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 also inhibit bHLH proteins and that the calcium 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) 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-100α, S-100β, 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.
**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 pmSP64-poly(A) (Promega). For overexpression in *E. coli*, constructs were cloned into a pET-20b derivative (Novagen) modified to include an N-terminal His6 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 MyoΔN.

The deleted SEF2–1 (amino acids 547–686), SEF2–1ΔN (amino acids 558–667), SEF2–1bHLH (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. pETCaM8, which contains the calmodulin-encoding sequences from pCaMRW (32) in a modified pET-3-1 vector (Novagen), was a kind gift from Dr. Peter Jones.

**Proteins**—*p*SP64-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 Histagged proteins were purified by metal chelation chromatography on a Ni-nitrotriacetic 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 heat-labile material. 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 heat-labile material. Calmodulin was dialyzed exhaustively against water and lyophilized, and the dry powder was stored at −20 °C.

**DNA Binding and Calmodulin Sensitivity Assay**—EMSA’s 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 CaCl2. 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 preincubated 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 D9K (35) where indicated.

**Synthetic Peptides**—Synthetic peptides were custom synthesized by the biotechnology facility at Upstate University (SEF2–1 peptide b), by AMS Biotechnology Ltd. (Taby, Sweden) (MyoD peptide b), or by Research Genetics (Huntsville, AL). The peptide sequences were as follows: peptide a, KERGMANNCLRGRGCYWARKDRC; peptide b, KERGMANNCLRGRGCYWARKDRC; peptide c, KLILLHQAVALILLSLEGY; and MyoD peptide b, DHCPKAATMRRERRLGCYY.

**Calmodulin Binding Assay**—Lyophilized recombinant calmodulin was resuspended at a concentration of 1 mg/ml in 10 mM NaHCO3, pH 10, and danylsated calmodulin (5-dimethylamino)napththalene-1-sulfonycalmodulin) was prepared using standard procedures (36).

**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 1 to M12 V. Analysis of the chimeric proteins showed that these regions N-terminal of helix 1 were important for determining the sensitivity of E12 to calmodulin; homodimers of M12 III, where the bHLH region...
Calmodulin- and S-100-inhibited bHLH Protein Sequences

**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 bHLH, 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 to 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. 2B). 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. 2B), 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. 2A 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 b bound to calmodulin to a much lower extent, and peptides H1 and H2 did not bind at the concentrations used (Fig. 2B). 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. 3A) 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. 3B, and the average results are summarized in Fig. 3C.

In a separate set of experiments, dansylated calmodulin was titrated with the above analyzed bHLH proteins, and the affinity constants ($K_i$) were determined using the simple binding model described under “Materials and Methods.” The numbers shown in Fig. 3C are the averages of three separate titrations, with the values agreeing to within 25%. Titration curves of three representative proteins, E12, MyoDAN, and MyoD, with strong, intermediate, and no calmodulin binding, respectively, are shown in Fig. 3D. 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. 3C). 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, $K_i$. 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

²S. Hermann, J. Onions, and T. Grundström, manuscript in preparation.
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 MyoDΔN-E12 and vice versa in E12N-MyoD. The origin of the directly N-terminal sequences was found to determine calmodulin binding and sensitivity. MyoDΔN-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-100αa, S-100αβ, S-100ββ, and Calbindin D_{28k}—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_{28k} 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-100αa, S-100αβ, S-100ββ, or calbindin D_{28k} and analyzed their effects on bHLH DNA binding activity. Representative results are shown in Fig. 4. The calmodulin-sensitive E12N and SEP2–1ΔN homodimers showed high sensitivity also to S-100 proteins but were unaffected by calbindin D_{28k}. Interestingly, a differential sensitivity was observed. SEP2–1ΔN showed the highest sensitivity toward the S-100 proteins, whereas E12N was more sensitive to calmodulin and S-100αa than to S-100αβ and S-100ββ. E12N/MyoDΔN heterodimers were resistant to calbindin D_{28k} and slightly sensitive to calmodulin, S-100αβ, and S-100ββ but surprisingly showed greater sensitivity to S-100αa. Homodimers of MyoDΔN dis-
played properties similar to those shown by the heterodimer with E12DN except that they displayed extremely efficient inhibition by S-100aa. The differential sensitivity seen for the E12DN/MyoDΔN heterodimer did not appear to be a property of all heterodimers between class A and class B proteins, since E12DN/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 unlabeled calmodulin or S-100ab. Relative affinities of calmodulin and S-100ab 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-100ab could compete with dansylated calmodulin for E12 binding, showing that there is a physical interaction between E12 and S-100ab. S-100ab was a slightly less efficient competitor than calmodulin. Conversely, the MyoDΔN complex with dansylated calmodulin was more efficiently competed by S-100ab than by calmodulin, suggesting that MyoDΔN has a much higher affinity for S-100ab than for calmodulin. Coupled with the finding that S-100ab 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-100ab could compete with the binding of dansylated calmodulin to the basic peptide of SEF2–1. We found that calmodulin and S-100ab competed with approximately equal efficiency (Fig. 6), strongly suggesting that the basic sequence is also the target for S-100ab.

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 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-100ab was a
severalfold better competitor than calmodulin for the complex with the MyoD peptide. Thus, the difference in S-100αβ 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-100αβ and calmodulin binding to the isolated MyoD basic sequence. This is consistent with the notion that the higher S-100αβ sensitivity of MyoDΔN is due to a stronger interaction of S-100αβ 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-100αα, and S-100ββ. The results with S-100αβ were intermediate to those with S-100αα and S-100ββ (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-100αα, but not S-100ββ, 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-100αα and S-100αβ 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-100ββ 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-100αα 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, 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 β to dansylated calmodulin is biphasic (Fig. 2B). This deviation from simple binding may be related to the deviations observed for bHLH proteins and will be discussed elsewhere.

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 KD 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

---

**FIG. 7.** Summary of localization of sequences modulating the sensitivities of constructed bHLH proteins toward calmodulin, S-100αα, and S-100ββ. 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-100αα, or S-100ββ as determined by EMSA. The binding conditions were as in Fig. 3B.

**SENSITIVITY**

|       | Calmodulin | S-100αα | S-100ββ |
|-------|------------|---------|---------|
| E12   | ++         | ++      | ++      |
| MyoD  | -          | -       | -       |
| E12ΔN | ++         | ++      | ++      |
| M12 II| ++         | ++      | ++      |
| M12 IV| -          | -       | -       |
| E12-MyoDbasic | ++ | +++      | +       |
| MyoD-E12basic | - | -       | -       |
| E12N-MyoD | ++ | ++      | ++      |
| MyoDN-MyoD | - | -       | -       |
| MyoDΔN | 102 - | 165     | +      | +++      |
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 α 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-100α or calmodulin binding to the bHLH domain of MyoD can influence its phosphorylation state. In this case, calmodulin and S-100α 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-100α 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-100α, which is expressed predominately 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.

Acknowledgment—We thank Dr. Peter Brodin for kindly sharing the calmodulin expression plasmid pETCaM.

REFERENCES

1. Littlewood, T. D., and Evan, G. I. (1994) in Protein Profile 1, 639–709
2. Murre C., McCaw, P. S., and Baltimore, D. (1989) Cell 56, 777–783
3. Henthorn, P., Kiledjian, M., and Kadesch, T. (1990) Science 247, 467–470
4. Corneliussen, B., Thornell, L., Hallberg, B., and Grundstrohm, T. (1991) J. Virol. 65, 6084–6093
5. Edmondson, D. G., and Olson, E. N. (1990) Genes & Dev. 4, 1450
6. Wright, W. E., Sassoon, D. A., and Lin, V. K. (1989) Cell 56, 607–617
7. Davis, R. L., Weintraub, H., and Lassar, A. B. (1987) Cell 51, 987–1000
8. Braun, T., Bober, E., Winter, B., Rosenthal, N., and Arnold, H. H. (1990) EMBO J. 9, 821–831
9. Miner, J. H., and Wald, B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1089–1093
10. Rhodes, S. J., and Konieczny, S. F. (1989) Genes & Dev. 3, 2050–2061
11. Braun, T., Buschhausen Denker, G., Bober, E., Tan, L., and Arnold, H. H. (1989) EMBO J. 8, 1065–1074
12. Johnson, J. E., Birren, S. J., and Anderson, D. J. (1990) Nature 346, 858–861
13. Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N., and Weintraub, H. (1995) Science 268, 836–844
14. Assar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991) Cell 66, 305–315
15. Rasmussen, H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 752–759
16. Pugh, B. W. (1985) Science 228, 285–289
17. Pugh, B. W. (1985) EMBO J. 4, 2301–2307
18. Simon, J., and Weintraub, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1089–1093
19. Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) Nature 315, 37–40
20. Tan, R.-Y., Mabuchi, Y., and Grabarek, Z. (1996) J. Biol. Chem. 271, 7479–7483
21. Schäfer, B. W., and Heizmann, C. W. (1996) Trends Biochem. Sci. 21, 134–140
22. Kimoto, H., Ishida, K., and Verma, I. M. (1987) Lab. Invest. 57, 489–498
23. Zimmer, D. B., and Van Eldik, L. J. (1987) Am. J. Physiol. 252, C285–C289
24. Kato, R., and Kimura, S. (1985) Biochem. Biophys. Acta 842, 146–150
25. Zimmer, D. B., Song, W., and Zimmer, W. E. (1991) Brain Res. Bull. 27, 157–162
26. Donato, R. (1986) Cell Calcium 7, 123–145
27. Baudier, J., Mochly Rosen, D., Weintraub, H., and Cole, R. D. (1987) Biochemistry 16, 5876–5883
28. Baudier, J., Bergeter, B., Ebert, C., Weintraub, H., Gagnon, J., and Garin, J. (1995) Biochemistry 34, 7834–7846
29. Baudier, J., Briving, C., Ziegler, B., Haglid, K., Sorskog, L., and Wallin, M. (1982) FEBS Lett. 147, 165–168
30. Baudier, J., and Cole, R. D. (1988) J. Biol. Chem. 263, 5876–5883
31. Corneliussen, B., Thornell, L., Hallberg, B., and Grundstrohm, T. (1994) Nature 368, 760–764
32. Wallersson, Y., Linsle, S., Brodin, P., and Grundstrom, T. (1993) Biochemistry 32, 7866–7871
33. Gaskell, M., Castiglione Morelli, M. A., Pfuhl, M., Motta, A., and Pastore, A. (1995) Eur. J. Biochem. 230, 752–759
34. Thornell, L., Hallberg, B., and Grundstrom, T. (1988) Mol. Cell. Biol. 8, 1625–1637
Calmodulin- and S-100-inhibited bHLH Protein Sequences

35. Brodin, P., Drakenberg, T., Thulin, E., Fersen, S., and Grundstrom, T. (1989) Protein Eng. 2, 353–357
36. Kincaid, R. L., Vaughan, M., Osborne, J. C., Jr., and Tkachuk, V. A. (1982) J. Biol. Chem. 257, 10638–10643
37. O’Neil, K. T., and DeGrado, W. F. (1990) Trends Biochem. Sci. 15, 59–64
38. Ma, P. C., Rouid, M. A., Weintraub, H., and Pabo, C. O. (1994) Cell 77, 451–459
39. Ellensberger, T., Fase, D., Arnaud, M., and Harrison, S. C. (1994) Genes & Dev. 8, 970–980
40. Anthony Cahill, S. J., Benfield, P. A., Fairman, R., Wasserman, Z. R., Brenner, S. L., Stafford, W. F., III, Altenbach, C., Hubbell, W. L., and DeGrado, W. F. (1992) Science 255, 979–983
41. Kligman, D., and Hilt, D. C. (1988) Trends Biochem. Sci. 13, 437–443
42. Fairman, R., Beran Steed, R. K., Anthony Cahill, S. J., Lear, J. D., Stafford, W. F., III, DeGrado, W. F., and Brenner, S. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10429–10433
43. Farmer, K., Catala, F., and Wright, W. E. (1992) J. Biol. Chem. 267, 5631–5636
44. Laue, T. M., Starovasnik, M. A., Weintraub, H., Sun, X. H., Snider, L., and Klevit, R. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 61824–61828
45. Babyychuk, E. B., Babyychuk, V. S., and Sobieszek, A. (1995) Biochemistry 34, 6366–6372
46. Mitsui, K., Shirakata, M., and Paterson, B. M. (1993) J. Biol. Chem. 268, 24415–24420
47. Berberich, S. J., and Cole, M. D. (1992) Genes & Dev. 6, 166–176
48. Sloan, S. R., Shen, C. P., McCarrick Walmsley, R., and Kadesch, T. (1996) Mol. Cell. Biol. 16, 6900–6908
49. Johnson, S. E., Wang, X., Hardy, S., Taparowsky, E. J., and Konieczny, S. F. (1996) Mol. Cell. Biol. 16, 1604–1613
50. Zhou, J., and Olson, E. N. (1994) Mol. Cell. Biol. 14, 6232–6243
51. Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal Ginard, B. (1993) Cell 72, 309–324
52. Kopan, R., Nye, J. S., and Weintraub, H. (1994) Development 120, 2385–2396
53. Thayer, M. J., and Weintraub, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6483–6487
54. Chen, C. M., Kraut, N., Groudine, M., and Weintraub, H. (1996) Cell 86, 731–741
Basic Helix-Loop-Helix Protein Sequences Determining Differential Inhibition by Calmodulin and S-100 Proteins
Jacqueline Onions, Stefan Hermann and Thomas Grundström

J. Biol. Chem. 1997, 272:23930-23937.
doi: 10.1074/jbc.272.38.23930

Access the most updated version of this article at http://www.jbc.org/content/272/38/23930

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 23 of which can be accessed free at http://www.jbc.org/content/272/38/23930.full.html#ref-list-1