Homer 2 antagonizes protein degradation in slow-twitch skeletal muscles

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Bortoloso E, Megighian A, Furlan S, Gorza L, Volpe P. Homer 2 antagonizes protein degradation in slow-twitch skeletal muscles. Am J Physiol Cell Physiol 304: C68–C77, 2013.—Homer represents a new and diversified family of proteins made up of several isoforms. The presence of Homer isoforms, referable to 1b/c and 2a/b, was investigated in fast- and slow-twitch skeletal muscles from both rat and mouse. Homer 1b/c was identical irrespective of the muscle, and Homer 2a/b was instead characteristic of the slow-twitch phenotype. Transition in Homer isoform composition was studied in two established experimental models of atrophy, i.e., denervation and disuse of slow-twitch skeletal muscles of the rat. No change of Homer 1b/c was observed up to 14 days after denervation, whereas Homer 2a/b was found to be significantly decreased at 7 and 14 days after denervation by 70 and 90%, respectively, and in parallel to reduction of muscle mass; 3 days after denervation, relative mRNA was reduced by 90% and remained low thereafter. Seven-day hindlimb suspension decreased Homer 2a/b protein by 70%. Reconstitution of Homer 2 complement by in vivo transfection of denervated soleus allowed partial rescue of the atrophic phenotype, as far as muscle mass, muscle fiber size, and ubiquitinazation are concerned. The counteracting effects of exogenous Homer 2 were mediated by downregulation of MuRF1, Atrogin, and Myogenin, i.e., all known genes to be upregulated at the onset of atrophy. On the other hand, slow-to-fast transition of denervated soleus, another landmark of denervation atrophy, was not rescued by Homer 2 replacement. The present data show that 1) downregulation of Homer 2 is an early event of atrophy, and 2) Homer 2 participates in the control of ubiquitinization and ensuing proteolysis via transcriptional downregulation of MuRF1, Atrogin, and Myogenin. Homers are key players of skeletal muscle plasticity, and Homer 2 is required for trophic homeostasis of slow-twitch skeletal muscles.

Homer; denervation; disuse; atrophy; protein degradation; slow-twitch skeletal muscles

A NOVEL ACTIVITY-DEPENDENT control of multiprotein signaling complexes has been shown in neuronal synapses and is mediated by a family of proteins termed Homers. Homers assemble proteins such as metabotropic glutamate receptors (mGluRs); transient receptor potential channels (TRPCs); intracellular Ca2+ channels, e.g., ryanodine receptor (RyR); inositol (1,4,5)-trisphosphate receptor (IP3R); scaffolding proteins, e.g., Shank and small GTPases, transcription factors; and cytoskeletal proteins (for a review, see Ref. 10). Homer 1b/c, 2a/b, 3a/b, known as constitutive isoforms, have been reported to cross-link multimers via the coiled-coil (CC) domain (17, 52), whereas Homer 1a has been considered an immediate early gene (IEG) product (4) able to antagonize CC-Homers. Although doubts have been cast on the unequivocal role of Homer 1a (48), Homers can be regarded as regulators of multimeric complexes involved in signal transduction.

In skeletal muscle, Homers have been detected at the protein (38, 39, 43, 44, 51) and mRNA levels (40, 44, 51). The regulatory equilibrium between constitutive Homers and up-regulated Homers could be particularly relevant not only in resting conditions but also during muscle adaptation to exercise and environmental stress. It is worth noting that mechanical (low-voltage electrical stimulation or low-frequency fatigue), metabolic (hypoxia), and hormonal stimuli all appear to be associated with induction of a number of IEGs, such as c-fos, c-jun, c-erb-1, and heat shock proteins (13). Postnatal differentiation of striated muscles and attainment of fiber-type specificity, fast- vs. slow-twitch skeletal muscles, entail complex genetic programs. Adaptive muscle changes entailing structural rearrangement and morphological remodeling, e.g., development, hypertrophy, and regeneration, are processes coupled to upregulation of several, distinct IEGs (14, 24), and, at least in the case of skeletal muscle regeneration, we recently reported a switch in Homer isoform composition, Homer 1a and Homer 3 being selectively upregulated (3).

Homer 2 enhances transcription of several slow-twitch muscle-specific genes, including myoglobin and slow troponin I promoter, via RyR/NFAT activation, according to Stiber et al. (46). Expression and accumulation of Homer 2 at the NMJ of human skeletal muscle fibers are modulated by neuronal activity, according to Salanova et al. (39). Thus the rationale of the present study was to ascertain whether Homer 2 plays a causative role in the atrophy process and in fiber type specialization of slow-twitch skeletal muscles, using in vivo models of skeletal muscle atrophy, i.e., denervation and disuse.

Here, we report that 1) Homer 2 is present only in slow-twitch skeletal muscle fibers, 2) denervation and disuse determine almost complete disappearance of Homer 2 at the protein and mRNA level, 3) Homer 2a/b expression is motor nerve dependent, and 4) in vivo, acute reintroduction of Homer 2, via cDNA electrotransfer, partially rescues the denervation atrophy phenotype, as judged by effects on muscle mass, fiber size, and ubiquitinization, but does not revert the slow-to-fast transition. Based on present and recent data, it appears that transition in Homer composition is relevant in skeletal muscle adaptation. In particular, Homer 2 is causally involved in atrophy of slow-twitch skeletal muscles, transcriptionally downregulates key atrophy genes, and exercises a negative feedback control on protein degradation.

MATERIALS AND METHODS

Tissue sources. All experiments were carried out on adult male Wistar rats (∼250 g of body wt) and CD1 mice (∼45 g of body wt) except those for disuse atrophy (see below). For denervation and regeneration experiments, rats were anesthetized with intraperitoneal injection of ketamine (1.5 mg/100 g), subjected to appropriate surgical
procedures, and killed by cervical dislocation at specified time points. Experimental protocols have been approved by University Review Boards. Soleus, a representative, predominantly slow-twitch skeletal muscle, and extensor digitorum longus (EDL) and tibialis anterior (TA), representative predominantly fast-twitch skeletal muscles, were used. For comparative purposes, in some experiments, we also used soleus and adductor magnus from New Zealand white rabbits; by conventional criteria such muscles are deemed pure slow-twitch and pure fast-twitch skeletal muscles.

Antibodies and cDNAs. The following primary antibodies were used for Western blots: polyclonal antibodies specific for Homer 1b/c were from Chemicon and for Homer 1a or Homer 2a/b from Santa Cruz for Western blots: polyclonal antibodies specific for Homer 1b/c were purchased from Invitrogen, respectively. Goat anti-rat IgG (Sigma), rabbit anti-goat IgG (Sigma), and goat anti-mouse IgG (Sigma) conjugated to alkaline phosphatase were used as secondary antibodies. cDNA coding for V5-tagged conjugates and anti-V5 were from Enzo Life Sciences and from Invitrogen from Chemicon and for Homer 1a or Homer 2a/b from Santa Cruz for Homer isoforms.

Denervation of hindlimb skeletal muscles of the rat. The sciatic nerve was cut unilaterally at the level of trochanter. About 0.5–1 cm of the perineurial nerve stump was removed to obtain a permanent denervation of the lower hindlimb. At 1, 7, and 14 days after denervation, rats were killed. Soleus, EDL, and TA muscles from the denervated limb were dissected out, frozen in liquid nitrogen, and utilized for further studies, the control muscles being the contralateral, innervated muscles.

Disuse atrophy. The hindlimb-unloading experimental protocol was performed following the recommendations provided by the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Council of Europe No. 123, Strasbourg, 1985) and authorized by the Animal Ethics Committee of the University of Padova and the Italian Health Ministry (103/2007B). Six-week-old female Wistar rats (140–160 g of body wt) were caged individually. Hindlimb muscles were unloaded using the tail-suspension model (7, 16). Female adult Wistar rats (~180 g of body wt) were used. The right soleus muscles were exposed and injected with 0.06 ml of a saline solution containing 50 μg of plasmid cDNAs. Electrodes were applied on both sides of the leg. Electroporation was carried out with a BTX ECM 830 square wave pulse generator using the protocol described by Nori et al. (29), i.e., pulse stimulation by field electrodes (220 V/linear cm) with six 20-ms pulses at 200-ms intervals. The damage evoked by the overall procedure was estimated in four distinct soleus muscles, 10 days after experimentation, by counting central nuclei whose presence is index of damage and of ensuing regeneration. Several transverse sections of each soleus were examined, and the number of central nuclei/100 fibers ranged from 0.02 to 0.12. Thus the extent of damage was deemed to be irrelevant. Denervation, where applicable, was carried out immediately after electroporation.

Homogenates from soleus and EDL skeletal muscles of rat and mouse. Homogenates of skeletal muscles were obtained as follows: frozen tissues were triturated in a mortar; homogenized with Polytron for 10 s at 20,000 RPM in 10 vol of 3% SDS, 1 mM EGTA, 20 μM phenylmethylsulfonyl fluoride, and 0.8 mM benzamidine, boiled for 5 min, and centrifuged at 18,000 g for 30 min to remove debris. Homogenates were kept at −20°C until use. Protein concentration was determined according to Lowry et al. (20).

SDS-PAGE. Western blot, and quantitative densitometry. SDS-PAGE on either 7.5% (in the case of Homer 1 b/c), 12.5% (in the case of Homer 1a/b), transfer to nitrocellulose, and Western blot were carried out essentially as previously described (29).

Homer were quantified by densitometry of immunoblots of homogenates. Densitometric analysis was performed with Scion Image for Windows, version Beta 4.0.2 (Scion, Frederick, MD; www.scioncorp.com). Each value for Homer isoforms is given as ratio to that of the corresponding contralateral sample.

RNA extraction and quantitative RT-PCR. Total RNA was extracted from either muscles or thin-sections using TRIzol (Invitrogen) extraction method or RNeasy Micro kit (Qiagen) according to the manufacturer’s instructions. RNA was eluted in RNase-free water and stored at −80°C until use. The concentration and quality of each sample were measured by NanoDrop (Thermo Scientific). Four hundred nanograms of RNA were converted to CDNA by using random hexamers and SuperScript II RT (Invitrogen) following the manufacturer’s instructions. RT was performed in a thermal cycler (Applied Biosystems, Foster City, CA): 25°C for 10 min, 42°C for 60 min, 95°C for 5 min, and 4°C for 5 min. All RNA samples were simultaneously converted to CDNA to minimize technical variability. Negative controls (no RNA or no RT enzyme) were simultaneously run for RNA and genomic DNA contamination.

Primers for quantitative PCR were already published or designed (∗) using Primer3 software (http://frodo.wi.mit.edu), Whitehead Institute for Biomedical Research) and their thermodynamic specificity was determined using BLAST sequence alignment (NCBI) and vector NTI (Invitrogen) software. All primers were purchased from Eurofins MWG Operon. Primer sequences were as follows: HOMER1∗: Fw GCACATATGATGAAAGCTCCTGAC, Rv GCTCAGTAGTGCCACACACC, HOMER2∗: Fw TCTTGCTTCTGGTGGTGTGTC, Rv CTGCGTAAACGGCTAAGGTA; ARGININ (Fbox32) (23): Fw GCAAAACGTCCACATTCCTC, Rv CTTGGGCTTAAAGAGACGGAGT, MARF1 (Triomphe)∗: Fw ACGTGCTTGCGGAGAACAC, Rv CTTGGTCTTCCTGGAACATC, MYOGENIN∗: Fw GTACAGCCGTGTAC, Rv ACATGCCGATCGAT, MYOSIN Myh7 (β slow) (37): Fw TATTGGGACGTCTGT, Rv GGACACGGTCTGAAAGGATG; MYOSIN Myh2 (fast 2A): Fw TGAAGGATAGAAGCAG, Rv GAATCCATGGGGACATG; MYOSIN Myh9 (23): Fw AGCATGCACTGCCATC, Rv CCTGGTTCTGGAGCCTGAGAACG; PPIA and TBP1 being the most stable genes to normalize cycle threshold (CT) values. All samples were run simultaneously with RNA- and RT-negative controls. The efficiency of each run was determined by a standard curve. Normalization was performed by ΔCT method. Data are expressed as means ± SE. Comparisons were made by using t-test.

Dual luciferase assay on rat soleus. Promoter activity of the slow myosin heavy chain (MHC) was measured using the Dual Luciferase Reporter Assay System (Promega), and luciferase activity was determined with an analytical luminometer. Relative luciferase units were calculated by determining the ratio of the intensity of the light produced by the firefly luciferase reporter plasmid to that produced by the Renilla luciferase pRL-TK plasmid.

Soleus muscles were injected with 60 μl of 0.9% NaCl containing 50 μg of firefly luciferase reporter construct, and 5 μg of pRL-TK vector and electroporated as described above. SOL muscles were homogenized in 0.5 ml of passive lysis buffer (Dual Luciferase Reporter Assay System; Promega), and luciferase activity was determined.

Confocal immunofluorescence. For soleus muscles, 6-μm longitudinal and transverse sections were obtained and incubated with primary antibodies at room temperature for 60 min as described previously (29). After extensive washing, muscle sections were incubated for 30 min with either Cy2-conjugated anti-mouse (Chemicon) mouse.
or rhodamine isothiocyanate anti-rabbit antibodies (Dako). Analysis was carried out in a Leica W5 microscope.

**Statistical analysis.** One-way ANOVA was used to evaluate the changes over time for both relevant proteins and muscles wet weight. All values are means ± SE. Post hoc comparison were performed with Fisher’s protected least significance difference test. Dunnett’s test was applied to compare each time point value with the postdenervation day 1 value. Correlations between changes in Homer isoforms and type 1 MHC content were assessed by linear regression analysis. Individual means were compared with a paired two-tailed t-test. Differences were considered statistically significant at the 0.05 level of confidence.

**RESULTS**

**Expression of Homer 1 and 2 isoforms in skeletal muscles.** Representative slow-twitch (soleus) and fast-twitch (EDL) skeletal muscles from both adult rat and mouse were analyzed for their content in Homer 1b/c and Homer 2a/b (Fig. 1A). Rat, mouse, and, in a few cases, rabbit muscles were used because conflicting results have been reported depending upon species (compare Ref. 51, for rat, with Ref. 43, for mouse).

Western blot analysis was carried out with antibodies specific for Homer 1b/c. The apparent molecular mass of the immunostained proteins (~47 kDa) was consistent with the calculated mass of Homer 1b/c (4, 17). Single, unique bands were detected in all lanes, and the amount of Homer 1b/c was virtually identical in muscles samples irrespective of tissue (slow and fast muscles) and species (rat and mouse).

Comparison of homogenates from soleus and EDL probed with anti-Homer 2a/b antibodies indicates that soleus muscles displayed by far the largest amount of Homer 2a/b; in EDL samples, from either rat or mouse, Homer 2a/b was barely detectable. Densitometric analysis of immunostained proteins yields a ratio of ~10:1 for soleus and EDL muscles, respectively (see also Fig. 1A).

Since slow-twitch skeletal muscles contained much larger amounts of Homer 2a/b than fast-twitch skeletal muscles, Homer 2a/b might be referable, in predominantly fast-twitch muscles, to the presence of slow-twitch fibers, i.e., ~5–6 and 4% in EDL of rat (26, 35) and mouse (28), respectively. In fact, when rabbit skeletal muscles were analyzed, e.g., soleus and adductor magnus as pure slow-twitch and pure fast-twitch skeletal muscles, respectively, no detectable Homer 2a/b was found in rabbit adductor (data not shown).

In summary, distribution of Homer 2a/b in skeletal muscles appears to be species independent and fiber type dependent. Published discrepancies (43, 51) might be accounted for by differences in antibody specificity, animal age, and type of skeletal muscle, e.g., it was not specified whether fast-twitch or slow-twitch muscles were used (compare Refs. 43, 51).

**Expression of Homer 1 and 2 isoforms in denervated soleus of the rat (denervation atrophy).** We next asked whether there are Homer isoform changes associated to skeletal muscle atrophy triggered by motor denervation. Soleus muscles were collected at 1, 7, and 14 days after denervation (see MATERIALS AND METHODS for details), and the content of Homer 1a, Homer 1b/c (Fig. 1B), and Homer 2a/b (Fig. 1C) was determined by Western blot of muscle homogenates and compared with that of innervated, contralateral soleus muscles. Relative levels of Homer 1a/b and Homer 1a, as judged by densitometry, were rather constant over time. Thus denervation did not significantly affect expression of

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**Fig. 1.** The presence of Homer isoforms in soleus and extensor digitorum longus (EDL) skeletal muscles (A) and rapid and conspicuous decrease of Homer 2a/b and of muscle mass in rat denervated soleus (model of denervation atrophy). A: Western blots were carried out with antibodies specific for either Homer 1b/c or Homer 2a/b, on homogenates (200 µg/lane) of predominantly slow-twitch and fast-twitch muscles from both rat and mouse (2 experiments for each muscle). Relative content of Homer 2a/b in EDL and soleus from rat and mouse was determined by densitometry of immunoblots performed with specific antibodies for each Homer isoform. C, inset: Western blots of soleus homogenates (200 µg) were decorated with antibodies specific for Homer 2a/b; denervated (d) and contralateral (c) control muscle obtained at 1 (1d), 7 (7d), and 14 (14d) postdenervation days. D: data of muscle mass (wet weight). In B–D, data are presented as ratios of denervated muscles over contralateral control muscles, and values are means ± SE; n = 4, **p < 0.01, significantly different from day 1 values.
Homer 1 isoforms (Fig. 1B). On the other hand, expression of Homer 2a/b sharply decreased after 1 day, the decrease being significant after 7 days; after 14 days, Homer 2a/b was barely detectable (note both Western blot and densitometric data in Fig. 1C). Therefore, denervation virtually abolished fiber type-specific expression of Homer 2a/b. Figure 1D shows, on the same time-scale, muscle mass decrease, i.e., the extent of muscle atrophy caused by denervation. Application of paired t-test indicates that decrease of either muscle mass or Homer 2a/b was not significant 1 day after denervation. However, the slope of Homer 2a/b reduction was steeper, suggesting that Homer 2a/b downregulation is as an early event in muscle atrophy.

Parallel studies were carried out by RT-PCR analysis. Levels of Homer 1 mRNA were unchanged upon denervation (Fig. 2), whereas Homer 2a/b transcription was quickly turned off. In fact, Homer 2a/b mRNA levels decreased sharply by ~40% within 24 h (not shown), attained a residual 10% by postdenervation day 3 and remained constant and low until postdenervation day 14 (Fig. 2).

Sharp decrease of Homer 2a/b in rat soleus following hindlimb suspension (disuse atrophy). Soleus muscles were obtained after 7-day hindlimb suspension of the rat, an established model of disuse inducing atrophy and slow-to-fast transition of postural muscles. A significant reduction of Homer 2a/b was observed (Fig. 3), in agreement with mRNA data obtained in similar experimental models (45).

Functional role of Homer 2 in skeletal muscle atrophy: gain-of-function studies. Based on present results and previous studies on different models of atrophy (12, 30–32, 39), it appears that Homers are expressed as part of the myogenic differentiation program, that Homer 2 expression is tissue-specific, and that Homer 2 transcription is downregulated in atrophy. The functional role of Homer in atrophy and skeletal muscle adaptation is unknown and was, thus, directly investigated. The specific issue was whether reintroduction of exogenous Homer 2 could reverse some of the adaptive changes induced by denervation, i.e., whether Homer 2 could rescue, at least in part, the denervation phenotype. To this effect, we relied on an established model of in vivo transient transfection of rat soleus via electroporation (9, 29).

First of all, qualitative expression and subcellular localization of recombinant Homer 2-V5 was assessed (Fig. 4), upon transient transfection with plasmid cDNAs coding for epitope

![Figure 2](https://example.com/figure2.png)

*Fig. 2. Time course of Homer 1 and Homer 2a/b transcripts of rat denervated soleus by quantitative (q)RT-PCR. qRT-PCR was carried out on muscles obtained at postdenervation days 3, 5, 7, and 14 and on contralateral control muscles. Data are normalized to those of the reference gene TBPI (see MATERIALS AND METHODS for details) and are given as means ± SE; n = 4. *P < 0.05.*

![Figure 3](https://example.com/figure3.png)

*Fig. 3. Rapid decrease of Homer 2a/b in rat soleus after 7-day of hindlimb suspension (model of disuse atrophy). Western blot of homogenates of soleus (200 μg) were decorated with antibodies for Homer 2a/b. Control soleus muscles were obtained from rats freely housed in cages. Densitometric data are presented as absolute optical density (OD) values and are means ± SE; n = 4. *P < 0.05.*

![Figure 4](https://example.com/figure4.png)

*Fig. 4. Reintroduction of Homer 2 in control and denervated soleus by in vivo transfection with plasmidic cDNA coding for epitope-tagged Homer 2-V5. A: electroporation (EP) and in vivo transfection of soleus muscles, with (den) or without sciatic nerve transection, were carried out with either pHomer 2-V5 (H2-V5) or pcDNA3 (vector). a: electroporated with pHomer 2-V5; b: electroporated with pcDNA3; c: electroporated with pcDNA3 and denervated; d: electroporated with pcDNA3 and denervated. Homogenates (200 μg) were analyzed by Western blots and decorated with antibodies specific for V5. Ponceau red staining of nitrocellulose membranes shows equal protein loading in each lane (loaded). B: immunolocalization of exogenous Homer 2-V5 following electroporation of rat soleus with pHomer 2-V5. *Nontransfected fibers. B, inset: a: immunofluorescence; b: phase contrast; c: merge image. Arrows point to the Z-line. C: transverse sections of soleus transfected with pHomer 2-V5. Bar = 10 μm.*
tagged V5-Homer 2 (pH2-V5). As judged by a series of Western blots of soleus homogenates (Fig. 4A), electroporation of innervated soleus with pH2-V5 resulted in the expression of an exogenous protein recognized by anti-V5 antibodies (lane a) and having an apparent molecular mass of ~45 kDa. Innervated soleus electroporated with the empty vector (vector) was negative for V5 reactivity antibodies (lane b). In denervated soleus, only transfection with Homer 2-V5 resulted in reconstitution of an exogenous Homer 2 complement (compare lane c with lane d). Immunofluorescence of longitudinal (Fig. 4B) and transverse (Fig. 4C) sections of rat soleus was carried out with antibodies specific for the V5 tag since no commercially available antibody for Homer 2 is adequate for immunofluorescence. As shown in Fig. 4B, recombinant Homer 2 displayed a regular sarcomeric pattern and localized at the Z line level (b and c), away from the A-I band where excitation-contraction coupling takes place. This finding is in perfect agreement with the sarcomeric distribution of endogenous Homer in rat skeletal muscle previously reported by Stiber et al. (46) and Salanova et al. (38, 39). Finally, Fig. 4C is a representative picture of the extent of transfection and shows several transfected fibers. Thus the electroporation procedure appears to be effective as far as expression and subcellular localization of recombinant Homer 2 is concerned.

The next question was whether Homer 2 replacement interferes with muscle mass reduction, a typical, macroscopic landmark of denervation atrophy. Figure 5, A and B, shows that exogenous Homer 2 partially counteracted the muscle mass reduction by ~20%. Figure 5A presents data normalized to the animal weight, whereas Fig. 5B compares the relative weight of denervated soleus in the absence and presence of exogenous Homer 2.

The subsequent question was whether Homer 2 replacement decreases muscle mass reduction by acting on fiber size. Denervation induced drastic decrease of cross-sectional area in both nontransfected (negative) fibers and transfected (positive) fibers, as shown in Fig. 5, C and D, respectively. Homer 2 replacement was able to partially reverse cross-sectional area decrease, as shown in Fig. 5D (compare fourth bar with third bar). Denervated and Homer 2-transfected (EP-Den H2) fibers were on average bigger than denervated and mock-transfected fibers (Fig. 5D) as well as contiguous, nontransfected fibers of the very same muscle (Fig. 5E) by ~30%.

Atrophy is mainly accomplished via activation of pathways for protein degradation. In this framework, the question was whether Homer 2 replacement antagonizes any protein breakdown pathways. As expected and as shown in Fig. 6A, the ubiquitin pathway is activated in denervated soleus. Electroporation per se did not change the ubiquitination pattern, whereas exogenous Homer 2 inhibited ubiquitination by ~60% (Fig. 6B). Figure 6C shows, in four pairs, that exogenous expression of Homer 2-V5 was comparable in denervated soleus (d) and contralateral nondenervated soleus (c). Thus Homer 2 appears to exert its effects by counteracting activation of proteolysis.

Homer 2 and transcriptional control in slow-twitch skeletal muscles during atrophy. Ubiquitin-ligases MuRF1, Atrogin (ATR), and Myogenin (MyoG) are genes postulated to be

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Fig. 5. Effect of Homer 2 reintroduction on muscle mass (A and B) and fiber cross-sectional area (CSA; C–E) of denervated soleus following in vivo transfection. A: weight of each soleus muscle was normalized to that of the rat. Bars at left represent measurements from normal soleus muscles electroporated with either pcDNA3 (empty vector, v) or pHomer 2-V5 (H2). Bars at right represent CSA measurements from denervated soleus muscles electroporated with either pcDNA3 (v) or pHomer 2-V5 (H2). Data are given as means ± SE; n = 4. *P < 0.05; ***P < 0.001. B: weight of each soleus was expressed as percentage of control. Data are given as means ± SE; n = 4. *P < 0.05. C–E: CSA was determined by morphometry, as described in MATERIALS AND METHODS. CSA was measured in nontransfected, also referred to as negative or not fluorescent, fibers (C) and transfected, also referred to as positive, fluorescent, fibers (D). Bars at left represent CSA measurements from normal soleus muscles electroporated with either pcDNA3 (v) or pHomer 2-V5 (H2); Bars at right represent CSA measurements from denervated soleus muscles electroporated with either pcDNA3 (v) or pHomer 2-V5 (H2). Data are given as means ± SE; n = 4. **P < 0.01; ***P < 0.001. In E, CSA was measured in positive and negative fibers of the same soleus. Data are given as means ± SE; n = 4. *P < 0.05.
10-fold induction and 2-fold induction were observed at denervated TA (6) and denervated soleus, although about MuRF1, ATR, or MyoG (Fig. 7, replacement on denervation-induced upregulation of either MyoG, and thus we investigated the effects of Homer 2 on several peculiar features of denervation-induced slow-to-fast transition in soleus, namely upregulation of type 2X MHC and downregulation of mitochondrial biogenesis.

Quantitative RT-PCR analysis was carried out in rat soleus and mRNAs referable to either fast 2A, β-slow, or 2X MHCs were measured 14 days after denervation in rat soleus. As expected, MHC isofrom transition was detected, i.e., fast 2A and β-slow MHCs were drastically downregulated whereas 2X MHC was sharply upregulated (Fig. 8A). Upon replacement of Homer 2 via transfection, no significant effect was observed on the expression of MHC isofroms (Fig. 8B). An additional insight was gathered by dual luciferase assay to measure directly the promoter activity of the slow MHC (Fig. 8C): as expected, the slow MHC promoter was downregulated upon denervation; on the other hand, either overexpression of Homer 2 (in control soleus) or Homer 2 replacement (in denervated soleus) did not influence the promoter activity of slow MHC.

PGC1-α, the master gene of mitochondrial biogenesis, is known to be downregulated in denervation. Homer 2 effects on regulation of mitochondrial biogenesis during denervation of soleus, were monitored by quantitative RT-PCR. Figure 8D shows that PGC1-α was halved at postdenervation day 14 and that Homer 2 replacement did not counteract such a decrease. Thus it appears that Homer 2 was not involved in mitochondrial biogenesis. Under the prevailing experimental conditions, Homer 2 does not appear to be involved in the characteristic slow-to-fast transition occurring upon denervation of soleus. On the other hand, the lack of effects of Homer 2 replacement on denervation-dependent slow-to-fast transition underscores the specific and clear-cut role of Homer 2 in counteracting protein breakdown and muscle atrophy.

DISCUSSION

Homer 2 in slow-twitch skeletal muscle fibers. Tissue differentiation, commitment, and maintenance of phenotypic specialization as well as plasticity are long-lasting and intriguing themes of skeletal muscle research. Motor innervation is one of the key factors controlling gene expression in skeletal muscle, and the low frequency firing pattern is the paramount determinant for promoting and maintaining the slow-twitch phenotype (13). Ca2+ plays a pivotal role as an intracellular messenger: it modulates the expression of specific muscle genes by transducing the tonic motor nerve activity into a program of gene expression specific of slow-twitch fibers (5) and is instrumental in hypertrophy/atrophy of both skeletal and cardiac muscles (5, 27). Homers might be involved in such pathways by acting on either RyR and IP3R Ca2+ release channels (11, 46, 49, 50) or TRPC (53) and, thus, dynamically affecting intracellular Ca2+ homeostasis.

Consistent with Rosenberg et al. (36), who mentioned a higher content of Homer 2a/b in slow oxidative myofibers, the present data clearly show that Homer 2a/b is expressed only in fully differentiated slow-twitch skeletal muscle fibers. Should Homer 1 and Homer 2 modulate intracellular Ca2+ homeosta-
sis via gating of both RyR and IP3R and through the assembly of macromolecular complexes also including TPRC, regulatory mechanisms must be different depending on the skeletal muscle phenotype. Differential expression of Homer isoforms, as shown by the present communication, would be coherent to this variable regulatory network.

Homer 2 in denervation and disuse atrophy of slow-twitch skeletal muscle fibers. Skeletal muscle atrophy is caused by different aetiologic factors, e.g., disuse, denervation, microgravity, starvation, aging, as well as by diseases (cancer cachexia, AIDS, and uremia) associated with inflammation (34). Interestingly, a small subset of genes is involved in all atrophy models whereas a larger number of genes is differentially expressed and, thus, specific for each type of atrophy (18). Denervation provokes not only muscle atrophy and loss of contractile force but also fiber type transition. In denervated rat soleus, both slow-twitch and fast-twitch fibers undergo rapid atrophy, muscle mass reduction (8), accompanied by slow-to-fast phenotype transition (15, 19). Several biochemical, metabolic, and functional features are modified, each with a different time course: full-fledged phenotype transformation occurs over a much longer time scale than atrophy, since it requires several weeks compared with a few days.

In the general context of phenotype determination, plasticity and adaptive responses, the role of Homer isoforms is being unraveled (3, 46), in particular the molecular mechanisms and intracellular pathways are under investigation. The specific biological question addressed by the present work is whether Homer 2 downregulation is causally involved in denervation-induced atrophy and fiber type specification.

Fourteen days after denervation of rat soleus, Homer 2a/b was found to be almost completely turned off. Sciatic nerve section and withdrawal of the tonic firing pattern of innervating motor neurons bring about, among other functional, biochemical, and molecular changes, the rapid and specific disappearance of Homer 2a/b. Since denervation-induced slow-to-fast transition is well established (15, 30), downregulation of Homer 2a/b is congruous and unambiguously supports the notion that Homer 2a/b is marker of the slow-twitch phenotype. Under the prevailing experimental conditions, lack of downregulation of both Homer 1a and Homer 1b/c would indicate that Homer 1 is not a slow-twitch specific marker. Homer 2a/b decrease might be due, at least in part, to the reduction of the number of the slow-twitch fibers characteristic of denervated soleus; however, such an interpretation does not hold since decrease of Homer 2a/b by far precedes that of type 1 muscle fibers taking place at least 3 wk after denervation (25, 30). Likewise, it does not appear plausible that Homer 2a/b reduction is due to acceleration of proteolytic processes since Homer 2a/b does not differ from other CC-Homer family members with regard to proteolytic sensitivity (1) and relative content of Homer 1 isoforms was not affected throughout the experimental time course of denervation.

Fig. 7. Upregulation of MuRF1 (A), Atrogin (ATR; B), and Myogenin (MyoG; C) following denervation of tibialis anterior (TA) and soleus (SOL) and downregulation of MuRF1, ATR, and MyoG of denervated SOL upon reintroduction of Homer 2 (D–F). qRT-PCR was carried out, as described in MATERIALS AND METHODS, on muscle samples from either TA or SOL (A–C) and on muscle samples from either mock-transfected and denervated SOL (vector) or transfected and denervated soleus (Homer 2), obtained at specific time points, following denervation (day (d)3, d5, d7, and d14), as indicated on the abscissa. Control (ctr) values were obtained on muscles that were not denervated. ctr values are different since it is known that "electric pulse associated with plasmid electroporation ... significantly increased atrogin-1 and MuRF-1 mRNA levels, whereas plasmid injection alone had no effect on gene expression" (52). Data are normalized to those of the reference gene TBP (see MATERIALS AND METHODS for details) and are given as means ± SE; n = 4–8. *P < 0.05.
The interesting issue is whether and how downregulation of Homer 2a/b is causally involved in denervation atrophy. The observation that neither Homer 1b/c nor Homer 1a was affected in denervation atrophy and similar findings in age-induced (31) and disuse (32, 39) atrophy, i.e., no other Homer isoform was decreased, whereas MHC type 2X was increased, compared with control (Ctr). B: in mock-transfected, denervated soleus (Ep-Den v) and in transfected, denervated soleus (Ep-DenH2), no significant change was detected in either MHC type 2A, β-slow, or 2X. C: promoter activity of slow MHC was monitored in a luciferase assay, as described in MATERIALS AND METHODS. Denervation decreased, as expected, slow MHC promoter activity, but Homer 2 replacement did not change this activity, as outlined in the graph on the left-hand side. D: activity of the PGC1α gene was measured by qRT-PCR, as described in MATERIALS AND METHODS. Denervation, as expected, decreased PGC1α activity (Den and Ep-Den v), but Homer 2 replacement (Ep-DenH2) was without any effect. qPCR data are given as means ± SE; n = 4 for control and denervated muscles; n = 9,13 for electroporated muscles. **P < 0.01, ***P < 0.001.

The interesting issue is whether and how downregulation of Homer 2a/b is causally involved in denervation atrophy. The observation that neither Homer 1b/c nor Homer 1a was affected in denervation atrophy and similar findings in age-induced (31) and disuse (32, 39) atrophy, i.e., no other Homer isoform was decreased, rule out the possibility that disappearance of Homer 2a/b is a nonspecific epiphenomenon of atrophy. On the other hand, the present data and previous findings by Pattison et al. (32), who reported downregulation of Homer 2 mRNA in disuse atrophy of rat soleus, ~86% decrease after 10 days of immobilization, and by Pattison et al. (31), who reported 40% reduction of Homer 2a/b mRNA in rat soleus muscles derived from aged rats where muscle atrophy is mainly but nor exclusively due to peripheral denervation (2, 33), indicate that neurological and nonneurological atrophic phenomena display selective Homer 2 downregulation. Both muscle activity and neurotrophic factors influence the molecular, biochemical, and mechanical properties of the muscle fiber (13); however, since Homer 2 is downregulated in atrophic muscle in the presence of intact neuromuscular junctions (31, 39 and Fig. 3), it seems that Homer 2 downregulation is also due to inactivity per se. The search for putative muscle-derived trophic factors controlling postsynaptic Homer 2 expression is worth future investigation.

Homer 2 contributes to the trophic control of protein turnover. Gain-of-function studies, implemented in vivo by electropora-
tion of plasmid cDNA coding for recombinant Homer 2 (Figs. 4–8), show that Homer 2 replacement in denervated soleus partially rescues the phenotype: in fact, exogenous Homer 2 counteracted decrease of muscle mass, of fiber size, and of ubiquitination via downregulation of specific genes that are known to be upregulated and to ignite atrophy, e.g., MuRF1, ATR, and MyoG. Since MyoG induction in denervated muscle contributes to the development of muscle atrophy via regulated expression of ubiquitin ligases ATR and MuRF1 (21), it appears that Homer 2 plays a two-level, negative feedback control in atrophying rat soleus. Mitigation of MuRF1, ATR, and MyoG and attenuation of ubiquitination are more pronounced than measured effects on muscle mass and fiber size: the variable extent of denervation phenotype rescue was probably accounted for by differences in parameters being analyzed, some of them simple (e.g., mRNA levels), others complex (e.g., muscle mass).

Homer 2 is causally involved only in this specific feature of phenotype adaptation, i.e., muscle trophysm, since the slow-to-fast transition, as judged by the same experimental approach, was not affected (Fig. 8). This finding indirectly confirms the notion that atrophy and fiber type transition are two independent processes occurring during both denervation and disuse.

Based on previous findings [i.e., Homer 2 expression during soleus regeneration (3)], it appears that Homer 2 is one of the molecular transducers for trophic control pathways in slow-twitch skeletal muscles. In the overall balance between protein synthesis and proteolysis, it appears that Homer 2 selectively interferes with the latter pathway. As shown here for the first time in denervation atrophy, Homer 2 antagonizes protein degradation via negative transcriptional control of MuRF1 and ATR. Although MuRF1 and ATR also control fiber size in fast-twitch fibers, the present findings indicate that Homer 2–dependent regulation might be restricted, given the fiber type-specific expression, only to slow-twitch fibers.

According to recent studies in disuse atrophy, muscle mass reduction is biphasic and due to an early and transient rise in protein breakdown that is followed by a sustained and conspicuous reduction in protein synthesis (22, 34). In this respect, it can be argued that Homer 2 disappearance, as shown here, might determine the shift in balance between protein synthesis and proteolysis in early phases, when protein breakdown is triggered.

How does Homer 2 exert its control on either MuRF1, ATR, or MyoG thus participating in regulation of slow-twitch muscle trophysm? Does Homer 2 act directly or, more likely, via changes of intracellular Ca2+ concentration? According to Pattison et al. (31), downregulation of Homer 2 mRNA in disuse atrophy may be part of a general adaptive response to “lower Ca2+ regulatory mechanisms” in skeletal muscle. Thus established and putative networks of regulatory mechanisms and intracellular pathways in which interplay between Ca2+ and Homer might occur should be investigated in future work.

In summary, the present study shows that Homer 2 is a marker of slow-twitch skeletal muscles and antagonizes protein breakdown. Homer 2 plays a role in muscle plasticity and transition between trophic states with upregulation of Homer 2 favoring growth and downregulation of Homer 2 favoring atrophy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: E.B. and P.V. conception and design of research; E.B., A.M., S.F., and L.G. performed experiments; E.B. interpreted results of experiments; E.B. drafted manuscript; E.B., A.M., S.F., and P.V. approved final version of manuscript; S.F. analyzed data; S.F. prepared figures; P.V. edited and revised manuscript.

REFERENCES

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