Id proteins negatively regulate the dimerization, DNA binding, and biological properties of basic helix-loop-helix proteins. In a search for novel factors that interact with Id1, we identified a component of the 26 S proteasome, S5a, that has previously been implicated only in the recognition of ubiquitinated polypeptides destined for proteolysis. S5a interacts strongly with Id1, less strongly with the basic helix-loop-helix proteins MyoD and E12, and not at all with other Id proteins. S5a restores DNA binding by MyoD-Id1 and E12-Id1 heterodimers, enhances DNA binding by MyoD and E12 homodimers, and reverses Id1-mediated repression of the muscle creatine kinase promoter during myogenic differentiation. Mutagenesis experiments showed that amino acids flanking the helix-loop-helix domain plus three residues in the first helix of Id1 impart S5a recognition. This requires only the NH2-terminal half of S5a. S5a thus appears to promote the positive regulation of myogenic genes through ubiquitin-independent mechanisms involving inhibition of Id1 and the enhancement of DNA binding by MyoD and E12. This latter property may permit the selection of novel promoter binding sites during myogenesis.

A large number of proteins involved in proliferation, differentiation, and oncogenesis contain the helix-loop-helix (HLH) motif. This was first identified as a region of homology shared by the myogenic determinant MyoD, the c-Myc oncprotein, and the products of the achaete-scute gene complex which function in Drosophila neurogenesis (1, 2). The HLH domain serves as a dimerization surface for other HLH family members with only certain pairings being permitted (3, 4). In most cases, this association results in the acquisition of sequence-specific DNA binding by the dimer, mediated by a short, highly basic segment of amino acids immediately adjacent to the HLH domain of each partner (5, 6). The canonical DNA sequence recognized by HLH proteins, CANNTG (7), is found in the regulatory regions of many genes whose transcription is regulated by HLH proteins (3, 8–14).

HLH proteins have been broadly classified into four groups based upon their structures, interactive properties, and patterns of expression (4). Class A proteins are widely or ubiquitously expressed and include products of the E2A gene (E12, E47, and E2-5), E2-2/ITF-2, HEB, and Drosophila daughterless (4, 15–23). Class B proteins are expressed in a tissue-restricted fashion and generally heterodimerize well with class A proteins to produce complexes that bind their cognate DNA sites more avidly than either partner alone (4). Examples of class B proteins include the myogenic factors MyoD, myogenin, Myf5, and Myf6/MRF4 and the products of the Drosophila achaete-scute complex (1, 4, 24–28). Class C proteins, which contain leucine zipper domains (29) adjacent to the HLH domain, include members of the Myc network (30–35), USF, TFE3, and TFEB (36–38). The last HLH class, class D, exemplified by the Drosophila emc gene product (39, 40), is comprised of proteins lacking a basic domain. These can dimerize with class A and class B proteins (41–43) but not at all with themselves or with class C proteins (4, 44). Such dimers are unable to bind DNA as they contain only a single basic domain (41, 43, 45, 46). Four mammalian proteins of this type have been described and are referred to as Inhibitors of DNA binding (Ids) (41, 43, 45–47).

The discovery of Id1 suggested that it might affect myogenic differentiation by acting as a naturally occurring dominant negative inhibitor of MyoD and E2A gene products prior to myoblast differentiation when these proteins are nevertheless expressed at high levels (41). As differentiation proceeds, Id1 is down-regulated, thus allowing for the formation of transcriptionally competent MyoD-E2A heterodimers. The constitutive expression of a transfected Id1 cDNA prevents the formation of MyoD-E2A dimers and inhibits myogenic differentiation (48).

Id proteins are expressed in a wide variety of tissues and, in many cases, more than one Id may be expressed by a single cell type (41, 43, 45, 46). Although not all possible combinations between Ids and class A and B HLH proteins have been tested, it appears that Id members can dimerize with most class A proteins and with some class B proteins, although with considerably lower affinity (41, 43, 46). As in myogenesis (48), Id1 levels have been shown to decline during hematopoietic cell maturation (41, 49), osteogenesis (50–52), and F9 murine teratocarcinoma differentiation (41). The enforced expression of Id1 inhibits B-lymphoid, myeloid, and erythroid differentiation (49, 53, 54). Id1 has also been shown to regulate indirectly several non-myogenic tissue-specific promoters (55, 56). Based on such observations, it has been proposed that Id proteins may serve to oppose the positive effects of HLH proteins on their target genes (42, 57, 58).

Using the Id1 HLH domain as a bait in a yeast two-hybrid screen (59–62), we have identified a non-HLH protein that

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interacts with Id1 but not with Id2 or Id3. In vitro, this protein can restore the DNA binding properties of MyoD-Id1 and E12-Id1 heterodimers and can enhance DNA binding by MyoD and E12 homodimers. In vivo, it can overcome the inhibitory effects of transfected or endogenous Id1 to promote muscle-specific gene expression. The protein appears to be the human homolog of S5a, a component of the regulatory subunit of the 26 S proteasome. These studies suggest novel roles for S5a in the control of gene expression by select HLH proteins.

EXPERIMENTAL PROCEDURES

Bacterial and Yeast Strains—Escherichia coli strain JEB181 was used to recover expression plasmids from yeast. The yeast strain Y153 (MATa leu2-3, 112, ura3-52, trpl-901, his3-D200, ade2-101, gal4D gal80D URA3; GAL-laZ, LYS2; GAL-HIS3) (63) was used for all transformations. The “built” plasmid, pGBT9, has been described previously (61, 62). The human B cell library in the pGAD424-derived pACT vector containing the yeast Gal4 transactivation domain (63) was obtained from CLONTECH. Yeast strains were grown in either YPD or SC medium (64).

cDNA Library Screening—Y153 was transformed to Trp prototrophy with pGBT9-Id1 using single-stranded DNA as a carrier (65). A single colony was grown at 30 °C in SC-Trp-Leu medium and transformed with a mixture of 40 μg of library plasmid and 5 mg of sheared salmon sperm DNA. Aliquots were taken from each transformation mix before plating and used to determine the transformation efficiency by plating on SC-Trp-Leu medium. The transformation mix was then plated on 150-mm Petri dishes containing SC-Leu-Trp T° his” medium plus 25 μm 3-aminotriazole (Sigma) and incubated at 30 °C for 3–5 days. His” colonies were then replica-plated onto minimal medium lacking leucine and containing ampicillin. Positive clones that grew after 2 days were grown in LB/Amp medium, and plasmid DNA was prepared by a standard alkaline lysis miniprep procedure (66).

Recovery of Plasmids from Yeast—Single colonies of yeast transformants growing on SC-Leu-Trp-His medium were isolated, and plasmid DNA was isolated from each and used to transform JEB181 by electroporation using a Gene Pulser (Bio-Rad) with the attached Pulse Controller set according to the manufacturer’s specifications. Transformations were plated on LB/Amp plates. The following day, bacterial colonies were replica-plated on minimal medium lacking leucine and containing ampicillin. Positive clones that grew after 2 days were grown in LB/Amp medium, and plasmid DNA was prepared by a standard alkaline lysis miniprep procedure (68).

Construction of Bait Plasmids—To construct the various pGBT9-bait plasmids, appropriate cDNAs were amplified by polymerase chain reaction with forward and reverse primers containing EcoRI and BamHI sites, respectively, and cloned into the corresponding sites in the pGBT9 poly linker. The specific coding sequences amplified and contained within the bait plasmids are as follows: Id1, a.a 73–138 (41); Id1F1, a.a 99–138; Mxl1, a.a 24–124 (34); MyoD, a.a 83–184 (1); Id2, a.a 72–139 (47); Id3, a.a 28–91 (45). Max, a.a 3–161 (31), was cloned as an EcoRI/ PstI fragment. Id1ΔH2 (a.a 73–117) was obtained by digestion of pGBT9-Id1 with PstI followed by religation. Additional full-length Id1 plasmids included pAS-E12 encoding amino acids 508–654 of E12 (13) (gift from Eric Olson).

In vitro oligonucleotide-mediated mutagenesis of Id1 and Id3 was performed in m13 by the method of Kunkel (69) using the T7 Mutagenex kit (Bio-Rad). Helix swaps were performed by first altering codons encoding Pro-Thr and Pro-Gly residues at the beginning of the loop with the codons CCC-GGG that introduced a unique region of Id1 and Id3, respectively, so that both encoded Pro-Gly with encoding Pro-Thr and Pro-Gly residues at the beginning of the loop. This allowed for the precise, in-frame replacement of one helix 1 region with another. All clones were sequenced in their entirety to confirm that only the desired mutations had been obtained.

Fusion Polypeptides for Protein-Protein Interactions and DNA Binding Assays—S5a (a.a 34–377) was expressed as a GST fusion protein and purified from isopropyl-1-thio-galactoside induced bacterial lysates by gel filtration and affinity chromatography. [35S]Methionine-labeled Id1, Id3, MyoD, E12, and Mxl1 were purified using a coupled TNT in vitro transcription/translation system (Promega, Madison, WI). Approximately 0.5–1.0 μg of GST-S5a or GST alone was then incubated with 2 μl of the Id1 translation reaction or 20 μl of the other reactions at 37 °C for 30 min. Following the addition of an equal volume of a buffer containing 20 mm Tris, pH 7.4, 500 mM NaCl, 10 mM EDTA, and 0.5% Nonidet P-40 for an additional 30-min period, 1 ml of 0.5 × buffer was added along with 50 μl of glutathione-agarose beads for 5 min. The beads were precipitated by brief centrifugation, and the washing was repeated three additional times before resolving the bound proteins by SDS-PAGE and processing for autoradiography.

NAD (a.a 70–978), E12 (a.a 287–412), Id1 (a.a 2–176), S5a (a.a 34–377) were expressed as histidine fusion proteins in E. coli prep4 cells using the QAexpress® series of plasmids pQE9-11 (Qiagen, Chatsworth, CA). Proteins were purified by nickel-agarose affinity chromatography (70) and used in electrophoretic mobility shift assays with a 32P-labeled double-stranded oligonucleotide containing a MyoD binding site derived from muscle creatine kinase (MCK) promoter (71). DNA binding and gel retardation assays performed in buffer containing 20 mM Heps, pH 7.6, 50 mM KCl, 1 mM EDTA, pH 8.0, 1 mM dithiothreitol, 10% glycerol, and 20 mM NaCl. Protein/DNA mixtures were incubated at 37 °C for 15 min, at 23 °C for 15 min, and then resolved on a nondenaturing 5% acrylamide gel (70).

Mammalian Two-hybrid Assay—A cDNA fragment encoding the Id1 HLH domain (a.a 73–138) was amplified by polymerase chain reaction and cloned into EcoRI + BamHI-digested pBlueScript/SK+ (Stratagene, La Jolla, CA). The Id1 sequences were excised with EcoRI and XhoI and cloned in the same sites in the pBSG424 vector (72). The S5a clone 14A cDNA insert was excised from the parental pACh yeast vector with Xhol and cloned in the SacI site of the vector pVL16P (73). Both plasmids were transformed into HeLa cells along with the pGal4-E72CAT reporter plasmid (73) and pSv2 β-galactosidase using a standard calcium phosphate-bisphosphate precipitation (71, 74). CAT assays were performed as described previously (74) and quantified with a Molecular Dynamics PhosphorImage. All transfections were performed in duplicate, using 2% galactose, 2% ethanol, 3% glycerol, and screened for survival of gene expression by select HLH proteins.

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Co-immunoprecipitation Assays—C2C12 myoblasts were labeled for 4 h with 100 μCi/ml [35S]methionine (Translabel, NEN Life Science Products) in cysteine/methionine-free Dulbecco’s modified minimal essential medium plus 10% supplemented calf serum, 20 mM Hepes, pH 7.2, 2 mM glutamine, 50 units/ml penicillin G, and 50 μg/ml streptomycin. C2C12 myoblasts were grown in the same medium except that fetal calf serum was used in place of supplemented calf serum.

DNA Sequence Analysis—All S5a cDNAs longer than the originally identified 14A clone were isolated by screening either the human B cell cDNA library in pACh or a human umbilical vein endothelial cell cDNA library cloned in Agt11 (76) with a 200-base pair cDNA fragment derived from the 5’ end of S5a clone 14A. An additional genomic clone was isolated from a human peripheral leukocyte library in the ZAP vector (gift of John Lowe, University of Michigan). All clones were sequenced using the dideoxy method with Sequence 2.0 according to the supplier’s directions (U. S. Biochemical Corp.). Sequence analyses and homology searches were performed using MacVector (IBI, New Haven, CT) or DNASTAR software (DNASTAR, Madison, WI).

Construction of Expression Plasmids for Full-length cDNAs—All full-length cDNA fragments were excised from their parental vectors, blunt-ended with the Klenow fragment of DNA polymerase, and ligated into HindIII-digested and blunt-ended pRMCM vector (Invitrogen, San Diego, CA). The 1.3-kb S5a cDNA depicted in Fig. 1 was cloned as an XhoI fragment in the SaI site of pBS/SK+, excised as a SacI/KpnI fragment, and blunt-ended before cloning into pReCMV. Full-length Id1 and Id3 cDNAs were isolated as NotI/EcoRI and EcoRI/XhoI fragments, respectively, and blunt-ended before cloning into pReCMV. Full-length Id1-Id3 cDNA was isolated from the pSV2+ β-gal plasmids as previously described (71). p3300 MCK-CAT was a gift from Steve Hauschka (71). Appropriate plasmids were transfected transiently into C3H10T1/2 fibroblasts or C2C12 myoblasts along with pSv2 β-gal plasmid as a control for transfection efficiency. Afterward, the cells were grown for 24 h, transferred to differentiation medium for 2 days, and assayed for β-galactosidase and CAT.
Id1 Protein Degradation Assay—A rabbit reticulocyte lysate degradation system was used as described previously (77). In experiments designed to demonstrate the ATP dependence of the system, AMP was added to a final concentration of 12 mM. 10 μl of in vitro translated 35S-labeled Id1 or Id2 was added to 25 μl of unprogrammed reticulocyte lysate, 5 μl of 50 X ATP-regenerating buffer (78), and 60 μl of dilution buffer (10 mM KCl, 30 mM Tris, pH 7.8, 5 mM MgCl2, 0.5 mM dithiothreitol). As a positive control for ubiquitin-mediated degradation, in vitro translated murine c-Myc protein was added to the above-described system (79). In some cases, affinity purified hexahistidine-tagged S5a (34–172) was first added to the reaction for 15 min and incubated at room temperature prior to the addition of other components. 20 μl aliquots were removed from the degradation assay at timed intervals and immediately diluted into 0.5 ml of TEG wash buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.2% Triton). Polyclonal anti-Id1, anti-Id2, or anti-Myc antibodies were added to a final dilution of 1:500. Following a 2-h incubation at room temperature, immunoconjugates were precipitated with protein A-Sepharose and washed exhaustively with additional TEG buffer three times. Samples were boiled and resolved by SDS-PAGE. The amount of 35S-protein remaining was quantified by phosphorimaging.

RESULTS

Id1 Interacts with S5a, a Component of the 26 S Proteasome Regulatory Subunit—The region between amino acids 73 and 138 of murine Id1 (41) was expressed in the pGBT9 yeast two-hybrid vector (62). The resultant construct (pGBT9-Id1) encoded the Id1 HLH domain, plus 14 additional NH2-terminal amino acids and 11 COOH-terminal amino acids, fused in-frame with the DNA binding domain of the yeast Gal4 transcriptional activator. The interaction of the Id1 fusion protein with a known dimerization partner was confirmed by expressing the above vector in yeast together with a vector encoding the Gal4 transactivation domain fused to the murine MyoD bHLH domain (pGAD424-MyoD). Together, the two plasmids imparted histidine prototrophy to the yeast strain (not shown) and an intense blue color upon in situ β-galactosidase assay (Table I, line 6). These results indicate that the Id1 protein was expressed and interacted specifically with a known natural target molecule. The yeast strain harboring the pGBT9-Id1 plasmid bait was next transformed with a human B cell cDNA library in the pACT vector (CLONTECH), and histidine prototrophs were selected in the presence of 25 μg/ml 3-amino triazone. The screening of approximately 4 × 106 yeast transformants yielded 28 candidate His+ clones. One clone, designated 14A, contained a 1.1-kb cDNA insert encoding an open reading frame contiguous with the Gal4 transactivation domain. This clone was reintroduced into the Y153 yeast strain together with various pACT or pGAD424 constructs to confirm the specificity of the observed interaction (Table I). As expected for a bona fide interactor, the expression of 14A produced intense β-galactosidase activity in yeast harboring the original pGBT9-Id1 plasmid (line 5); no β-galactosidase activity was observed in yeast expressing either the “empty” parental pGBT9 vector (line 3) or expressing an in-frame fusion with the class C HLH-zipper protein Mxi1 (34) (line 9). Weaker interactions between 14A/MyoD and 14A/E12 were seen (lines 26 and 30, respectively) and were of a strength comparable to that observed between MyoD and itself (line 25). An intriguing observation was that neither Id2 nor Id3 HLH domains (45, 47) interacted with 14A (lines 13 and 16) despite scoring strongly for interaction with MyoD (lines 14 and 17). This suggested that the 14A protein could discriminate between closely related Id HLH sequences.

Deletions within the Id1 HLH domain were used to determine whether the complete motif was required for its interaction with 14A. Fusion proteins lacking either the first or second α-helix of Id1 (Id1ΔH1 and Id1ΔH2) failed to interact with 14A (lines 19 and 22), thus indicating that the interaction between 14A and Id1 requires an intact Id1 HLH domain.

Using a 200-base pair restriction fragment derived from the 5′ end of the original 1.1-kb 14A cDNA, we rescreened the pACT B cell cDNA library as well as a ggt11 cDNA library derived from HeLa cells. Of the 14A-related clones identified, we sequenced several that contained additional 5′ sequence. The length of the most complete clone was 1286 nt (Fig. 1). Conceptual translation of the cDNA indicated that it encoded a protein of 377 amino acids and that clone 14A began at residue 34. Two closely spaced potential AUG initiation codons were identified at nt 27 and 54, respectively. The first of these resides within a reasonable, although not ideal, context for translational initiation (80). Because recent work suggests that scanning 40 S ribosomal subunits can initiate protein synthesis at such non-optimal sites but can also bypass them and initiate at a nearby, downstream AUG codon as well (81), we are unable to state unequivocally that the position 27 AUG is the only initiator codon. Since the region upstream of nt 27 contains no terminator, there exists the possibility of a more proximal initiation codon not included in the cDNA sequence depicted in Fig. 1. Arguing against this, however, is the observation that this cDNA is actively transcribed and translated when cloned in a Bluescript vector (not shown). Northern blot and primer extension analyses also suggest that the cDNA is nearly full length and contains approximately 185–190 nt of

| Bait construct | Target construct | β-Gal expression |
|----------------|------------------|-----------------|
| pGBT9 only     | pACT only        | –               |
| pGBT9 only     | pGAD only        | –               |
| Id1            | pGAD only        | –               |
| Id1            | 14A              | + + +           |
| Id1            | MyoD             | + + +           |
| pGBT9 only     | MyoD             | –               |
| Mxi1           | pGAD only        | –               |
| Mxi1           | 14A              | –               |
| Mxi1           | Max              | + + +           |
| pGBT9 only     | Max              | –               |
| Id2            | pGAD only        | –               |
| Id2            | 14A              | –               |
| Id2            | MyoD             | + + +           |
| Id3            | pGAD only        | –               |
| Id3            | 14A              | –               |
| Id3            | MyoD             | + + +           |
| Id3            | Mxi1             | + + +           |
| Id1ΔH1         | pGAD only        | –               |
| Id1ΔH1         | 14A              | –               |
| Id1ΔH1         | MyoD             | –               |
| Id1ΔH2         | pGAD only        | –               |
| Id1ΔH2         | 14A              | –               |
| Id1ΔH2         | MyoD             | –               |
| MyoD           | pGAD only        | –               |
| MyoD           | 14A              | +               |
| MyoD           | E12              | + + +           |
| pGBT9 only     | E12              | –               |
| E12            | pGAD only        | –               |
| E12            | 14A              | +               |

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| Table I | Summary of interactions between pGBT9-encoded “baits” and pACT or pGAD-encoded “targets” |
|---------|-----------------------------------------------------------------------------------------|
| pGBT9 bait and pACT or pGAD target expression vectors were constructed by directional cloning of polymerase chain reaction-amplified segments of the indicated cDNAs. Correct orientations and reading frames were confirmed by DNA sequencing. In most cases, expression of each pGBT9 construct was demonstrated by the ability of its encoded protein to interact with a known pACT or pGAD-encoded target sequence (for example, see lines 6, 10, and 14). None of the individual fusion proteins transactivated when expressed individually (cf. lines 3, 4, 12, and 15). In situ β-galactosidase (β-gal) assays were performed essentially as described (66). –, no evidence for color change of the yeast colony after an overnight incubation at 37 °C; +, weak color observed with > 2 h incubation; + +, intense color observed within 1–2 h; ++, + +, intense color observed at 30 min – 1 h; + + +, intense color observed within 15–30 min. |
| Bait construct | Target construct | β-Gal expression |
|----------------|------------------|-----------------|
| pGBT9 only     | pACT only        | –               |
| pGBT9 only     | pGAD only        | –               |
| Id1            | pGAD only        | –               |
| Id1            | 14A              | + + +           |
| Id1            | MyoD             | + + +           |
| pGBT9 only     | MyoD             | –               |
| Mxi1           | pGAD only        | –               |
| Mxi1           | 14A              | –               |
| Mxi1           | Max              | + + +           |
| pGBT9 only     | Max              | –               |
| Id2            | pGAD only        | –               |
| Id2            | 14A              | –               |
| Id2            | MyoD             | + + +           |
| Id3            | pGAD only        | –               |
| Id3            | 14A              | –               |
| Id3            | MyoD             | + + +           |
| Id3            | Mxi1             | + + +           |
| Id1ΔH1         | pGAD only        | –               |
| Id1ΔH1         | 14A              | –               |
| Id1ΔH1         | MyoD             | –               |
| Id1ΔH2         | pGAD only        | –               |
| Id1ΔH2         | 14A              | –               |
| Id1ΔH2         | MyoD             | –               |
| MyoD           | pGAD only        | –               |
| MyoD           | 14A              | +               |
| MyoD           | E12              | + + +           |
| pGBT9 only     | E12              | –               |
| E12            | pGAD only        | –               |
| E12            | 14A              | +               |
The predicted size of the full-length protein (45.5 kDa) is in excellent agreement with the size of the largest product observed following in vitro translation of the full-length cDNA (approximately 47 kDa, not shown). Secondary structure analysis of the full-length protein showed that, while it contained several regions of α-helical content, particularly in its COOH-terminal region, it did not contain a bHLH domain (not shown). It does contain two putative nuclear localization signals (amino acids 123–129 and 361–367) (82).

A computerized BLAST search of the GenBank database revealed significant homology between the protein identified by us and the S5a subunit of the regulatory 19 S proteasome from Arabidopsis and Drosophila (Fig. 2). In a third case, sequencing of chromosome VIII of yeast (85) revealed the presence of a hypothetical open reading frame (YHR200w) whose conceptual translation product is 55% identical to the product encoded by our cDNA. This suggests that YHR200w encodes the yeast S5a homolog. Finally, the recent cloning of the human S5a subunit (86) shows it to be nearly identical to the one described here.

One difference between our sequence and that of other S5a subunits reported (including the human protein) is the presence in our cDNA clone of a GTG instead of an ATG initiation codon at nt 9 (Fig. 1). This results in the absence of six NH2-terminal amino acids that are present in the other S5a proteins (Fig. 2). We cannot attribute this difference to cloning or sequencing artifacts as we were unable to identify an ATG initiation codon in several independently derived cDNAs as well as in a genomic clone. Despite these differences, we conclude that the cDNA that we have cloned encodes the human S5a protein or a nearly identical polypeptide. Northern blotting revealed the presence of an approximately 1.6-kb transcript in each of the eight different tissues examined (not shown).

In Vitro Association between Id1 and S5a—The above yeast studies strongly suggested that S5a might be an authentic and specific partner for Id1 and perhaps for MyoD and E12 as well. We therefore sought independent ways to establish these putative associations and determine their functional consequences. One approach was to ask whether S5a protein would interact with Id1 in vitro. We therefore expressed and purified a GST-S5a fusion protein derived from the original 14A cDNA clone and asked if it could specifically associate with several of the proteins previously demonstrated to interact with S5a in yeast (Table I). As shown in Fig. 3A, GST-S5a bound 35S-labeled in vitro translated, full-length Id1, MyoD, and E12 but not

2 G. Anand and E. V. Prochownik, unpublished observations.
Further experiments were performed to confirm the above results and to investigate the nature of the protein associations involving MyoD, Id1, and S5a. Full-length MyoD was co-translated with full-length Id1, Id2, or Id3 in a reticulocyte lysate containing \(^{35}\)S-methionine. Labeled Id-MyoD dimers were then immunoprecipitated by the addition of a polyclonal antibody specific for the appropriate Id protein followed by the addition of Protein A-Sepharose beads. After extensive washing of the immune complexes, the pellets were resuspended in buffer and further incubated with purified GST-S5a. Immune complexes were removed by centrifugation, and the \(^{35}\)S-labeled MyoD released into the supernatants was resolved by SDS-PAGE and visualized by autoradiography. As seen in Fig. 3B, MyoD was displaced only from the MyoD-Id1 complexes and not from the MyoD-Id2 or MyoD-Id3 complexes. SDS-PAGE of immune complexes after incubation with S5a showed Id2 and Id3 to still be associated with MyoD, whereas virtually all of the Id1 was absent. These experiments confirm those presented in Fig. 3A that show a specific association between S5a and Id1. Just as importantly, they indicate that the binding of S5a to Id1 results in the disruption of pre-existing Id1-MyoD heterodimers rather than in the formation of ternary complexes.

To determine the functional consequences of the interactions shown above, each of the proteins was expressed in E. coli as hexahistidine fusion and purified by nickel-agarose affinity chromatography (70, 87). We then examined how their DNA binding properties were affected by similarly purified His\(^{6}\)-tagged S5a. As seen in Fig. 4A, and as previously reported (3, 5), MyoD bound a \(^{32}\)P-labeled double-stranded oligonucleotide from the muscle creatine kinase promoter in a concentration-dependent manner (lanes 4–6). Also in keeping with prior observations (41), addition of Id1 extinguished MyoD binding (lanes 7 and 8). Although S5a protein by itself showed no tendency to bind DNA, even at high concentrations (lane 2), its addition to non-binding MyoD-Id1 complexes not only restored DNA binding in a concentration-dependent manner (lanes 9 and 10) but strikingly augmented DNA binding by MyoD by as much as 50-fold (compare lanes 4 and 10). S5a also enhanced DNA binding by MyoD in the absence of Id1 (lanes 11 and 12). The virtually identical appearance of the bands in lanes 10 and 12 suggested that, in the former case, all MyoD, including that previously complexed with Id1, was interacting with S5a. The presence of more slowly migrating protein-DNA complexes in lanes 9–12 is consistent with a direct association between MyoD homodimers and S5a. The remarkable concentration-dependent nature of the S5a-augmented DNA binding is indicative of cooperativity and suggests either that S5a itself must multimerize prior to binding MyoD or that MyoD can bind a variable number of S5a molecules, with each addition resulting in a synergistic increase in DNA binding. Either possibility is consistent with the apparent reduction in the electrophoretic mobility of the protein-DNA complexes seen with increasing amounts of added S5a (A, lanes 9 and 10, and 11 and 12).

Similar studies were performed with purified, His\(^{6}\)-tagged E12 protein (Fig. 4B). As in the case of MyoD, E12 homodimeric binding to the \(^{32}\)P-labeled probe was concentration-dependent and was inhibited by Id1 (lanes 4, 5, 7, and 8). The addition of S5a to either E12-Id heterodimers or to E12 homodimers alone greatly augmented DNA binding, although the extent of this effect (up to 10-fold) was not as great as that seen with MyoD (lanes 6, 10 and 11). Once again, complexes of slower mobility

not Id3 or Mxi1 (lanes 1–5). Control experiments, performed with purified GST alone, showed no binding of any of the proteins (lanes 6–10). Although roughly comparable amounts of captured \(^{35}\)S-proteins are shown, approximately 10-fold more MyoD and E12 were required to achieve this degree of binding. This is in good agreement with the results in yeast where much weaker interactions of S5a with MyoD and E12 were seen (Table 1).
were observed suggesting a direct interaction between E12 and S5a.

The effect of S5a on binding by MyoD-E12 heterodimers was next examined (Fig. 4C). At the concentrations employed, homodimeric DNA binding by either MyoD or E12 alone was weak and its detection required prolonged autoradiographic exposure (lanes 3 and 4). Synergistic DNA binding was achieved, however, in the presence of equimolar amounts of the two proteins (lane 5) and was abrogated in a concentration-dependent manner by the addition of Id1 (lanes 6 and 7). As was the case for each of the homodimers (A and B), the addition of S5a reversed the inhibitory effect of Id1 and augmented DNA binding by the MyoD-E12 heterodimer by only about 3-fold. This was only slightly greater than the 1.5-2-fold non-specific enhancement of DNA binding that S5a conveyed to purified Max protein (Fig. 4D, lane 2). Finally, a labeled oligonucleotide containing a mutant MyoD binding site (5) failed to be bound by any of the proteins tested, either in the absence or presence of excess S5a (not shown). This indicated that the action of S5a requires sequence-specific binding by HLH proteins.

Specific antibodies were next used in “supershift” experiments to identify the components of the various DNA binding complexes. As previously demonstrated, the addition of S5a to MyoD resulted in more pronounced DNA binding (Fig. 4E, compare lanes 3 and 5). Supershifted complexes were observed when either anti-MyoD or anti-S5a antibodies were added to the reaction after DNA binding had been allowed to occur (lanes 8 and 9). No supershift was observed, however, with an anti-E12 antibody (lane 10). Similarly, S5a once again enhanced DNA binding by purified E12 (compare lanes 4 and 6). The complex was supershifted with either anti-E12 or anti-S5a antibodies but not with an anti-MyoD antibody (lanes 4 and 5) but not by anti-Id1 antibodies (lane 7).


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Id1 and S5a Associate in Mammalian Cells—Further evidence to support an Id1-S5a association was obtained using a
in vivo such as E12 and E47. These results also demonstrate that the expression, presumably through its ability to dissociate complex between Id1 and either MyoD or class A bHLH proteins such as E12 and E47. These results also demonstrate that the behavior of S5a is precisely that predicted from its in vitro properties.

The induction of myogenesis in most in vitro models typically involves the removal of serum to induce growth arrest. This is followed by the down-regulation of Id1 mRNA and protein (48). Based upon the results of the above experiments, one prediction is that, by sequestering and inactivating endogenous or overexpressed Id1, S5a might be able to drive myogenic differentiation independently of the requirement for serum with-

To confirm the above detected association between S5a and Id1, we performed co-immunoprecipitation experiments of metabolically labeled endogenous proteins from C2C12 myoblasts. Under stringent conditions, polyclonal anti-Id1 or anti-S5a antibodies precipitated only their respective proteins from labeled cell lysates thus confirming the specificities of the antibodies (Fig. 5, lanes 4 and 5). Using non-stringent conditions (48), cell extracts were first precipitated with either preimmune or anti-Id1 antisera (lanes 2 and 6). The immune complexes were then reprecipitated under stringent conditions in RIPA buffer with anti-S5a antibody. We detected S5a protein only when cell extracts were first precipitated with anti-Id1 antibody in the first, non-stringent step. In reciprocal experiments, cell extracts were precipitated under non-stringent conditions with preimmune or anti-S5a antibody (lanes 3 and 7) followed by precipitation under stringent conditions with anti-Id1 antibody. Id1 protein was detected only in the immune complex formed with anti-S5a. Together with the mammalian two-hybrid results presented above, these results provide evidence for the in vivo association between endogenous Id1 and S5a.

### Full-length S5a Reverses the Inhibitory Effect of Id1 on the MCK Promoter in Vivo

In the previous experiments (5, 24), MyoD augments MCK-CAT expression in a concentration-dependent manner, whereas co-expression of Id1 abrogates this effect (41, 90). Based upon our results indicating that S5a could dissociate Id1 heterodimeric complexes and augment DNA binding by MyoD and E12 homodimers (Figs. 3 and 4), we predicted that the overexpression of full-length S5a in vivo would exert a positive effect upon the MCK promoter.

As expected, MyoD stimulated the expression of an MCK-CAT vector in a concentration-dependent manner in C3H10T1/2 cells induced to undergo myogenic differentiation (Fig. 7, columns 2 and 3) whereas co-expression of Id1 abrogates this effect (41, 90). The inhibitory action of Id1 was almost completely reversed by co-expressed full-length S5a (column 4). In keeping with its observed in vitro effects (Fig. 4A), S5a also enhanced the activity of transfected MyoD alone by 3–4-fold (column 5). S5a alone had no discernible effect on the activity of the MCK promoter (column 6). These results indicate that full-length S5a can significantly influence myogenic gene expression, presumably through its ability to dissociate complexes between Id1 and either MyoD or class A bHLH proteins such as E12 and E47. These results also demonstrate that the in vivo behavior of S5a is precisely that predicted from its in vitro properties.
To investigate the validity of this prediction, we used either C3H10T1/2 fibroblasts or C2C12 myoblasts. In the first case, C3H10T1/2 cells were transfected with a MyoD expression plasmid along with the MCK-CAT reporter and maintained under conditions that prevented differentiation (10% serum) for the next 2 days. As shown in Fig. 8 (column 1), these cells expressed low levels of CAT, consistent with their undifferentiated state. However, when S5a was co-expressed, CAT activity was enhanced 8-fold over background (column 2). The relative level of CAT conversion was comparable to that seen when the cells were induced toward myogenic differentiation by serum withdrawal in the absence of co-transfected CMV-S5a (Fig. 7, compare columns 1 and 3). The addition of a 2-fold molar excess of a CMV-Id3 expression vector to the transfection totally suppressed CAT activity, whereas a comparable amount of CMV-Id1 was only partially effective (compare columns 3 and 4). We interpret these results as showing that, by sequestering and inhibiting Id1, S5a is able to provide an intracellular environment conducive to the expression of a muscle-specific promoter, even in the absence of a differentiation stimulus. The strong down-regulation of the MCK promoter by Id3 presumably reflects its inability to be inhibited by co-expressed S5a, whereas the weak down-regulation of the promoter by Id1 results from its more effective sequestration by co-transfected S5a. In other experiments, we have demonstrated that 5–10 times more Id1 than Id3 DNA is required to achieve comparable degrees of S5a inhibition.

Essentially identical results were obtained with C2C12 myoblasts (Fig. 8, columns 5–8). As in the case of C3H10T1/2 cells, the overexpression of S5a was sufficient to induce the MCK promoter nearly 10-fold over background. Strong down-regulation by Id3 and weak down-regulation by Id1 were again seen (columns 7 and 8). These experiments demonstrated that the phenotypic effects imparted by S5a could be recapitulated in an in vivo system containing only endogenous levels of HLH proteins.

**Structural Features That Distinguish Id1 from Id3**—The HLH regions of all known Id family members are closely related (45, 46). Nevertheless, it is apparent that S5a readily discriminates Id1 from other Id proteins (Table I and Fig. 3). To investigate the basis for this remarkable specificity, we constructed a series of helix “swap” and point mutants of Id1 and Id3 and assessed their ability to interact with S5a in yeast (Fig. 9). Control experiments showed that all Id constructs inter-
acted equally well with E12, a known dimerization partner. As previously observed, the Id1 HLH domain interacted strongly with S5a, whereas the Id3 HLH domain was completely inactive (compare lines 1 and 2). Testing of hybrid HLH domains indicated that the specificity for S5a interaction lay in either the first helix of Id1 and/or in the region immediately upstream of helix 1 (compare lines 3 and 4). Using site-directed mutagenesis, we first changed individual amino acids in helix 1 of Id1 to those found at the corresponding position in Id3 (lines 5–7). In all three cases, interaction with S5a was severely compromised; even the conservative Lys98 → Arg mutation almost completely eliminated interaction with S5a (line 7). These results suggested that each of the three amino acids that distinguish helix 1 of Id1 from Id3 were necessary for the S5a interaction. In addition deletions of the Id1 upstream flanking region (lines 13–15) indicated that amino acids adjacent to helix 1 are also important for the S5a interaction. Testing of two segments of S5a, consisting of amino acids 34–172 and 173–377, mapped the Id1-interacting domain to the NH2-terminal portion of the S5a molecule (lines 4 and 5).

The first helices of the Id1 and Id3 HLH domains differ by only three amino acids (45). Using site-directed mutagenesis, we first changed individual amino acids in helix 1 of Id1 to those found at the corresponding position in Id3 (lines 5–7). In all three cases, interaction with S5a was severely compromised; even the conservative Lys98 → Arg mutation almost completely eliminated interaction with S5a (line 7). These results suggested that each of the three amino acids that distinguish helix 1 of Id1 from Id3 played a critical role in recognition by S5a. In a similar manner, neither individual mutants nor a double mutant in helix 1 of Id3 was able to allow S5a to interact (lines 8–11). Somewhat surprisingly, this was also true of the triple helix 1 Id3 mutant (line 12). These results suggested that additional residues upstream of helix 1 of Id1 were important for S5a interaction. Therefore, a series of five amino acid deletions was introduced into the Id1/3 helix swap mutant. Testing each of these revealed that an additional region necessary for S5a interaction with Id1 lay within the five amino acid stretch immediately preceding the helix 1 domain (line 15). From these experiments, we conclude that the structural features that allow S5a to distinguish Id1 from Id3 consist of three amino acids within helix 1 and no more than five amino acids immediately adjacent to this region.

Preliminary deletion analysis of S5a was also performed to identify the region of the molecule necessary for its interaction with Id1. Restriction fragments encoding amino acids 34–172 and 173–377 were cloned in the pGAD424 vector and introduced into yeast along with each of the Id1 and Id3 mutants shown in Fig. 9. The results of these experiments demonstrated that the NH2-terminal segment of S5a was capable of all of the interactions seen with the nearly full-length molecule (amino acids 34–377, clone 14A). In contrast, the region between amino acids 173 and 377 was incapable of interacting with any of the Id proteins. 

Effect of S5a on 26 S Proteasome-mediated Id1 Degradation—The intracellular degradation of many proteins involves ubiquitination and subsequent ATP-dependent processing by the 26 S proteasome (77, 91). The 26 S proteasome contains two complexes, consisting of a 20 S proteolytic component and a
Inhibition of Id1 by S5a

The protein characterized in this report was identified through its interaction with the HLH domain of Id1 and appears to be S5a or a close homolog. In yeast two-hybrid screens, S5a interacts strongly with Id1, less strongly with MyoD and E12, and not at all with the class C HLH-zipper protein Mxi1. S5a also appears to be highly specific for Id1, being incapable of interacting with the highly homologous Id2 or Id3 HLH domains. Deletion mapping indicated that Id1 requires an intact HLH domain to associate with S5a.

A number of studies were performed to confirm the specificity of S5a’s interactions and to assess their consequences. The results of our in vitro studies can be summarized as follows. 1) A GST-S5a fusion protein was able to bind 35S-labeled Id1, MyoD and E12. 2) S5a specifically promoted the dissociation of pre-formed MyoD-Id1 complexes. 3) In electrophoretic mobility shift assays, His6-tagged S5a reversed the loss of DNA binding that accompanied the formation of MyoD-Id1 or E12-Id1 heterodimers. 4) S5a greatly enhanced the DNA binding of MyoD and E12 homodimers while having a less pronounced effect on MyoD-E12 heterodimers. These in vitro findings together with antibody supershift experiments indicate that S5a enhances DNA binding of bHLH proteins by promoting their dissociation from Id1 as well as by increasing their intrinsic DNA binding as homodimers.

In vivo, an interaction between Id1 and S5a was detected using both yeast and mammalian two-hybrid assays as well as by a more traditional co-immunoprecipitation approach (Figs. 5B and 6). The co-expression of S5a also completely reversed the inhibitory effect of Id1 on myogenic gene expression in C3H10T1/2 fibroblasts following the induction of differentiation. Perhaps most convincingly, the overexpression of S5a was also sufficient to overcome the inhibitory effects of Id1 in either C3H10T1/2 cells or C2C12 myoblasts without the need for induced differentiation (Fig. 8). In the latter case, the expression of only endogenous levels of MyoD and Id1 by these cells indicates that Id1’s role in inhibiting the myogenic program can be overridden simply by the expression of S5a. Since myogenic differentiation in cultured cells is typically accompanied by down-regulation of Id1 and withdrawal from the cell cycle following the removal of growth factors (45, 94, 95), the results presented in Fig. 8 would suggest that the overexpression of S5a can substitute for this more classical differentiation stimulus. We suggest that either changes in Id1 levels (following serum removal) and/or its inhibition by S5a are sufficient to promote this process.

In vitro association experiments (Fig. 3A) as well as yeast two-hybrid data (Table I) indicate a much stronger association of S5a with Id1 than with either MyoD or E12 proteins. On the other hand, electrophoretic mobility shift experiments (Fig. 4A) demonstrated a strong and specific enhancement of MyoD DNA binding by S5a. E12 DNA binding was affected as well although to a lesser degree. These observations are not necessarily at odds as it is possible that relatively weak associations between the homodimeric bHLH proteins and S5a result in greatly increased affinity of the former proteins for the oligonucleotide probe. Our in vivo experiments also do not allow us to determine how much of the increased reporter plasmid activity following co-expression of S5a is due to Id1 inhibition versus enhanced MyoD or E12 binding. Further work will be required to determine the relative contribution to myogenic

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3 M. Rechsteiner, personal communication.
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differentiation of each of the S5a activities we have observed.

In addition to our results indicating that S5a causes a displacement of Id1 from its heterodimeric partners, the foregoing experiments suggest two additional non-mutually exclusive mechanisms by which S5a could function to enhance DNA binding by MyoD and E12: 1) S5a might increase the affinity of the homodimer for its DNA target; 2) S5a might promote the dissociation of higher order oligomers to dimers. This would be especially important in the case of MyoD since only small amounts of it exist in dimeric form in solution, with the majority of it occurring in tetrameric and higher order non-binding complexes (96–98). At the present time, we are unable to distinguish between these models. However, since S5a acts to promote DNA binding by both MyoD and E12, the second model seems less likely given that E12 has been reported to exist primarily in dimeric form in solution (96–98).

A substantial amount of evidence suggests that Id proteins participate in terminal differentiation as well as in events related to cell cycle progression and withdrawal. Well-documented declines in Id1 levels accompany myogenic, myeloid, erythroid, and osteogenic differentiation (41; 49; 51–53). Simultaneous reductions in Id1 and Id2 levels occur during erythroid and B cell terminal differentiation (18). In non-differentiating in vitro systems, Id1 and Id3 transcript levels have been reported to decline as cells enter the G0 state following contact inhibition or deprivation of growth factors (41; 45; 50). Id1, Id2, and Id3 are rapidly induced following mitogenic stimulation of quiescent cells, thus mimicking the behavior of immediate-early response genes (45; 94). More direct evidence in favor of a role for Id proteins in cell cycle progression has been obtained by demonstrating that suppression of individual or multiple Id proteins can delay the G1 to G2 transition (94; 95; 99).

The link between cell cycle withdrawal, terminal differentiation, and reduction in Id protein levels raises the question of how some HLH proteins might function prior to the occurrence of these events when many cells express tissue-specific genes and retain limited proliferative capacity in the face of high Id protein expression. Myogenic proteins are expressed in a temporal albeit overlapping manner, with MyoD and Myf5 being expressed earlier than others in committed myoblasts (100–103). Evidence that these factors are functional at such early times is provided by work indicating the presence of positive autoregulatory and feedback loops involving their cognate genes (103–105). The ability of proteins such as S5a to promote the dissociation of Id1-containing heterodimers and to enhance DNA binding by residual class A and B homodimers may provide a means by which certain classes of myogenic factors could be activated prior to terminal differentiation. This could also explain how some rhabdomyosarcoma cells are able to express skeletal muscle-specific genes (106, 107) despite high rates of proliferation and Id1 protein expression (108). By virtue of their enhanced DNA binding capacity in association with S5a, MyoD and E12 homodimers might contribute more significantly to the transcriptional control of myogenic genes than would otherwise be inferred from their low endogenous levels and intrinsic DNA binding capabilities. Given that the DNA target sites for MyoD and E2A product homodimers are distinct from those for MyoD-E2A heterodimers (7), S5a also has the potential for indirectly influencing the expression of a subset of genes distinct from those regulated by the above heterodimers.

Another potential role for S5a stems from the realization that individual cells may simultaneously express multiple class A and class B HLH proteins as well as more than one Id (43; 46; 100–103). Although not all combinations have been rigorously tested, most evidence indicates that all Id proteins are capable of interacting with all four myogenic factors (43, 45). Thus the ability of S5a to interact only with Id1 provides a potentially attractive means by which only certain inactive heterodimers could be reactivated.

S5a bears a striking functional resemblance to a previously characterized cellular factor capable of enhancing the DNA binding of MyoD and E2A homodimers and heterodimers (108). Although the effect of this factor on complexes formed with Id1 was not reported, it is of note that it promoted DNA binding by minimal MyoD and E2A bHLH domains.

Proteasomes are multisubunit proteolytic complexes, found both in the nucleus and cytoplasm (77; 91; 109; 110). The basic core element of the proteasome consists of a 20 S component containing 14 subunits with at least five different proteolytic activities. A 19 S regulatory complex, comprised of at least 15 subunits, interacts with the 20 S core to constitute the 26 S proteasome which generally functions in the ATP-dependent recognition and degradation of ubiquitinated protein substrates. Among the proteins known to be subject to this pathway are those involved in cell cycle regulation, growth, and differentiation (79; 111; 112).

The recently cloned S5a subunit of the 19 S regulatory complex binds multi-ubiquitin chains after their attachment to target proteins (84; 113). It has subsequently been demonstrated that S5a interacts with hydrophobic residues in ubiquitin and has led to the proposal that a conserved, hydrophobic region in the COOH-terminal half of S5a contains the domain responsible for this interaction (78). Until now, no evidence has existed to implicate S5a in the recognition of non-ubiquitinated proteins. However, our results suggest that S5a may play a role in modulating Id1 function by interacting directly with the unmodified protein. The extent to which this interaction influences either ubiquitin-dependent or -independent Id1 proteolysis appears minimal and nonspecific based upon the experiments presented in Fig. 10.

In summary, through the use of two hybrid screening, we have determined that S5a can associate with Id1, a member of the class D HLH family, as well as with E12 and MyoD, members of the class A and B bHLH families, respectively. These interactions lead to the dissociation of Id1 heterodimers and to enhanced DNA binding by the class A and B family members. S5a does not appear to specifically accelerate the ubiquitin-independent degradation of Id1 by the proteasome. Taken together, these novel properties of S5a contribute to a profound capacity to regulate terminal myogenic differentiation.

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