β-Enolase Is a Marker of Human Myoblast Heterogeneity prior to Differentiation

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In this report, we define a muscle-specific marker, β-enolase, that distinguishes proliferating myoblasts from different stages of development. Enolase exists as multiple isoforms and in the course of cardiac and skeletal muscle development the β isoform progressively replaces the α isoform. In skeletal muscle, this change in gene expression, unlike most developmental changes in myogenic gene expression, is evident in undifferentiated myoblasts. Whereas myoblasts from fetal tissues express α-enolase mRNA, β-enolase is the predominant mRNA expressed by myoblasts from postnatal tissues. Our results are consistent with the idea that distinct precursor myoblasts contribute to the diversity of fiber types characteristic of muscle tissue at different stages of development.

INTRODUCTION

During the development of vertebrate muscle, diverse fiber types arise in specific temporal and spatial patterns (Condon et al., 1990). This diversity in fiber type could arise at the level of the differentiated myofiber or from fusion of myoblasts that differ intrinsically. Heterogeneity among myoblasts at different developmental stages is suggested by differences in morphology and sensitivity to growth factors, acetylcholine, and phorbol esters (White et al., 1975; Cossu et al., 1987, 1988). In addition, myoblasts induced to differentiate in vitro can express different isoforms of myosin heavy chain and myosin light chain (Mouly et al., 1987; Feldman and Stockdale, 1991). However, molecular markers that distinguish dividing myoblast populations prior to differentiation have been lacking.

In this report we demonstrate that β-enolase, a muscle-specific enzyme, is a molecular marker of myoblast heterogeneity that accompanies development. Moreover, β-enolase expression in myoblasts appears independent of the helix-loop-helix (HLH) family of myogenic regulators (MyoD, myogenin, myf-5, MRF4). These results suggest that as yet unidentified muscle-specific regulators play a key role in myogenesis.

MATERIALS AND METHODS

Isolation and Culture of Human Myoblasts

Muscle tissues were dissociated and myoblasts separated from nonmyogenic cells using the 5.1H11 antibody to neural cell adhesion molecule (N-CAM) and the fluorescence-activated cell sorter (FACS, Becton-Dickinson), as described previously (Webster et al., 1988). Human muscle samples were from the vastus lateralis of fetuses (Weeks 11 and 22 of gestation) obtained subsequent to therapeutic abortion, a neonate (premature birth at Week 31 of gestation) obtained at autopsy, and an adult (5 years postnatal) following corrective orthopedic surgery. Myoblasts from the 14-week fetus and from the neonate were cloned prior to analysis. All samples were procured in accordance with guidelines set by the Stanford Human Subjects Committee and were from individuals with no known myopathies or other abnormalities affecting muscle development.

For growth, human myoblasts were plated in a growth factor-rich medium (GM) consisting of Ham’s nutrient mixture F-10 with 15% fetal bovine serum (Hyclone), 0.5% chick embryo extract (GIBCO), and 1% penicillin/streptomycin (GIBCO) on dishes that had been precoated with a solution of type I collagen (0.15% calf skin collagen, Sigma). For differentiation, confluent myoblast cultures were exposed to a differentiation medium (DM) consisting of Dulbecco’s modified Eagle’s medium (DME) with 5% horse serum (Hyclone), 10−6M bovine insulin, and 10−6M dexamethasone (Sigma).

Mutagenesis of Mouse C2C12 Cells and Selection of the NQO-1 Cell Line

The mouse muscle cell line C2C12 (Rian et al., 1983) and the NQO-1 mutant cell line derived from it were cultured as follows. For growth, myoblasts were plated in modified GM: DME with 15% defined/supplemented bovine calf serum (Hyclone) and 5% fetal bovine serum.
For differentiation, myoblasts were exposed to DM containing DME with 2% horse serum.

For mutagenesis, C2C12 cells were treated with 0.1 μg/ml of 4-nitroquinoline oxide (Sigma) in a phosphate-buffered saline solution (PBS) for 1 hr at 37°C. Mutagenized cells were plated at a density of 1 x 10⁶ cells per 100-mm culture dish and differentiation-defective variants were selected on the basis of colony size and morphology after 10 days in DM. The NQO-1 mutant clone was large, containing cells that readily grew in DM, but did not fuse to form myotubes. The NQO-1 phenotype has proved stable for more than 20 passages by morphological and biochemical criteria.

**Rat Tissue RNA Isolation and Northern Analysis**

Muscle tissues were dissected from Wistar rats and RNA was isolated from the tissues and from cultured cells by the method of Chirgwin et al. (1979) for Northern analysis as described previously (Peterson et al., 1990). Filters were washed under high stringency in 0.1 x SSC, 1.0% SDS 2 x 30 min each at 65°C. Low stringency washes were 0.5 x SSC, 1.0% SDS 30 min at 42°C.

Probes were prepared by random primer synthesis (Feinberg and Vogelstein, 1983) using an Amersham kit. The enolase probe was derived from pCP2A, a cDNA clone isolated from a C2C12 cDNA expression library. Sequence data for this cDNA clone are available from EMBL/GenBank/DDBJ under Accession Number X62667. A 1.6-kb BamHI fragment from pCP2A hybridized only to β-enolase transcripts under high stringency wash conditions. Under low stringency conditions, this probe hybridized to both α- and β-enolase transcripts. The cardiac actin-specific probe was a 130-bp BamHI fragment from pBSa, (Sassoon et al, 1988), while the coding region probe recognizing all actin transcripts was a 500-bp EcoRl fragment originally derived from pHMaA-PX as described (Peterson et al., 1990). A 4.6-kb XbaI/SalI fragment from human hereulin/MRF4 (Miner and Wold, 1990) was also used. All other probes have been described previously (Peterson et al., 1990).

**RESULTS**

To examine the tissue distribution and developmental pattern of expression of β-enolase mRNA, Northern analysis of RNA isolated from rat cardiac and skeletal muscle tissues was performed (Fig. 1). Hybridization of a 1.6-kb β-enolase cDNA under low stringency allowed detection of both muscle-specific β-enolase mRNA and the slightly larger, ubiquitous α-enolase mRNA. A progressive replacement of α- by β-enolase mRNA was characteristic of cardiac muscle development. The switch from α- to β-enolase expression occurred earlier during skeletal than cardiac muscle development. In the adult, β-enolase mRNA preferentially accumulated in fast twitch skeletal muscle such as the EDL relative to slow twitch skeletal muscle.

We determined whether the β-enolase expression observed in skeletal muscle tissues at different developmental stages reflected differences among the undifferentiated myoblasts derived from those stages. It had been shown previously that β-enolase mRNA was expressed in myoblasts from an established muscle cell line (Lamandé et al., 1989). We isolated myoblasts from human muscle tissues by the method of Webster et al. (1988) which has been shown to provide an enrichment of myoblasts to greater than 99% of the cell population. Northern blot analysis under low stringency demonstrated that myoblasts isolated from embryonic muscle expressed predominantly α-enolase mRNA, myoblasts from neonatal muscle expressed both α- and β-enolase mRNA, and myoblasts from adult muscle expressed predominantly β-enolase mRNA (Fig. 2). These results demonstrate that β-enolase mRNA is a predifferentiation marker that is developmentally regulated.

For these studies, it was critical to establish that the myoblasts analyzed at each developmental stage were of comparable purity and state of differentiation. To test directly the myogenic nature of the cells, the accumulation of MyoD mRNA was monitored (Fig. 2). In each undifferentiated myoblast sample, high levels of MyoD mRNA accumulated. Although myotubes were not ap-

**FIG. 1.** A progression from α- to β-enolase mRNA expression accompanies both cardiac and skeletal muscle development. Total RNA (10 μg) from rat cardiac and skeletal muscle tissues derived from different stages of development were analyzed by Northern blot. Both embryonic (Emb, Day 18 of gestation) and neonatal (Neo, 1 week after birth) skeletal muscle RNA samples were from whole legs, whereas adult (Ad, 2 months after birth) samples were from the soleus (Sol) and extensor digitorum longus (EDL). Two different exposures of the same autoradiogram are shown (48 hr and 1 week). Hybridization to the enolase probe was performed under low stringency to detect both α- and β-enolase mRNAs. The bottom panel shows the 18s ribosomal RNA bands visualized by ethidium bromide staining to indicate the amount of RNA in each lane.
El4 E22 Neo Ad

DM El4

tiated myotubes in DM and did not express mRNAs encoding muscle-specific gene products (Fig. 3a). Moreover, MyoD, myogenin, myf-5, and MRF4 mRNAs, although expressed in parental C2C12 myoblasts, were undetectable in NQO-1 cells (Fig. 3b). Despite the absence of expression of the entire family of HLH regulators in NQO-1 cells, wild-type levels of $\beta$-enolase mRNAs accumulated.

Fig. 2. $\beta$-enolase mRNA expression is detectable in proliferating myoblasts from postnatal but not from embryonic muscle. Human myoblasts were isolated from muscle tissues at four developmental stages: E14 (Week 14 gestation), E22 (Week 22 gestation), Neo (premature birth, Week 34 gestation), and Ad (5 years postbirth). At each stage, total RNA (20 $\mu$g) from proliferating myoblasts was analyzed by Northern blot with the indicated probes. Total RNA (20 $\mu$g) from E14 myoblasts allowed to differentiate for 5 days (MyoD and enolase panels) and from adult myoblasts after 1 day in DM (actin panel) were included. The blot was hybridized under low stringency with the enolase probe to detect both $\alpha$- and $\beta$-enolase mRNAs and under high stringency with MyoD and actin probes. c, cytoskeletal actin mRNAs; s, sarcomeric actin mRNAs.

Following differentiation in culture, $\beta$-enolase mRNA accumulation in myotubes derived from Embryonic Week 14 myoblasts was similar to that of myotubes from all stages (Fig. 2, right, and data not shown). In addition, the levels of MyoD mRNA declined in all samples upon exposure to DM. Thus, $\beta$-enolase mRNA expression differs among proliferating myoblasts from different stages, but this difference disappears following fusion and differentiation into postmitotic myotubes.

Since $\beta$-enolase was expressed in proliferating myoblasts, it seemed likely that expression was independent of the activity of the HLH family of muscle regulators. To test this possibility directly, we analyzed the expression of $\beta$-enolase mRNA in the myogenic cell line C2C12, originally derived from adult murine muscle, and in a mutant cell line, NQO-1, derived from it by chemical mutagenesis. NQO-1 cells did not fuse to form differen-
tiated myotubes in DM and did not express mRNAs encoding muscle-specific gene products (Fig. 3a). Moreover, MyoD, myogenin, myf-5, and MRF4 mRNAs, although expressed in parental C2C12 myoblasts, were undetectable in NQO-1 cells (Fig. 3b). Despite the absence of expression of the entire family of HLH regulators in NQO-1 cells, wild-type levels of $\beta$-enolase mRNAs accumulated.

DISCUSSION

The present study provides evidence that $\beta$-enolase is a muscle-specific gene expressed in undifferentiated myoblasts. $\beta$-enolase mRNA accumulation was unchanged in mutant C2C12 myoblasts in which the expression of the HLH family of myogenic regulators had been abolished, consistent with data from several groups that the HLH myogenic regulators are inactive as transcription factors for muscle-specific genes in proliferating myoblasts (Mueller and Wold, 1989; Brennan et al., 1991).

Although $\beta$-enolase mRNA is a molecular marker of undifferentiated myoblasts, it is not uniformly ex-
pressed in all myoblast populations. We have shown that β-enolase is expressed in growing myoblasts derived from postnatal tissues, but absent from embryonic myoblasts. Thus, this change in myoblast gene expression may represent the emergence of adult satellite myoblasts. Currently, it is unclear whether embryonic myoblasts are the direct progenitors of adult myoblasts that arise at a precise time either in response to an endogenous clock or to an exogenous cue. Alternatively, embryonic and adult myoblasts could derive from discrete populations of myoblasts that migrate into the limb at different times during development, or migrate together but initiate proliferation at different times in response to different signals. The use of retroviral vectors to mark muscle cells and monitor their fate in vivo (Hughes and Blau, 1990) should make it possible to distinguish among these possibilities and establish the etiology of embryonic and adult myoblast types and their contribution to muscle fiber diversity.

The β-enolase gene also appears to be differentially regulated in myotubes. High level accumulation of β-enolase transcripts in adult fast twitch muscle relative to slow twitch muscle suggests that the contractile activity of the fibers influences β-enolase expression. Although we have shown that myoblasts from all stages tested accumulate comparable levels of β-enolase transcripts following differentiation in culture, Barbieri et al. (1990) have reported that cultured myotubes derived from mouse embryos at earlier developmental stages than studied here do not accumulate significant levels of β-enolase mRNA, suggesting additional control during development. A molecular dissection of the β-enolase gene should provide insight into the cis-regulatory DNA sequences and trans-acting factors that mediate the complex pattern of tissue- and stage-specific expression.

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