequence—Calmodulin inhibits DNA binding of bHLH proteins through binding to their basic sequence. To analyze if S-100 proteins act in a similar way, we first investigated whether S-100 could bind to bHLH proteins. For technical reasons, an indirect approach was chosen. Dansylated calmodulin was half-saturated with the bHLH protein, and the preformed complex was then challenged with increasing concentrations of unmodified calmodulin or S-100$\alpha\beta$. Relative affinities of calmodulin and S-100$\alpha\beta$ were estimated from their ability to compete with dansylated calmodulin for the binding of the bHLH protein. Representative results are shown in Fig. 5. S-100$\alpha\beta$ could compete with dansylated calmodulin for E12 binding, showing that there is a physical interaction between E12 and S-100$\alpha\beta$. S-100$\alpha\beta$ was a slightly less efficient competitor than calmodulin. Conversely, the MyoDΔN complex with dansylated calmodulin was more efficiently competed by S-100$\alpha\beta$ than by calmodulin, suggesting that MyoDΔN has a much higher affinity for S-100$\alpha\beta$ than for calmodulin. Coupled with the finding that S-100$\alpha\beta$ inhibits the DNA binding of MyoDΔN more efficiently than calmodulin (Fig. 4), this supports the notion that S-100 proteins, like calmodulin, inhibit the DNA binding of bHLH proteins by binding to their basic sequence.

The direct correlation between S-100 interaction with the bHLH domain and inhibition of DNA binding indicates that S-100 proteins also interact with the basic sequence, as shown for calmodulin (Fig. 2). Alternatively, there could be another target within the bHLH domain for the S-100 proteins. We therefore analyzed whether S-100$\alpha\beta$ could compete with the binding of dansylated calmodulin to the basic peptide of SEF2–1. We found that calmodulin and S-100$\alpha\beta$ competed with approximately equal efficiency (Fig. 6), strongly suggesting that the basic sequence is also the target for S-100$\alpha\beta$.

In agreement with the intrinsic calmodulin sensitivity of the bHLH domain of MyoD, the basic peptide of MyoD was found to bind to dansylated calmodulin. The binding strength was approximately the same as for the basic peptide of SEF2–1 (data not shown). Competition analysis of this complex showed a

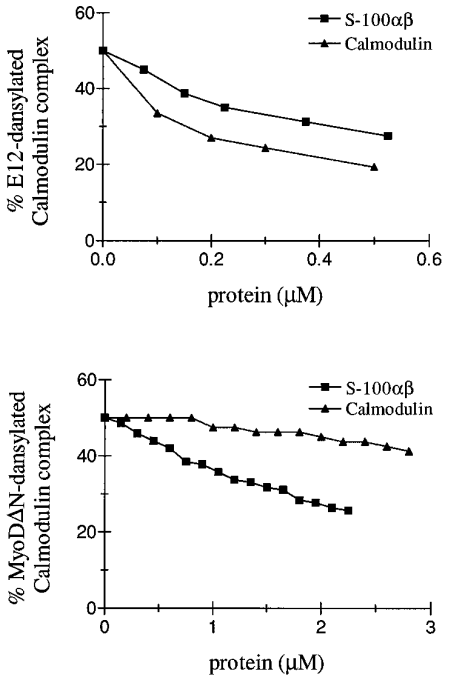

FIG. 5. **S-100$\alpha\beta$ and calmodulin competitions of E12 and MyoDΔN binding to calmodulin.** Dansylated calmodulin (75 nM) was half-saturated with homodimers of E12 (*upper panel*) or MyoDΔN (*lower panel*). Preformed complexes were then challenged with increasing concentrations of unlabeled calmodulin (*triangles*) or S-100$\alpha\beta$ (*squares*), and the remaining emitted fluorescent light at 490 nm was recorded.

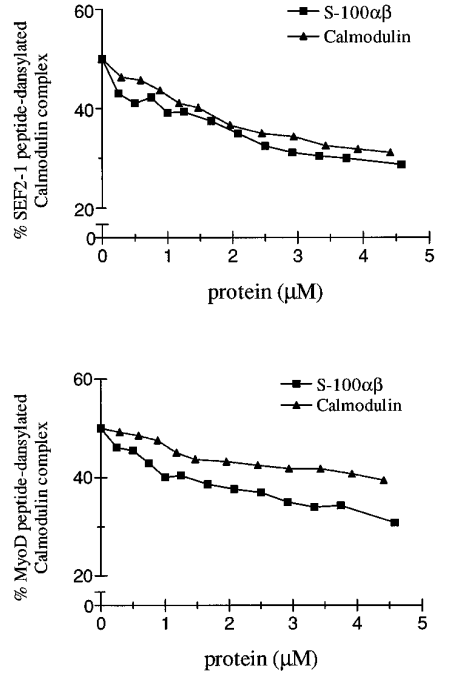

FIG. 6. **Calmodulin and S-100$\alpha\beta$ competition of the calmodulin binding of the basic sequences of SEF2–1 and MyoD.** Dansylated calmodulin (560 nM) was half-saturated with peptide b of SEF2–1 (*upper panel*) or MyoD (*lower panel*). Preformed complexes were then challenged with increasing concentrations of unlabeled calmodulin (*triangles*) or S-100$\alpha\beta$ (*squares*), and the remaining emitted fluorescent light at 490 nm was recorded.

lower effect of calmodulin than expected from the binding strength of dansylated calmodulin, indicating that dansylated calmodulin might bind somewhat more strongly than nondansylated calmodulin to this peptide (Fig. 6). S-100$\alpha\beta$ was a

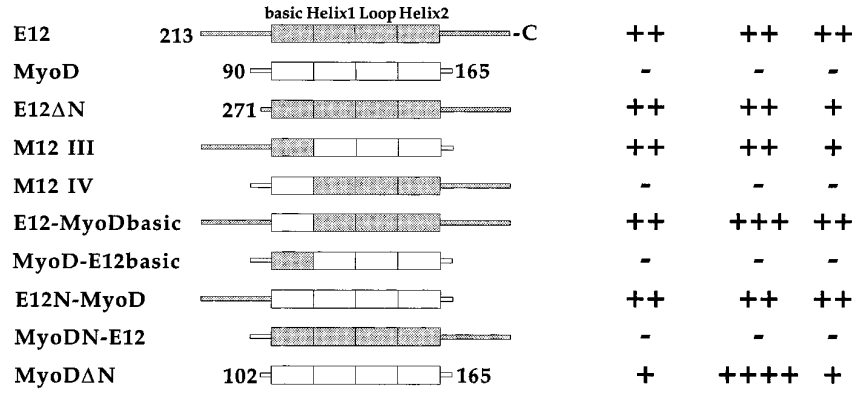

FIG. 7. **Summary of localization of sequences modulating the sensitivities of constructed bHLH proteins toward calmodulin, S-100$\alpha\alpha$, and S-100$\beta\beta$.** The chimeric constructs used are represented schematically together with a semiquantitative grading of the sensitivities of the DNA binding of recombinant protein homodimers to 2.5 μM calmodulin, S-100$\alpha\alpha$, or S-100$\beta\beta$ as determined by EMSA. The binding conditions were as in Fig. 3*B*.

severalfold better competitor than calmodulin for the complex with the MyoD peptide. Thus, the difference in S-100$\alpha\beta$ and calmodulin sensitivity of MyoDΔN (Fig. 4) and in the strengths of their binding to the bHLH domain of MyoD (Fig. 5) was also seen when comparing S-100$\alpha\beta$ and calmodulin binding to the isolated MyoD basic sequence. This is consistent with the notion that the higher S-100$\alpha\beta$ sensitivity of MyoDΔN is due to a stronger interaction of S-100$\alpha\beta$ than calmodulin with its basic sequence.

S-100 Binding Is Differentially Modulated by Directly N-terminal Sequences—Having shown that sequences N-terminal to the bHLH domain can modulate the effects of calmodulin, we wanted to determine whether this was also the case for S-100 proteins. Fig. 7 summarizes the results from EMSA quantification of the sensitivities of various chimeric bHLH proteins toward calmodulin, S-100$\alpha\alpha$, and S-100$\beta\beta$ proteins. The results with S-100$\alpha\beta$ were intermediate to those with S-100$\alpha\alpha$ and S-100$\beta\beta$ (data not shown). In general, the findings with the S-100 proteins are similar to those with calmodulin presented in Fig. 3. N-terminal MyoD sequences, which render intrinsically calmodulin-sensitive bHLH domains resistant to calmodulin, also block the S-100 sensitivity of bHLH domains (Fig. 7). There are, however, quantitative differences. Whereas all constructs with the E12 basic sequence exhibited relatively small differences in the sensitivities to the S-100 proteins compared with calmodulin, the differences were large for certain constructs having the MyoD basic sequence. E12-MyoDbasic and MyoDΔN showed a much higher inhibition of DNA binding by S-100$\alpha\alpha$, but not S-100$\beta\beta$, compared with calmodulin. In contrast, E12N-MyoD, which has the same MyoD basic sequence, is inhibited to an equal extent by calmodulin and S-100 proteins. These results indicate that the differential sensitivity is not a mere result of higher affinity of the MyoD basic sequence for S-100$\alpha\alpha$ and S-100$\alpha\beta$ compared with calmodulin (*cf.* Figs. 4–6) but also reflects a differential inhibition by N-terminal sequences. It is notable that the relatively small S-100$\beta\beta$ inhibition of E12ΔN and MyoDΔN was increased by the addition of the directly N-terminal E12 sequences in E12N-MyoD and E12, indicating that in these cases the N-terminal sequence of E12 may have a positive effect. Interestingly, MyoDΔN showed a very high sensitivity toward inhibition by S-100$\alpha\alpha$ that was not seen with any of the other protein combinations.

DISCUSSION

We have previously shown that the calcium sensor protein calmodulin is able to inhibit the DNA binding of the class A bHLH proteins (E proteins) E12, E47, and SEF2–1, but heterodimers between class A and class B proteins are unaffected (31). Here we have demonstrated a direct physical interaction between calmodulin and the bHLH proteins, which leads to the inhibition of DNA binding. Mutational analysis using the calmodulin-sensitive protein E12 and the calmodulin-resistant protein MyoD showed that the binding between calmodulin and the bHLH protein occurred within the bHLH domain and could be further localized to the DNA binding basic sequence. An alternative mapping approach using synthetic peptides covering the bHLH domain of another E protein, SEF2–1, confirmed this result. We observed a large difference between the binding constants determined for the bHLH domain of SEF2–1 and the peptide covering the basic sequence. The weaker binding of the peptide could be due to conformational differences. In the absence of calmodulin, the isolated basic peptide does not form any structure,[3] whereas in the context of a complete bHLH domain an α-helical structure might be present or be more easily induced by calmodulin (38–40). It appears that the binding of peptide b to dansylated calmodulin is biphasic (Fig. 2*B*). This deviation from simple binding may be related to the deviations observed for bHLH proteins and will be discussed elsewhere.[2]

Although repeated titrations of dansylated calmodulin with bHLH proteins gave reproducible results, they systematically diverged from the theoretical titration curve for simple binding. The dissociation constants obtained therefore have a high degree of uncertainty (see Fig. 3). While we have given the dissociation constants obtained through this fit, the reported values should be treated with due caution. Small differences do not necessarily reflect a difference in binding strength. This is especially the case for strong binding where the reported K_D is below the calmodulin concentration. Here, small differences in the titration data will lead to comparatively large changes in the fitted dissociation constant.

The three-dimensional structures of the DNA complexes of two bHLH proteins, MyoD (38) and E47 (39), demonstrate that the HLH region is responsible for dimerization of the proteins, while the basic sequence binds the specific DNA sequence. It is therefore possible that calmodulin binding to the basic sequence of E12 or SEF2–1 makes it inaccessible for interaction with the DNA sequence. This is in agreement with previous results showing that when bound to calmodulin, the bHLH domain is unable to bind DNA and when bound to DNA the

[3] G. Larsson, S. Hermann, T. Grundström, and S. Wijmenga, unpublished observation.

protein is unable to bind calmodulin (31).

The mutation and peptide analyses showed that the basic sequence of the calmodulin-resistant MyoD is also capable of binding calmodulin. Calmodulin resistance of the DNA binding of MyoD resides in sequences directly N-terminal to the basic sequence. Upon deletion of the N-terminal sequences, MyoD can bind to, and is inhibited by, calmodulin. The mechanism of this negative effect of the directly N-terminal sequences is not known. They might form a tertiary structure that is incompatible with calmodulin binding. Another possibility is a direct interaction between the basic sequence and the more N-terminal sequences, inhibiting calmodulin binding. Additional investigations are needed to further understand the mechanism of this inhibition.

Analysis of members of another family of calcium-binding proteins possessing EF-hand structures, the S-100 proteins, showed that these could also inhibit the DNA binding of certain bHLH proteins. Despite the high degree of similarity between S-100α and S-100β (41), there were large differences in the levels of inhibition of bHLH proteins by homo- and heterodimers of these proteins. The S-100 proteins also target the basic sequence of the bHLH domain, and, as is the case for calmodulin, binding of S-100 is modulated by directly N-terminal sequences of bHLH proteins.

How do calmodulin and S-100 proteins regulate bHLH transcription factor activity? One possibility could be that binding to the basic sequence simply blocks DNA binding. This simple model, however, does not completely explain the data; Fig. 3 shows several cases where the inhibition constant is higher than the binding constant estimated with the assumption of a simple binding. More elaborate models are therefore necessary. There are complex equilibria between bHLH proteins that form part of their regulatory system. It is generally believed that bHLH dimers are the DNA-binding and transcriptionally active moieties. In addition to the many possible bHLH homo- and heterodimers within a cell, other oligomerization states are possible and have been shown to influence the activities of the proteins (42–44). A regulation of enzyme activity through oligomeric-type modifications has been described for another calmodulin target, myosin light chain kinase (45). It is therefore feasible that the binding of calmodulin could change the equilibria between the oligomerization states of bHLH proteins. Changes in the tendency to oligomerize may differ between bHLH proteins, which could, for example, result in smaller effects of calmodulin binding for some proteins that appear to bind as well as other more sensitive proteins.

bHLH proteins can be regulated by phosphorylation events (46–50). Calmodulin and S-100 proteins influence the activities of many protein kinases and phosphatases. It is therefore tempting to speculate that calmodulin, in addition to the effect on DNA binding, might also act as a "recruiting factor," attracting proteins that alter the phosphorylation status of bHLH proteins, which in turn could alter its transcriptional activity. Baudier and co-workers (28) recently reported that S-100$\alpha\alpha$ or calmodulin binding to the bHLH domain of MyoD can influence its phosphorylation state. In this case, calmodulin and S-100$\alpha\alpha$ acted like "exclusion factors," inhibiting the phosphorylation of MyoD by protein kinase C.

Why does MyoD have both a highly S-100- and calmodulin-sensitive basic sequence and a nearby sequence that blocks this sensitivity? This would be meaningless if the block by the nearby sequence was always complete. However, Baudier and co-workers (28) have recently reported that full-length MyoD shows S-100$\alpha\alpha$ sensitivity, although it is much smaller than the extreme sensitivity we obtain for MyoDΔN. Thus, sequences further away in MyoD appear to moderate the effect of the inhibitory sequence. Furthermore, a number of proteins, including pRB, Notch, and I-mf, have been reported to affect the activities of bHLH proteins including MyoD, and in some cases also E47, through their bHLH domains (51–54). It is therefore possible that S-100 or calmodulin sensitivities of bHLH proteins are affected by such protein interactions. Reciprocally, S-100 protein or calmodulin binding could potentially, in addition to the effect on the DNA binding, also affect an important protein interaction of the bHLH domain.

We have pointed out above that S-100$\alpha\alpha$, which is expressed predominantly in muscle cells, has a high affinity for the myogenic protein MyoD. This could reflect a tissue-specific regulation involving high amounts of the particular S-100 that has high affinity for the specific bHLH proteins made in those cells.

From the data we have presented, it is clear that calmodulin and S-100 proteins can inhibit the activity of bHLH proteins. This inhibition correlates with their binding to the basic sequence within the bHLH domain. Sequences directly N-terminal to the bHLH domain are responsible for the differential calmodulin and S-100 resistance of certain bHLH proteins. We have proposed several mechanisms by which binding to the basic sequence could lead to inhibition of DNA binding and perhaps also other functions of the bHLH domain. The mechanisms responsible for the actions of calmodulin and S-100 proteins toward bHLH transcription factors *in vitro* and *in vivo* await further investigation.

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