Transition of Homer isoforms during skeletal muscle regeneration

Elena Bortoloso,1,2 Nadia Pilati,1 Aram Megighian,3 Elisa Tibaldo,1 Dorianna Sandonà,1 and Pompeo Volpe1,2

1Dipartimento di Scienze Biomediche Sperimentali dell’Università di Padova, 2Istituto Interuniversitario di
Miomologia, and 3Dipartimento di Anatomia e Fisiologia Umana dell’Università di Padova, Padova, Italy

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Bortoloso, Elena, Nadia Pilati, Aram Megighian, Elisa Tibaldo, Dorianna Sandonà, and Pompeo Volpe. Transition of Homer isoforms during skeletal muscle regeneration. Am J Physiol Cell Physiol 290: C711–C718, 2006.—Homer represents a new and diversified family of proteins that includes several isoforms, Homer 1a, 1b/c/d, 2b, and 3; some of these isoforms have been reported to be present in striated muscles. In this study, the presence of Homer isoforms 1a, 1b/c/d, 2b, and 3 was thoroughly investigated in rat skeletal muscles under resting conditions. Transition in Homer isoforms composition was studied under experimental conditions of short-term and long-term adaptation, e.g., fatigue and regeneration, respectively. First, we show that Homer 1a was constitutively expressed and was transiently upregulated during regeneration. In C2C12 cell cultures, Homer 1a was also upregulated during formation of myotubes. No change of Homer 1a was observed in fatigue. Second, Homer 1b/c/d and Homer 2b were positively and linearly related to muscle mass change during regeneration, and third, Homer 3 was not detectable under resting conditions but was transiently expressed during regeneration although with a temporal pattern distinct from that of Homer 1a. Thus a switch in Homer isoforms is associated to muscle differentiation and regeneration. Homers may play a role not only in signal transduction of skeletal muscle, in particular regulation of Ca2+ release from sarcoplasmic reticulum (Ward CW, Feng W, Tu J, Pessah IN, Worley PF, and Schneider MF. Homer protein increases activation of Ca2+ sparks in permeabilized skeletal muscle. J Biol Chem 279: 5781–5787, 2004), but also in adaptation.

fatigue; immediate early gene; muscle adaptation; myogenesis

A novel activity-dependent control of association of multimeric receptor complexes has been shown in neuronal synapses for the metabotropic glutamate receptors (mGluR), and appears to be mediated by a family of proteins termed Homers (4, 35, 39). Homer 1a is considered an immediate-early gene (IEG) product (4), whereas Homers 1b/c/d, 2a/b, and 3a/b are constitutively expressed and self multimerize, through the coiled-coil (CC) domain, and cross-link mGluR (39). CC-Homers appear to couple mGluR to inositol trisphosphate receptor (IP3R), one of the endoplasmic reticulum Ca2+ release channels, whereas IEG Homer 1a disrupts the complex and seems to act as a natural dominant negative competitor with CC-Homers (35). Although doubts have been cast on the univocal role of Homer 1a (see Ref. 34), Homers can be regarded as regulators of multimeric complexes involved in signal transduction (4, 35, 39). Studies (34) in nonneuronal and neuronal cultured cells have revealed that Homer may regulate the expression and function of mGluRs at five steps: 1) targeting, 2) surface expression, 3) clustering, 4) physical linkage to other synaptic complexes, and 5) modulation of activity.

Complementary data seem to suggest a role of Homers in signal transduction of skeletal muscle, and particularly in the regulation of Ca2+ release from SR. Several proteins in the 40- to 47-kDa range referable to CC-Homer have been detected in muscle detergent extracts, as judged by Western blot analysis (31, 33, 39). The data (33) reported the constitutive expression of mRNAs for Homer 1, 2, and 3 in murine skeletal muscles; the complete open reading frame and full-length cDNA clones of both Homer 1a and Homer 1c were obtained from rat skeletal muscle (30). Homer 1 isoforms have been localized by immunofluorescence at the Z band of skeletal muscle fibers (29, 36). Moreover, Homers were bound to bind to a proline-rich motif of IP3R (35), which is also present in ryanodine receptor (RyR), the predominant Ca2+ release channel of striated muscles (11, 32), and, on the basis of immune precipitation and glutathione S-transferase pull-down experiments, a direct association of Homer 1b/c and RyR1 was postulated (10, 29, 37). Finally, Homer 1 isoforms have been reported to influence in vitro the gating kinetics of RyR1 (although with somewhat conflicting results; see Refs. 10, 15, 37).

In skeletal muscle, the functional role of Homer would be to regulate multimeric complexes involved in signal transduction (33), to act as adapter for RyR (10, 15, 37) and to bind partner for IP3R (29). In particular, the catalytic activity of recombinant Homer 1 isoforms (Homer 1a and Homer 1c) on RyR may provide one of the mechanisms linking Ca2+ signals with dynamic regulation of Homer-mediated protein clustering (10).

The regulatory equilibrium between the constitutive Homers and the ex novo synthesis of their natural dominant competitor IEG Homer could be particularly relevant not only in skeletal muscle resting conditions, but also during pathophysiological conditions, e.g., muscle adaptation, fatigue, regeneration, or hypoxia. It is worth noting that low-voltage electrical stimulation (20) or low-frequency fatigue (9) of skeletal muscle, and experimental models of ischemia (3), all appear to be associated with induction of several IEGs, such as c-fos, c-jun, egr-1, and HSPs. Furthermore, adaptive muscle changes entailing structural rearrangement and morphological remodeling, e.g., development, short-term plasticity, hypertrophy, and regeneration, processes coupled to upregulation of several, distinct IEGs (13, 20), might include a switch in Homer isoform composition. Sarcoplasmic reticulum (SR) biogenesis and triad formation take place in an orderly fashion and are dependent on regulated expression of marker proteins and their selective intracellular targeting (26). A significant temporal relationship between Homer transition and skeletal muscle differentiation might be intriguing. It must be mentioned that a differential Homer expression has been already reported during brain development (31).
In the present study, we undertook a first attempt to determine and characterize stimuli that induce Homer 1a transcription in mammalian striated muscles, the long goal being to unravel mechanisms and intracellular pathways involved in short- and long-term adaptation.

**MATERIALS AND METHODS**

**Tissue sources.** Experiments were carried out on adult male Wistar rats (~250 g body wt). For marcare (bupivacaine) injections, fatigue experiments, or transfection experiments, rats were anesthetized with ketamine (1.5 mg/100 g) and euthanized by cervical dislocation. Experimental protocols have been approved by University of Padua Review Boards. Soleus (SOL) a representative slow-twitch skeletal muscle, and extensor digitorum longus (EDL), a representative fast-twitch skeletal muscle, were used.

**Antibodies.** The following primary antibodies were used for Western blot analysis: polyclonal antibodies specific for either Homer 1b/c, 1a, 2a/b, or 3 (Santa Cruz Biotechnology), monoclonal antibodies for myosin heavy chain (MHC) (MF20; Developmental Studies Hybridoma Bank), and monoclonal antibodies for α-sarcomorphic actin (Sigma). Rabbit anti-goat IgG and goat anti-mouse IgG (both from Sigma) conjugated to alkaline phosphatase were used as secondary antibodies.

**cDNA cloning of Homer 1a and cloning of HA-tagged Homer 1a cDNAs.** The full-length cDNA referable to Homer 1a (612 bp) was cloned as previously described (30). The nucleotides coding for the nine amino-acid of hemagglutinin (HA) tag were added by PCR at the S’ end; EcoRI-HindIII inserts were cloned into pcDNA3.1 vectors for expression in eukaryotic cells (36).

**Fatigue.** A longitudinal opening through skin and fascia was made close to the right knee, and the common peroneal nerve was exposed. The ends of two flexible, multistranded steel wires (model AS 632, Cooner Wire, Chatsworth, CA) with their Teflon insulation removed for the distal 20–25 mm were fixed by sutures on each side of the common peroneal nerve at the level of the knee. The electrodes did not touch the nerve (38). After the electrodes were placed, the opening was sutured, and the proximal ends of insulated wires, exiting through the suture, were connected to a stimulator (Grass S44) through a stimulus isolation unit (Grass). The efficacy of nerve stimulation was checked with the use of supramaximal single stimuli (0.1-ms duration), which usually elicited a strong foot dorsiflexion due to EDL activation. Muscles were then submitted to a fatigue protocol, consisting in a train of short tetani at low frequency (40 Hz, 300-ms duration, 0.3 s⁻¹) for either 30 or 60 min (12), which reduced force development to ~15–20% of the initial value. At the end of the fatigue protocol, both fatigued (right) and control, not fatigued (left) EDL were quickly dissected out, weighted, and frozen in liquid nitrogen.

**Bupivacaine-induced necrosis and regeneration of adult rat skeletal muscle.** The right soleus muscles were exposed and injected with 0.5 ml of 0.5% bupivacaine, as described previously (22). At different time points (between 0.25 and 21 days postinjection), the muscles were quickly removed and frozen in liquid nitrogen for biochemical analysis.

**Electroporation and transfection of soleus muscles.** Soleus muscles were exposed and injected with 60 µl of a saline solution containing 50 µg of either Homer 1a or Homer 1a-HA cDNAs. Electroporation was carried out with a BTX ECM 830 square-wave pulse generator using the protocol of Mathiesen (19), with slight modifications (21); pulse stimulation by field electrodes (220 V/linear cm) was applied for six 20-ms pulses at 200-ms intervals. The rats were euthanized 3 days after surgery; the muscles were quickly removed and frozen in liquid nitrogen for biochemical analysis.

**Cell cultures.** C2C12 myoblasts (MB) were grown in DMEM high glucose (Sigma) supplemented with 10% fetal calf serum (Sigma) (proliferation medium). Cells were seeded on 100-mm-diameter petri dishes. When confluence was reached (day 0), C2C12 myoblasts were induced to differentiate and fuse into multinucleated myotubes once the proliferation medium was changed to DMEM with 2% horse serum (Sigma; differentiation medium).

**Homogenates from C2C12 myotubes, from soleus and EDL skeletal muscles, and from cerebrum.** C2C12 MB and myotubes were cultured as described above, harvested in PBS, and lysed with the β-Gal lysis buffer (β-Gal ELISA Kit, Roche) for 30 min at room temperature. After centrifugation at 18,000 for 15 min, the protein concentration of supernatants was determined by the Bradford assay (Bio-Rad). Supernatants were kept at ~20°C until use.

**Homogenates of rat skeletal muscles and cerebrum were obtained as follows:** frozen tissues were triturated in a mortar, homogenated in 10 volumes of 3% SDS, 1 mM EGTA, boiled for 5 min, and centrifuged at 18,000 for 15 min to remove debris. Homogenates were kept at ~20°C until use. Protein concentration was determined according to Lowry et al. (18).

**SDS-PAGE, Western blot analysis, and quantitative densitometry.** SDS-PAGE on 12.5% gels, transfer to nitrocellulose, and Western blotting were carried out essentially as previously described (22).

**Homers and myofibrillar proteins were quantified by densitometry of immunoblots of homogenates from fatigued EDL, bupivacaine-treated soleus, transfected soleus, and corresponding controlateral muscle, as well as from C2C12 MB and myotubes.** Densitometric analysis was performed with Image for Windows software (version Beta 4.0.2; Scion, Frederick, MD; www.scioncorp.com). Each value for the Homer isoforms, MHC, or α-actin in regenerating muscles is given as ratio to the corresponding controlateral sample, with the exception of Homer 3; in the latter case, absolute optical density (OD) values are provided because Homer 3 was undetectable in control muscles. Because morphological and histochemical recovery is attained at 3–4 wk after bupivacaine injection (see Ref. 5 for a review) and because Homers and myofibrillar proteins had returned to control levels by postinjection day 21, we assessed the time course change by testing the differences between each time point and the value at postinjection day 21 (see also Statistical analysis).

In C2C12 MB/myotubes, the value for Homer 1b/c/d, 1a, and 3 is given relative to that obtained just before the induction of differentiation (day 0 value). Changes in Homer expression were assessed by comparing each experimental value (between day 1 and day 8) to that obtained in proliferating myoblasts (see also Statistical analysis).

**RNase protection assay.** Antisense RNA probes were produced using T7 polymerase (Roche Molecular Biochemicals) on linearized Homer 1a and Homer 1c in the presence of biotinylated ribonucleotide. RNasePAs were carried out using the RPA III kit (Ambion) hybridizing 10–50 µg of total RNA from each tissue to 600 pg of biotinylated RNA probe. After Rnase digestion, the protected RNA probe was run on 5% polyacrylamide/8 M urea denaturing gels and then electrotransferred on positively charged nylon membranes (Amersham). The biotinylated RNA probes were detected by using the BrightStar BioDetect kit (Ambion). Undigested RNA probes were also run on the same gel as size markers. The Homer 1a probe was made up of 247 nucleotides and the expected size of the Homer 1a protected fragment is 179 nucleotides; the same probe is also able to protect a Homer 1b/c fragment of 146 bases. Quantitation was carried out by densitometric analysis performed with an imaging densitometer (model G5-670, Bio-Rad). The OD value of Homer 1a fragment was normalized to that of Homer 1b/c. The time course of changes of Homer transcripts was determined by comparison of each value to that obtained from control muscles (see Statistical analysis).

**Statistical analysis.** One-way ANOVA was used to evaluate the changes over time for both relevant proteins and mRNA. All values are means ± SE. Post hoc comparison were performed with Fisher’s protected least-significant difference test. Dunnett’s test was applied to compare each time point value with either the postinjection day 21 value in the case of soleus regeneration, or the MB value in C2C12 differentiation, or the control value in RNasePA. Correlations be-

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twitch and slow-twitch skeletal muscles, as well as homoge-
Homer 1a, was further tested using homogenates from fast-
apparent constitutive expression of Homer 1a, deemed as an
the rat, both mRNA (30) and protein analysis revealed the
mass of Homer 1b/c and Homer 1a, respectively (4, 17).
recovery, because mRNA coding for Homer 1d (an additional
alternative splicing variant of Homer 1) is specifically ex-
ersive between long forms of Homer 1; how-
molecular weight of the immunostained proteins (not shown).
RNasePA analysis (not shown) indicated that there was no
difference between fatigued and controlateral, control muscles,
with respect to both Homer 1b/c and Homer 1a.
Western blot analysis (Fig. 1) was carried out with antibod-
ies specific for either Homer 1b/c or Homer 1a. The apparent
oral weight of the immunostained proteins (~47 and
~27 kDa, respectively) was consistent with the calculated
mass of Homer 1b/c and Homer 1a, respectively (4, 17).
Antibodies for Homer 1b/c yielded single, unique bands, being
unable to discriminate between long forms of Homer 1; how-
ever, because mRNA coding for Homer 1d (an additional
alternative splicing variant of Homer 1) is specifically ex-
pressed in skeletal muscle (28), we cannot conclude that the
~47-kDa-labeled polypeptide might also include Homer 1d.
Therefore, we will refer to the immunoreactive Homer 1 long
forms as Homer 1b/c/d hereafter. Western blot analysis
showed that, relative to controlateral, control muscles,
Homer 1a and Homer 1b/c/d were virtually unchanged in
fatigued EDL after tetani applied for either 60 min (Fig. 1)
or 30 min (not shown). Average densitometric data, derived
from four distinct experiments for each group, were not
significantly different (see Fig. 1).
Expression of Homer 1a in both fast-twitch and slow-twitch
skeletal muscles under resting conditions. In EDL muscles of
the rat, both mRNA (30) and protein analysis revealed the
apparent constitutive expression of Homer 1a, deemed as an
IEG product (4). The specificity of the probe, antibodies for
Homer 1a, was further tested using homogenates from fast-
twitch and slow-twitch skeletal muscles, as well as homoge-

RESULTS
Because the transition in Homer isoform composition is
thought to occur during adaptation of skeletal muscle, two
well-established and distinct in vivo experimental systems,
fatigue and muscle regeneration, were investigated.
Skeletal muscle fatigue: expression of Homer 1 isoforms in
EDL, a fast-twitch skeletal muscle of the rat. Transcription and
transduction of Homer 1 isoforms in skeletal muscle might be
influenced by fatigue: thus we measured the content of Homer
1a and of long Homer 1 isoforms, both at mRNA and protein
levels, in EDL fatigued by repeated tetani applied for either 30
or 60 min, as detailed in MATERIALS AND METHODS.
A linear regression analysis. Differences were considered statistically
significant at the 0.05 level of confidence.

Anti-Homer 1a antibodies clearly recognized Homer 1a in
rat brain homogenates (Fig. 2, lane 7), chosen as positive
controls because the short Homer 1 isoform has been already
detected by Western blot analysis in brain lysates (31) and in
cultured cortical neurones (1); comigrating immunostained
bands, detected by the same antibodies in all skeletal muscle
samples (Fig. 2; lanes 2–6 and 8), were thus referable to Homer
1a. It is worth noting that although the amounts of Homer 1a in
brain and muscle samples were comparable, slow-twitch mus-
cles showed higher levels of Homer 1a than fast-twitch mus-
cles (cf. lanes 3, 4, 6, and 8).

Soleus muscles of the rat were transiently transfected by
electroporation with cDNAs coding for either recombinant
Homer 1a or epitope-tagged (HA)-Homer 1a: the very same
~27-kDa protein band was recognized by both anti-Homer 1a
and anti-HA antibodies in homogenates from Homer 1a-HA
transfected soleus (Fig. 2, lane 2). Notably, in the nontrans-
sected, controlateral soleus, a slightly less intense band was
evidenced by anti-Homer 1a antibodies (Fig. 2, lane 3),
whereas no labeling was observed after incubation with anti-
HA antibodies (Fig. 2, lane 1).

Finally, the comparison between homogenates of Homer
1a-transfected soleus and of controlateral muscle (Fig. 2, lanes
5 and 4) showed that the immunoreactive ~27-kDa band is
increased, as expected, in transgenic muscles. Taken together,
these data demonstrated that anti-Homer 1a antibodies specif-
cally recognize the “short” Homer 1 isoform and that Homer
1a appears to be “constitutively” expressed in skeletal muscle.

Homer 1a and Homer 1b/c/d protein levels in regenerating
soleus skeletal muscle. We next asked whether there are
changes in either Homer 1a or Homer 1b/c/d associated to
skeletal muscle regeneration. The biphasic-induced muscle
degeneration/regeneration model was implemented in the so-
leus of adult rat and the content of Homer 1 isoforms was
monitored by Western blot analysis on muscle homogenates
obtained from 0.25 to 21 days postinjection.

Relative levels of both isoforms were different over time but
they displayed distinct expression patterns (Fig. 3). With re-
gard to Homer 1a, a nonsignificant decrease in protein level
was observed at 6-, 12-, and 24-h postinjection. Homer 1a
expression then significantly increased and attained levels that
were two- to threefold higher than those detected in control
muscles between 6 and 10 days (see Fig. 3, inset, lane 1 vs.
returned to control levels at day 21 postinjection. Homer 1a decreased at postinjection day 15 and are presented as ratios of bupivacaine-treated muscles over controlateral indicated on the x-axis. Data are obtained from densitometry of Western blots and are given as means ± SE (n = 4–6 for both Homer 1 isoforms). *P < 0.05, significantly different from postinjection day 21. Inset, Western blot of homogenates of SOL (300 μg) obtained from control (lanes 2 and 4) and from postinjection day 10 (lanes 1 and 3) decorated with antibodies for either Homer 1a (lanes 1 and 2) or Homer 1b/c (lanes 3 and 4).

Fig. 3. Changes of Homer 1a and Homer 1b/c/d in regenerating SOL of the rat. Homogenates were obtained from SOL muscles at specified time points as indicated on the x-axis. Data are obtained from densitometry of Western blots and are presented as ratios of bupivacaine-treated muscles over controlateral control samples for Homer 1a or Homer 1b/c/d, and are given as means ± SE (n = 4–6 for both Homer 1 isoforms). *P < 0.05, significantly different from postinjection day 21. Inset, Western blot of homogenates of SOL (300 μg) obtained from control (lanes 2 and 4) and from postinjection day 10 (lanes 1 and 3) decorated with antibodies for either Homer 1a (lanes 1 and 2) or Homer 1b/c (lanes 3 and 4).

Homer 1a decreased at postinjection day 15 and returned to control levels at ~21 days postinjection.

Relative levels of Homer 1b/c/d were, instead, always below control values, being ~14% at 6-h postinjection, and gradually increased until 21 days postinjection, when control values were fully attained. At postinjection day 10, Homer 1b/c/d levels were only slightly lower than in control (see Fig. 3, inset, lane 3 vs. lane 4).

Up to 3 days postinjection, necrosis is the prevalent phenomenon so that reduction of protein levels relative to control is plausible. Later and up to postinjection day 15, when regeneration is in full-blown, relative differences between Homer 1a and Homer 1b/c/d became conspicuous.

RNasePA in regenerating soleus skeletal muscle. Differences in protein content might be due to either increased transcription, reduced turnover of mRNA and protein, or both. Some clues to this effect were sought by RNase protection assay. Plot of Fig. 4 shows the Homer 1a-to-Homer 1b/c ratio as a function of postinjection time. Compared with control (0 on the x-axis), the ratio was significantly twofold higher up to 1 day postinjection, and virtually back to baseline by postinjection day 3. Because the Homer 1b/c/d protein content increased steadily and monotonically (cfr. Fig. 3), if we assume that the relative mRNA had increased accordingly, it would appear as though transcription for Homer 1a was upregulated after bupivacaine injection, i.e., when activation of satellite cells occurs.

Homer 2 and Homer 3 in regenerating soleus skeletal muscle. The present data suggest that Homer 1 isoforms are differentially expressed in regenerating soleus; thus we also monitored the expression pattern of other Homer family members (Homer 2 and Homer 3) during muscle regeneration.

Expression of Homer 2a/b in skeletal muscle is still controversial (31, 39), whereas it is undoubtedly detectable in cerebellum extracts (31, 39). In preliminary Western blot experiments, the reactivity to Homer 2a/b antibodies of homogenates from untreated soleus and cerebellum was investigated; both homogenates displayed a doublet (~42 and ~47 kDa), referable to the two long forms of Homer 2, 2a, and 2b, respectively (31), the latter being predominant in soleus (not shown). Soleus muscle homogenates from regenerating and controlateral samples were then analyzed by being immunoblotted with antibodies specific for Homer 2a/b. Homer 2b appeared to be the predominant isoform in regenerating and untreated soleus (Inset of Fig. 5A, lanes 1 and 2, respectively); Homer 2a was indeed only barely detectable and for this reason only the expression pattern of Homer 2b has been described. Relative levels of Homer 2b were reduced by ~80% up to 3 days postinjection, then gradually increased and returned to control values ~10 days postinjection (Fig. 5A) with a pattern formally similar to that of Homer 1b/c/d.

Homer 3 was not detectable in control soleus (Fig. 5B, inset, lane 3), whereas regenerating muscles strongly reacted to anti-Homer 3 antibodies and displayed a single immunoreactive band at ~47 kDa (Fig. 5B, inset, lane 4). Homer 3 content dramatically increased at postinjection day 3 and remained elevated until postinjection day 6, the increase being ~8-fold compared with postinjection day 21 (Fig. 5B). Expression likewise decreased steeply, i.e., very low at postinjection day 10 (Fig. 5B, inset, lane 5) and almost undetectable at postinjection day 21.

Variation of α-actin and MHC protein content in soleus during the degeneration/regeneration process. Initial changes in Homer proteins levels may be merely, or at least in part, because of muscle necrosis induced by bupivacaine. Evidence for the extent of muscle necrosis was gained by measuring the relative content of two structural muscle-specific proteins by immunoblot analysis (Fig. 6A). Both α-actin and MHC showed a dramatic reduction between 6 h and 3 days postinjection, then rapidly increased by postinjection day 6 and returned to control values between 15 and 21 days postinjection. These findings might be taken as evidence that the maximum decrease in muscle mass takes place before/on postinjection day 3, and that the increase of both Homer 1/b/c/d and Homer 2b is related to muscle mass increase later than postinjection day 3: in both cases, plots of MHC vs. Homer 1b/c/d (Fig. 6B) and of MHC vs. Homer 2b (Fig. 6C) and linear regression analysis show a positive relationship with R = 0.97 and R = 0.95, respectively. No significant relationship was found between Homer 1a and
MHC (R = 0.02, data not shown), i.e., the pattern of Homer 1a is unrelated to muscle mass increase. On the other hand, regression analysis applied to Homer 3 vs MHC (Fig. 6D) shows a negative relationship with $R = 0.92$, i.e., Homer 3 linearly decreased as muscles mass approached steady-state conditions at postinjection day 21.

**DISCUSSION**

We provide here definitive evidence for the constitutive expression of Homer 1a in skeletal muscle, and show, for the first time, that expression of Homer 1a is transiently upregulated during regeneration. Complementary experiments on C2C12 cell cultures show that Homer 1a is upregulated during formation of myotubes. Thus, Homer 1a might be involved in a variety of functions both in resting conditions and during long-term adaptation, at least that encompassing regeneration. On the other hand, no evidence of Homer 1a involvement is observed in fatigue, one experimental condition of short-term adaptation.

Moreover, we provide evidence for the transient and rapidly decaying expression of Homer 3 during regeneration and observe a time course different from that of Homer 1a; thus the role of the Homer family proteins in regeneration appears to be pleiomorphic.
Expression of Homer 1 in skeletal muscle. After bupivacaine treatment, from postinjection day 3 to postinjection day 21, Homer 1b/c/d displayed an expression pattern superimposable to that of myofibrillar proteins (Figs. 3 and 6, A and B) and of creatine phosphokinase, a myogenic marker of myotube formation, monitored under similar experimental conditions (8). Not only myogenic cells (satellite cells, myoblasts) and myotubes but also regenerating muscle fibers appear to express long isoforms of Homer 1. Homer 1b/c/d never overshoots expression levels of control, controlateral samples, and, thus its increase appears to parallel restoration of muscle mass. A similar pattern was displayed also by C2C12 myoblasts and myotubes: thus long isoforms of Homer 1 have undoubtedly a myogenic origin.

The paradigm for Homer 1a transcription, as hypothesized by Brakeman et al. (4) in neural tissue has become less stringent because Homer 1a does not only appear to behave as an IEG product, but it also appears to be constitutively expressed: Homer 1a protein was detected in control brain lysates (31) and in quiescent cultured hippocampal neurons (17); moreover, Homer 1a was detected in cultured cortical neurones upon inhibition of proteasome activity (1).

Within this framework, it is not surprising that we found the constitutive expression of Homer 1a both in fast-twitch and in slow-twitch skeletal muscles of the rat. The present finding fits well with our own previous finding concerning the presence of mRNA coding for either Homer 1a or Homer 1c in skeletal muscle (30). We did not assess quantitatively the relative level of Homer 1a vs. Homer 1b/c/d in each muscle type, but it is clear that Homer 1b/c/d was expressed in comparable amounts, whereas Homer 1a was more represented in slow-twitch muscles (about twofold).

Thus, in skeletal muscle, as in other tissues, Homer 1a might play additional roles besides the classic one, i.e., competitive antagonist of Homer 1b/c: it might have a role of its own in cell regulation.

During muscle regeneration and after postinjection day 3, Homer 1a increased markedly compared with controlateral samples and remained 2.5-fold higher until postinjection day 10. Therefore the increase of Homer 1a is not positively correlated to muscle mass increase and could be the result of several concomitant processes affecting transcription as well as protein stability and turnover: 1) satellite cells are known to be activated and to start proliferation on postinjection days 4–5 (14), whereas at postinjection day 10, myotubes and regenerating fibers constitute the vast majority of the cell population; 2) in C2C12 cell cultures, Homer 1a expression increases as proliferation and differentiation occur (Fig. 7); and 3) proteasome activity increases between 3 and 7 days during muscle regeneration but then rapidly returns to control levels (7). In this respect, it is noteworthy that only Homer 1a, but not Homer 1b/c, -2, or -3, has been reported to be degraded by proteasomes and that this mechanism is responsible for the rapid turnover rate of Homer 1a protein observed both in cultured neurons and upon expression in heterologous cell lines (1). If Homer 1a escapes proteasome-mediated degradation by some yet unknown mechanism, protein accumulation ensues (1). Indeed, treatment with phorbol esters led to a moderate upregulation and increased synaptic localization of Homer 1a, even in the absence of transcriptional activity (1). Moreover, upon blockade of proteasomes, Homer 1a mRNA levels rose markedly in primary neuronal cell cultures (24).

Even if the possible contribution of inflammatory cells cannot be ruled out in the initial phases, Homer 1a accumulation in regenerating soleus is likely associated to proliferation of satellite cells (day 3 onward) as well as to differentiation of myotubes (day 10 onward); interestingly, postnatal brain development, up to 5 wk, has been reported to be associated to increase of mRNA coding for Homer 1a (4).

The long-lasting Homer 1a accumulation might be due not only to increase of mRNA levels (Fig. 4) but also to decreased protein turnover should proteasome activity be reduced. Compared with upregulation of Homer 1a in hippocampus by seizure-induced neuronal activation, i.e., mRNA peaks around 1 h and protein at 4 h (4), our findings are quite different, mRNA peaks ~12–24 h, protein accumulates after day 3 up to day 7.

Thus, there appears to be two pools of Homer 1a, the constitutive pool, and the inducible pool, that might be referable to the IEG one and include transient expression during muscle differentiation. Interestingly, in hippocampal cultures, Okabe et al. (23) argued that the composition of Homer-mediated clustering of protein complexes is highly dynamic and postulated that Homers rely on two regulatory systems for their distribution and interaction with other proteins: 1) a local, protein synthesis-independent signaling cascade that rapidly redistributes Homers within dendritic compartments, and 2) an inducible, Homer 1a-dependent system, linked to stimulus-transcription coupling, that regulates the global state of signaling complex formation with a slower, yet long-lasting, time course.

With regard to the putative role of Homer 1 during muscle differentiation, a tentative although speculative role might be derived from models put forward in nonmuscle cells. It is known that Homer 1 isoforms control plasma membrane (PM) targeting of mGluR5 in neurons: Homer 1b caused intracellular retention of the receptor, whereas Homer 1a reversed the Homer 1b-mediated effect, triggering PM localization of the receptor (2). Moreover, it has been demonstrated that Homer 1b inhibits PM localization of mGluR5 in heterologous cells, causing mGluR5 to be retained in the endoplasmic reticulum.
In contrast, mGluR5 alone or mGluR5 coexpressed with Homer 1a successfully travels through the secretory pathway to PM (27). These data are consistent with a model, in which the activity-regulated increased expression of Homer 1a disrupts the binding of Homer 1b to mGluR and leads to increased trafficking of mGluR to PM. Transient upregulation of Homer 1a during muscle differentiation might be involved in protein redistribution related to SR biogenesis and/or formation of peripheral junctions and triads.

Change of Homer 3 expression during regeneration. Interestingly, no constitutive expression of Homer 3 was detected in control soleus muscle. This finding is in qualitative agreement with previous findings by Soloviev et al. (33), who reported that mRNA for Homer 3 is the least represented among Homer transcripts in skeletal muscle of the adult mouse, and by Xiao et al. (39) and by Shiiraishi et al. (31) who reported no expression of Homer 3, as judged by Western blot analysis of skeletal muscles of the adult mouse, and by Xiao et al. (39) and by Shiiraishi et al. (31) who reported no expression of Homer 3, as judged by Western blot analysis of skeletal muscles of the adult rat and mouse, respectively. On the other hand, during regeneration, there was a conspicuous and sustained expression of Homer 3 between postinjection day 3 and day 6. Compared with Homer 1a upregulation, Homer 3 buildup started earlier and its decay was inversely related to increase of muscle mass (see Figs. 5B, and 6, A and D).

Consistently, in C2C12 cell cultures, Homer 3 expression was already at plateau when Homer 1a started its ascending phase (Fig. 7). Taken together, these data indicate that Homer 3 might be characteristic of satellite cell activation and of the following early phases of myoblast proliferation (6). Thus upregulation of Homer 3 and that of Homer 1a are clearly distinct phenomena. The reasons for such a different behavior are unknown.

Interestingly, transient expression of Homer 3 has been already observed during postnatal development of the brain (31). In cultured T cells, on serum stimulation, Homer 3 has been reported (16) to be overexpressed and to translocate to the nucleus, thus regulating T cell activation via interaction with specific transcription factors. It is uncertain whether Homer 3 is indeed an IEG in skeletal muscle. Expression pattern of Homer 2b during regeneration. Expression pattern was similar to that of Homer 1b/c/d, and, thus, Homer 2 could be expressed by myogenic cells during regeneration and as a constitutive isoform in adult muscle. Our observations are interesting in relation to previous findings by Pattison et al. (25), who reported downregulation of Homer 2 mRNA in disuse atrophy, an adaptive phenomenon characterized by reduced number and size of muscle fibers. Thus increase of Homer 2b during regeneration might be associated to mass recovery (cfr. Fig. 6C) and be also instrumental to transition toward hypertrophy.

Final remarks. The relevant biological question stemming from the present work is whether Homer (either 1a and/or 3) transcription is causally involved in differentiation and regeneration of skeletal muscle, or is an epiphenomenon associated to activation of specific myogenic programs. The distinct expression pattern displayed by Homer 1a and Homer 1b/c/d, as well as the temporal dissociation of upregulation transiently shown by Homer 1a and Homer 3, would indicate a role for both Homer 1a and Homer 3 in muscle differentiation and regeneration, whereas Homer 1b/c/d and Homer 2b appear to be part of the muscle growth program. If Homer 1a and Homer 3 were to play a role in differentiation and regeneration, as judged by data obtained in C2C12 cell cultures, then Homer 3 might be mainly involved in early phases of myogenesis up to myoblast differentiation and Homer 1a in later phases of myogenesis, i.e., formation and maturation of myotubes.

Negative results obtained in fatigue experiments do not rule out a role for Homer 1a; in fact, other patterns of fatigue might do so and additional investigations are in order.

Finally, a variety of functional studies (10, 15, 37) would have no relevance at all, under resting conditions, if Homer 1a were an IEG only. The constitutive expression of Homer 1a, as shown here, corroborates the putative role of Homer 1a in skeletal muscle, i.e., Homers may play a role in signal transmission of skeletal muscle, in particular regulation of Ca^{2+} release from SR.

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