An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene

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ABSTRACT An mRNA encoding a helix-loop-helix protein that we have named HLH462 is induced in mouse 3T3 cells as part of the immediate early transcriptional response to growth factors and other signaling agents. The RNA is present in a number of mouse tissues and in the developing mouse fetus. The HLH462 gene has been mapped by interspecific backcross analysis to the distal region of mouse chromosome 4. In its helix-loop-helix region HLH462 is closely related to the Id protein and the Drosophila emc protein. Like Id, HLH462 lacks a basic region required for DNA binding, and it inhibits the DNA-binding activities of other helix-loop-helix proteins. On the basis of its structural and functional similarity to Id, we suggest that HLH462 may inhibit the activities of helix-loop-helix transcription factors during the cellular growth response and during development.

When growth factors or other extracellular ligands bind to cell surface receptors, they induce sequential changes in gene expression in target cells. In mouse 3T3 cells the first genes activated by serum growth factors include those that code for a diverse group of transcription regulators: Jun and Fos family members, zinc-finger proteins, and serum response factor (SRF) (1, 2). Such immediate early transcription factors are thought to regulate genetic programs that mediate cellular responses to extracellular signals.

In this report we describe the induction in mouse 3T3 cells and the presence in the developing mouse fetus and in adult tissues of mRNA that codes for a protein (called HLH462) that belongs to a recently recognized class of transcription factors, the helix-loop-helix proteins (3). The distinguishing feature of this class is an amino acid sequence motif predicted to form a helix-loop-helix structure through which the proteins dimerize (3). Most members of the helix-loop-helix protein family so far described also have a basic DNA-binding domain adjacent to the dimerizing region, but others do not (4-6). Among the latter is the protein Id, implicated in the suppression of mammalian muscle cell differentiation (5) and the Drosophila emc protein, a negative regulator of sensory organ development on the fly's body surface (4, 6). These proteins are thought to act by forming inactive dimers with positively acting helix-loop-helix transcription factors (5). HLH462 shows extensive amino acid sequence similarity to Id and the Drosophila emc protein in the helix-loop-helix domain, and like Id it lacks a basic DNA-binding region but can interact with other helix-loop-helix proteins. Based on this structural and functional similarity, we hypothesize that HLH462 may negatively regulate the activity of other helix-loop-helix transcription factors during the cellular response to growth factors and during development.

MATERIALS AND METHODS

Cell Culture. BALB/c 3T3 cells were maintained and stimulated with serum or other inducing agents as described (7).

DNA Sequencing. HLH462 cDNA and genomic DNA fragments were cloned into pBluescript II KS (Stratagene) and sets of 5' and 3' deletions were prepared. The resulting subclones were sequenced as double-stranded plasmids by the dideoxynucleotide chain-termination method (8) using 2'-deoxyadenosine 5'-[α-35S]triphosphate and Sequenase (United States Biochemical).

Primers and S1 Nuclease Assay. These procedures were carried out as described (9). The primer used was a 32P-labeled oligonucleotide complementary to nucleotides 132-155 of the cDNA (Fig. 1). Partial sequence of the genomic fragment used to prepare the probe for S1 analysis is shown in Fig. 1.

In Vitro Transcription and Translation. In vitro transcription and translation (10) used either T7 or T3 RNA polymerase for transcription. The HLH462 cDNA template was a cDNA clone containing nucleotides 44-969 (see Fig. 1). cDNA clones for MyoD1 and E12 were generously supplied by H. Weintraub (Hutchinson Cancer Research Center, Seattle) and D. Baltimore (Rockefeller University, New York), respectively.

RNA Blot Hybridization. This procedure was performed as described (11) with 15 μg of total cellular RNA per lane. DNA fragments used as probes were 32P-labeled by nick-translation (Boehringer Mannheim).

DNA-Binding Assays. Double-stranded oligonucleotides corresponding to the MyoD binding site in the muscle creatine kinase (MCK) enhancer (5) or the Zif268 binding site (MUT.3, ref. 12) were labeled as described (12). Binding to the MCK enhancer site was carried out as described (5) except that sonicated salmon sperm DNA was used at 50 μg/ml as nonspecific competitor, and reactions were carried out in a total volume of 25 μl with 12.5 fmol of 32P-labeled DNA probe. Binding to the Zif268 site was carried out as described (12) except that EDTA was omitted from the binding buffer. Competition assays were carried out in a total volume of 25 μl with 5 fmol of labeled probe (12). Competitors used were double-stranded oligonucleotides corresponding to Zif268 sites (12) or the Zif-like site within the HLH462 promoter (Fig. 1). For competition analysis using the SRE-like site within the HLH462 promoter, HELa cell nuclear extract was used as a source of SRF protein (13). Double-
stranded unlabeled oligonucleotides corresponding to the SRE-like site in the HLH462 promoter or a previously described double-stranded oligonucleotide site from the c-fos promoter (13) were preincubated with the extract before addition of the probe. Gel analysis of protein-DNA complexes was carried out as described (12, 13).

Interspecific Backcross Mapping. Interspecific backcross progeny were generated by matings (C57BL/6J × Mus spretus)F1 females and C57BL/6J males (14). A total of 205 N2 progeny were obtained; a random subset of these N2 mice were used to map the Hlh462 locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (15). All blots were prepared with Zetabind nylon membrane (AMF Cuno). The Hlh462 probe (the 940-base-pair (bp) mouse cDNA) was labeled with [α-32P]dCTP by using a nick-translation kit (Boehringer Mannheim). Washing was generally done to a final stringency of 75 mM NaCl/7.5 mM sodium citrate, pH 7.4/0.1% SDS at 65°C. An ~8.2-kilobase (kb) fragment was detected in BamHI-digested C57BL/6J DNA; an ~9.5-kb fragment was detected in BamHI-digested M. spretus DNA. Probes and restriction fragment length polymorphisms for LyVE, Fgr, Del-1, and Anf have been described (16). Recombination distances were calculated (17) using the computer program SPRETUS MADNESS, developed by D. Dave (Data Management Services, Frederick, MD) and A. M. Buchberg (National Cancer Institute–Frederick Cancer Research and Development Center). Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS AND DISCUSSION

Nucleotide Sequence of HLH462 cDNA and 5' Flanking Genomic Sequence. Lau and Nathans (7) previously reported the isolation of a cDNA (clone 3CH 462) derived from an immediate early mRNA of about 1 kb induced in BALB/c...
Fig. 2. Comparison of the predicted amino acid sequence of HLH462 with the sequences of selected helix–loop–helix proteins. Protein sequences are shown in the single-letter code. Amino acid identities relative to the HLH462 sequence are shaded. Sources of the other sequences: Id (5), MyoD1 (24), E47 (3), c-Myc (25), and Emc (4, 6). Except for Emc (Drosophila), all sequences are derived from murine cDNA clones.

3T3 cells by serum, platelet-derived growth factor (PDGF), or fibroblast growth factor. Using this partial cDNA as a probe, we isolated independent cDNA clones of ≈1 kb (hereafter called HLH462 cDNA) and sequenced each strand of one of these isolates and partially sequenced another. The sequence of 940 nucleotides has a single long open reading frame beginning at the first ATG and encodes 119 amino acids (Fig. 1). There is a poly(A)-addition signal 18 nucleotides from the poly(A)tail and four TATA or ATTTT sequences near the end of the cDNA that may be related to the short half-life of HLH462 mRNA (7, 18). The nucleotide sequence surrounding the first ATG triplet fits the consensus sequence for an efficient translation start signal (19).

The 5′ end of the HLH462 mRNA missing in the cDNA clone was defined by reverse transcription of RNA from 3T3 cells stimulated with serum for 3 hr in the presence of cycloheximide, using an antisense oligonucleotide primer corresponding to nucleotides 155–132 of the cDNA (data not shown). These results were confirmed by S1 nuclease mapping using a 32P-labeled single-stranded DNA probe synthesized on an HLH462 cloned genomic fragment starting with the above-described primer (data not shown). We conclude from these experiments that HLH462 mRNA is 955 nucleotides in length exclusive of the poly(A)tail, and its predominant 5′ end maps on the genomic sequence as shown in Fig. 1.

Also shown in Fig. 1 is the mouse genomic sequence extending to 400 nucleotides upstream of the transcription start site. There is a TATA sequence at −29, a possible Zif268 binding site at (12) at −88, two overlapping Sp1 sites (20) at −90 and −94 that also overlap the Zif268 site, a possible SRE (21, 22) at −351, and a possible CRE (23) at −375. To determine whether the SRE-like element and the Zif268-like site bind their respective proteins, synthetic oligonucleotides corresponding to these sequences were tested for their ability to inhibit the binding of SRF to the c-fos SRE (21, 22) or to inhibit the binding of Zif268 to its cognate site (12). In the case of the SRE-like sequence, an oligonucleotide containing this sequence failed to compete with the c-fos SRE for binding to SRF; therefore it is not likely to be a functional SRE. However, a double-stranded oligonucleotide (GATCCCGGGCCGCTTGGATC) containing the putative Zif268 site upstream of the HLH462 gene did compete with a known site for binding by the Zif268 protein (data not shown). We conclude that, like a number of other immediate early genes, the HLH462 gene (Hilm62) has an upstream binding site for Zif268 and may be regulated by it.

The HLH462 Protein. The long open reading frame of HLH462 cDNA encodes a protein of 119 amino acids recognizable as a helix–loop–helix protein by comparison with other members of this family of transcription factors (Fig. 2). Between residues 40 and 81 HLH462 shows 69% identity with the helix–loop–helix sequence of Id (5) and clear relatedness to the helix–loop–helix sequences of MyoD (24), the Drosophila emc protein (4, 6), E47 (3), and c-Myc (25). Like Id and the Drosophila emc protein (4, 5), HLH462 lacks a basic region, which in other helix–loop–helix proteins is required for specific binding to DNA (26, 27). Outside the protein regions shown in Fig. 2 there is only limited similarity between the amino acid sequence of HLH462 and those of the other proteins.

To determine whether the HLH462 protein interacts with other helix–loop–helix proteins as predicted from its amino acid sequence, we determined the effect of HLH462 synthesized in vitro on the DNA-binding activity of a mixture of MyoD and E12 (Fig. 3). HLH462 inhibited the binding of MyoD/E12 to an oligonucleotide containing the MCK enhancer sequence, but it had no effect on the binding of an unrelated protein (Zif268) to its binding site (12). We infer that HLH462, like Id (4), can dimerize with other helix–loop–helix proteins and inhibit their DNA-binding activity.

Induction of HLH462 mRNA in 3T3 Cells. The HLH462 gene is rapidly and transiently activated in BALB/c 3T3 cells by serum or PDGF, resulting in an increased mRNA level (ref. 7 and Fig. 4). As is the case with other immediate early mRNAs, HLH462 mRNA is superinduced by growth factor in the presence of inhibitors of protein synthesis, and its mRNA has a short half-life (7). Other agents also produce a rise in HLH462 mRNA in 3T3 cells (Fig. 4), including ...
Fig. 4. Northern blot analysis showing the response of HLH462 mRNA in BALB/c 3T3 cells to various signaling agents. Total RNA was prepared at various times after stimulation with 20% fetal bovine serum (FBS), PDGF at 5 ng/ml (BB homodimer, Collaborative Research), 30 mM phorbol 12-myristate 13-acetate (PMA; Sigma) 10 μM forskolin (Sigma), 1 μM calcium ionophore A23187 (Sigma), or 60 mM KCl plus 10 mM CaCl₂. Following electrophoresis of 10 μg of total RNA and blotting onto nitrocellulose, filters were probed with full-length HLH462 cDNA. Lanes 1, RNA from unstimulated cells; lanes 2, RNA from cells stimulated for 0.5 hr.; lanes 3, 1 hr; lanes 4, 2 hr (FBS, PDGF) or 3 hr (PMA, forskolin, A23187, KCl + CaCl₂); lanes 5, 6 hr. Only the relevant region of each autoradiogram is shown. All lanes had approximately equal amounts of ribosomal RNA, as determined by staining with ethidium bromide.

particularly phorbol 12-myristate 13-acetate and forskolin. Depolarization concentrations of KCl, or calcium ionophore, produced less marked increases in HLH462 mRNA levels. [The change in mobility of HLH462 RNA with time seen in Fig. 4 is presumably due to sequential shortening of the poly(A) tail.] That HLH462 mRNA is induced by diverse signaling agents suggests that the gene can be activated by a variety of signal-transduction pathways.

HLH462 mRNA in Mouse Tissues. To determine how widely expressed the HLH462 gene is, we examined a number of mouse tissues for the presence of HLH462 mRNA by Northern blotting (Fig. 5). The mRNA is most abundant in lung, intestine, brain, and thymus and is undetectable in testis. Most striking is its presence in readily detectable amounts in RNA of whole mouse embryos from day 10 to 14 (Fig. 5), after which its level is much reduced. These results suggest that HLH462 may play a regulatory role in several tissues of the adult animal and during development.

Chromosomal Location of the HLH462 Gene. The mouse chromosomal location of the HLH462 gene was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J X M. spreitus)F₁ X C57BL/6J] mice (14). An ~9.5-kb M. spreitus-specific BamHI restriction fragment (see Materials and Methods) was used to follow the segregation of the HLH462 gene in backcross mice. The mapping results indicated that Hlh462 is located in the distal region of mouse chromosome 4, tightly linked to Fgr and Dsi-I (Fig. 6). The additional loci included in this analysis are Lmyc, Lck, and Anf. Although 116 mice were analyzed for every marker, up to 122 mice were scored for some markers. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are as follows: centromere–Lmyc–5/122–Lck–6/122–Fgr–1/122–Dsi-I–1/119–Hlh462–5/116–Anf. The recombinant frequencies (expressed as genetic distances in centimorgans ± the standard error) are Lmyc–(4.1 ± 1.8)–Lck–(4.9 ± 2.0)–Fgr–(0.8 ± 0.8)–Dsi-I–(0.8 ± 0.8)–Hlh462–(4.3 ± 1.9)–Anf.

Fig. 5. HLH462 mRNA in mouse tissues and embryos. (Upper) Distribution in adult mouse tissues. Lanes: 3CH, RNA from serum-starved BALB/c 3T3 cells treated with 20% fetal bovine serum for 3 hr in the presence of cycloheximide; B, brain; LU, lung; TH, thymus; H, heart; TE, testis; K, kidney; T, intestine; M, muscle; S, spleen; LI, liver. (Lower) HLH462 mRNA from whole mouse embryos. Lanes: 3CH, as above; 10–18, RNA from embryos at days 10–18 of gestation. 3CH samples contained 0.1 μg of RNA; all others, 15 μg of RNA. Only the relevant part of each autoradiogram is shown.

Fig. 6. Position of the Hlh462 locus on mouse chromosome 4. (Upper) Segregation patterns of Hlh462 and flanking markers in 116 backcross animals that were typed in common for Hlh462. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J X M. spreitus)F₁ parent. Black boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of an M. spreitus allele. The number of offspring inheriting each type of chromosome is listed below each column. Transmission ratios distortion, the inheritance of more M. spreitus alleles than expected, has been reported previously for the distal region of chromosome 4 (16). (Lower) Partial chromosome 4 linkage map showing the location of Hlh462 in relation to linked genes. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of these loci (except Dsi-I and Hlh462) on human chromosome 1 are shown to the right.
Comparison of the interspecific backcross map with the composite intraspecific backcross map (16) indicates that Hlh462 is located in a region of chromosome 4 in which several mutations have been localized. It will be interesting to determine whether any of these mutant animals show changes in the structure or expression of the HLH462 gene. We note also that the segment of mouse chromosome 4 to which Hlh462 maps is homologous with the p36 region of human chromosome 1 (Fig. 6). Since translocation breakpoints associated with human neoplasia have been assigned to 1p36 (28), it will be important to determine if the human HLH462 gene maps to this locus and whether its expression is perturbed in neoplastic cells containing a 1p36 breakpoint.

CONCLUSION

HLH462 resembles Id and the Drosophila emc protein in its helix–loop–helix domain and in its lack of a DNA-binding basic region. Like Id, it interacts with other HLH proteins and inhibits their DNA-binding activities. Therefore HLH462 would be expected to inhibit the transcriptional effects of helix–loop–helix transcription factors with which it can form heterodimers. In the context of the genetic program induced in fibroblasts by growth factors, HLH462 may dimerize with other helix–loop–helix proteins and thereby limit their activities. In view of the recent observations that expression of MyoD cDNA in cultured cells suppresses DNA synthesis induced by serum (29) and the proliferation of both normal and transformed cells (30), a specific hypothesis to be tested is that HLH462 antagonizes the actions of an endogenous growth-suppressing helix–loop–helix transcription factor present in quiescent cells. Similarly the observation that HLH462 mRNA is abundant in the early stages of embryonic development of the mouse and sharply decreases thereafter raises the possibility that HLH462 suppresses certain developmental processes until the later stages of fetal maturation. Insight into the role of HLH462 in cell proliferation and development may come from identifying proteins in quiescent cells and in relevant tissues of the early fetus that interact with HLH462.

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