BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 6–10 (1997)
ARTICLE NO. RC977414

Interleukin-15 Stimulates C2 Skeletal Myoblast Differentiation

LeBris S. Quinn, Kathleen L. Haugk, and Susan E. Damon

Geriatric Research, Education, and Clinical Center, VA Puget Sound Health Care System, American Lake, Tacoma, Washington 98493, and Department of Medicine, University of Washington, Seattle, Washington 98195

Received August 22, 1997

Interleukin-15 (IL-15) is a cytokine which is highly expressed in skeletal muscle, and which stimulates muscle protein accretion in cultured skeletal muscle fibers. Using parental C2 skeletal myoblasts, no significant effects of IL-15 on skeletal muscle differentiation were observed. To test the hypothesis that IL-15 may stimulate skeletal muscle differentiation if the strong differentiation-inducing effects of autocrine insulin-like growth factor (IGF) production were inhibited, a C2 myoblast subline (C2-pBP4) was stably transfected with an expression vector for rat IGF binding protein-4 (IGFBP-4). Differentiation responses to autocrine and exogenous IGFs in C2-BP4 myoblasts were reduced 3- to 4-fold in C2-BP4 cultures compared to C2-pLXSN cultures, a subline transfected with a control plasmid. Addition of IL-15 to C2-pBP4 myoblasts doubled the number of differentiated muscle cells which arose. These findings indicate that IL-15 can stimulate myogenic differentiation in conditions in which the strongly differentiative effects of the IGFs are inhibited. The differentiative activity of IL-15 may be of physiological significance in conditions in which IGF concentrations are low or in which the IGFs are sequestered by binding proteins.

Interleukin-15 (IL-15) is a recently discovered cytokine with activities that are similar to those of IL-2, such as stimulation of natural killer (NK) cell activity and stimulation of lymphoid cell proliferation (1). These functions are mediated by the β and γ subunits of the IL-2 receptor, plus an IL-15-specific α subunit (2,3). IL-15 is an approximately 15 kDa protein which displays no sequence homology to other cytokines (1). IL-15 can be classified as a cytokine not only by virtue of its activities and receptor, but also by its predicted tertiary structure, a four-helix bundle (1).

Multiple tissue Northern blots revealed that IL-15 is highly expressed in skeletal muscle tissue (1). Previous work by our laboratory demonstrated that IL-15 is anabolic for differentiated mouse C2 and bovine primary skeletal muscle cells in culture (4). We showed that after cultured myogenic cells underwent differentiation and fusion into multinucleated muscle cells known as myotubes, the addition of IL-15 increased myosin heavy chain (MHC) accumulation in these cells (4). However, we were unable to detect any significant effects of added IL-15 on proliferation or differentiation rates of cultured skeletal myoblasts, the dividing muscle precursor cells (4). With the myogenic cell types utilized in that study, it was possible that the strong differentiation-promoting effects of autocrine insulin-like growth factor (IGF-I and IGF-II) production (5-7) may have masked any differentiation-stimulating effects of IL-15.

In the present study, we investigated the issue of possible differentiation-stimulating effects of IL-15 utilizing a subline of C2 myoblasts (C2-pBP4) which were transfected with an expression plasmid for rat IGF binding protein-4 (IGFBP-4). IGFBP-4 inhibits IGF action by sequestration of IGF, preventing binding of both IGF-I and IGF-II to the type-1 IGF receptor (8,9). C2-pBP4 myoblasts underwent differentiation at lower rates compared to myoblasts transfected with a control plasmid (C2-pLXSN), and were refractory to the differentiation-stimulating effects of exogenous IGF-I. Addition of IL-15 to C2-pBP4 myoblasts doubled the number of differentiated cells which arose, indicating that the differentiation-stimulating effects of IL-15 can be discerned in conditions in which the influence of the IGFs on this process are reduced. The differentiative activity of IL-15 may be of physiological significance in conditions in which IGF concentrations are low or in which the IGFs are sequestered by binding proteins.

Abbreviations: IL-15, interleukin-15; IGF-I and IGF-II, insulin-like growth factors -I and -II; IGFBP, IGF binding protein; FCS, fetal calf serum; DME, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSC, sodium chloride/sodium citrate; EtBr, ethidium bromide; MRF, muscle regulatory transcription factor.

0006-291X/97 $25.00

MATERIALS AND METHODS

Materials. Simian recombinant IL-15 was obtained from Immunex Research and Development Corporation, Seattle, WA. MF-20, a monoclonal antibody directed against muscle-specific myosin heavy chain, was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA. The rat IGFBP-4 cDNA was a gift from Dr. S. Shimaski, Whittier Institute, La Jolla, CA. C2 cells were obtained from Dr. D. Yaffe, Weizmann Institute, Rehovat, Israel. Des(1-3)IGF-I was obtained from Diagnostic Systems Laboratory (DSL), Webster, TX. The LXSN plasmid was obtained from Dr. B. Osborne, University of Washington, Seattle, WA. Other materials (culture medium, chemicals, serum, secondary antibodies, molecular biology reagents and kits) were from standard commercial sources.

Plasmid construction and cell culture. The Moloney murine leukemia virus-based LXSN plasmid (10) expresses an inserted gene (X) from the retroviral long terminal repeat (L). The plasmid also contains the neomycin phosphotransferase gene (N) controlled by an internal SV40 promotor (S). To construct the LBP4SN plasmid (pBP4), a 0.989 kb cDNA fragment containing the full length coding sequence of rat IGFBP-4 was ligated into the *EcoR*1 and *Xho*1 sites, oriented 5′ to 3′, of pLXSN. The IGFBP-4 cDNA included 189 bp of the 5′ untranslated region, the 761 bp coding sequence, and 42 bp of the 3′ untranslated region. Unmodified pLXSN was used as a control vector. C2 myoblasts cultured at low densities in 10% fetal calf serum (FCS; Hyclone) in Dulbecco's modified Eagle's medium (DME; Sigma, St. Louis, MO) were transfected with pLXSN and pBP4 using Lipofectin (GIBCO, Grand Island, NY). Two days after transfection, cells were selected in 1.0 mg/ml Geneticin (G418; GIBCO) and polyclonal populations expanded, pooled, and used for analysis.

For Northern blot analysis of IGFBP-4 mRNA expression, C2-pLXSN and C2-pBP4 cells were cultured for 24 h in 10% FCS/DME, or 3 additional days in 0.5% FCS/DME, to harvest myoblast and myotube RNA, respectively. Total RNA was extracted using guanidinium thiocyanate-phenol-choroform (11). Ten micrograms total RNA was electrophoresed through a 1.2% agarose/2.2 M formaldehyde gel, stained with 2 μg ethidium bromide (EtBr) and blotted onto a Genescreen nylon membrane (New England Nuclear-Dupont, Boston, MA). Blots were probed with a rat IGFBP-4 *EcoR*1/*Hind*111 845 bp cDNA fragment radiolabeled using a random priming kit (Promega, Madison, WI). Blots were hybridized for 16 h at 42°C then washed 30′ in 2× SSC, 30′ in 2× SSC and 1% SDS at RT, and 30′ in 0.2% SSC and 1% SDS at 65°C and exposed to X-ray film.

Assays for muscle differentiation and proliferation. To assay muscle differentiation, parental C2 myoblasts, C2-pLXSN myoblasts, or C2-pBP4 myoblasts were plated onto 35 mm culture dishes in 10% FCS/DME at 2 $\times 10^5$ cells per dish and allowed to attach overnight. The next day, the cells were changed to 0.5% FCS/DME with various growth factors. Human/simian recombinant IL-15 (Immunex) was used at 10 ng/ml; human recombinant IGF-I (UBI, Lake Placid, NY) and des(1-3)IGF-I (DSL) were used at 1-100 ng/ml. Myoblasts were cultured in 0.5% FCS/DME with various growth factors for 1-4 days, with medium changes and replenishment of growth factors on alternate days. Muscle differentiation was assessed by immunofluorescent staining for muscle-specific myosin heavy chain (MHC) using the MF-20 monoclonal antibody (12) and FITC-labeled secondary antibodies as previously described (13). Nuclei were counterstained with EtBr, which fluoresces red/orange in the FITC filter (13). The numbers of total nuclei per field and the numbers of nuclei within MHC-positive cytoplasm per field were counted in ten fields per dish. Each experiment was repeated three times independently. Differentiation was also assessed by Western blot analysis of MHC expression. Cells were cultured as above and harvested in SDS reducing sample buffer. Protein samples were resolved in 7.5% SDS-PAGE gels, transferred to nitrocellulose, and probed for MHC using the MF-20 antibody as described previously (13). Signals were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL), and quantified using an image analyzer equipped with MCID version 4.2 software (Imaging Research, St. Catharines, Ontario, Canada).

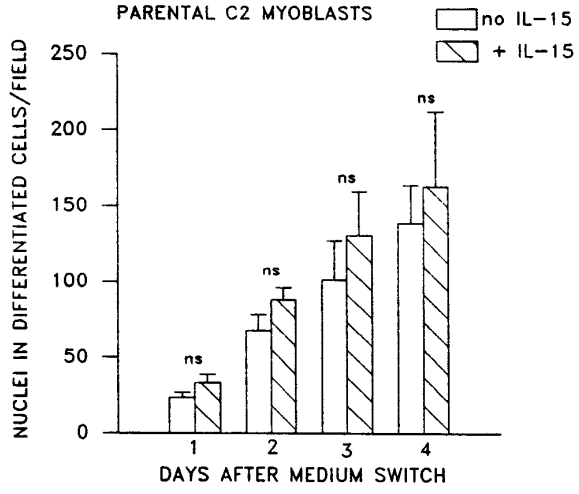

FIG. 1. Effects of 10 ng/ml IL-15 on differentiation in parental C2 myoblast cultures. No significant effects of IL-15 on differentiation were observed. Differentiation was assessed by immunofluorescent staining for MHC. At each time point, mean numbers of differentiated cells in the group treated with IL-15 were compared to those in the control group; ns denotes not significantly different, t-test.

RESULTS

Effects of IL-15 on Differentiation of Parental C2 Myoblasts

Parental C2 myoblasts were cultured in low serum medium ± 10 ng/ml IL-15 for 1-4 days to determine the effects of IL-15 on myogenic differentiation. Differentiation was assayed immunocytochemically by counts of nuclei within MHC-stained cytoplasm. Immunocytochemical assay of differentiated cells (as opposed to soluble biochemical assays of muscle differentiation markers) eliminated from consideration any post-differentiative effects of IL-15 on muscle anabolism.

On each day of culture, no significant differences (t-tests) in the rate of differentiation were observed between C2 myoblasts cultured with or without IL-15 (figure 1). Previous studies (4) indicated that IL-15 was not mitogenic for C2 myoblasts, nor did it inhibit myoblast proliferation.

Although not significantly different, a consistent pattern of slightly higher numbers of differentiated nuclei were detected in the IL-15-treated cultures on each day of analysis. A similar pattern was observed with primary bovine myogenic cultures (not shown). Since IL-15 has been shown to increase MHC accumulation in differentiated myotubes in culture (4), this could be due to slightly faster rates of immunocytochemical detection of newly-formed muscle cells in the cultures. Alternatively, the lack of a significant effect of IL-15 on differentiation in parental cells could be due to

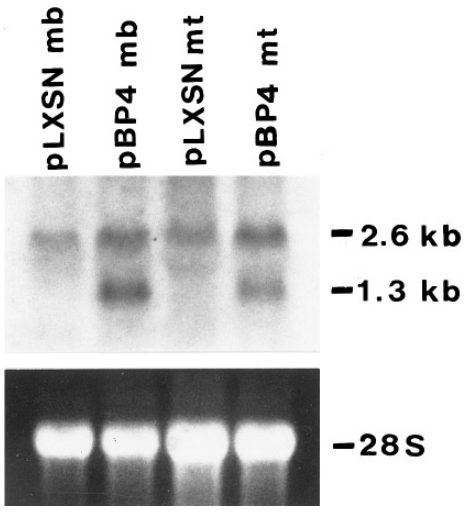

FIG. 2. Northern blot for IGFBP-4 expression in C2-pLXSN (control) and C2-pBP4 myoblasts (mb) and myotubes (mt). The endogenous IGFBP-4 transcript (2.6 kb) is expressed in all four cell types; C2-pBP4 myoblasts and myotubes also express an additional 1.3 kb transcript from the introduced gene. Lower panel is ethidium bromide (EtBr) of 28S ribosomal RNA band (loading control).

masking by the strong differentiation-stimulating effects of IGF-I and IGF-II, which are produced in large amounts by cultured C2 myoblasts in an autocrine fashion (5-7). The next series of experiments were designed to test this hypothesis.

Characterization of C2-BP4 and C2-pLXSN Myoblasts

In order to reduce the effects of autocrine IGF-I and IGF-II on C2 myoblast differentiation, C2 myoblasts were transfected with an expression plasmid (pLBP4SN) and with a control plasmid (pLXSN) to produce two polyclonal sublines of C2 myoblasts (C2-pBP4 and C2-pLXSN) after selection in antibiotics. Both C2-pBP4 myoblasts and myotubes express a 1.3 kb IGFBP-4 mRNA transcript from the construct, in addition to the endogenous 2.6 kb IGFBP-4 mRNA transcript expressed by C2-pLXSN myoblasts and myotubes (figure 2).

As measured by total MHC production, C2-pBP4 myoblast cultures were less sensitive to the differentiation-stimulating effects of exogenous IGF-I than were C2-pLXSN myoblast cultures (figure 3,A). At 0 and 1 ng/ml IGF-I, very little MHC was detected in C2-pBP4 cultures compared to the controls. The addition of 10 and 100 ng/ml IGF-I progressively stimulated MHC accumulation in C2-pBP4 cultures, presumably by exceeding the binding of endogenous and exogenous IGFs by the overexpressed IGFBP-4. Figure 3,B shows that C2-pBP4 and C2-pLXSN myoblasts were equally responsive to the differentiation-stimulating effects of des(1-3)IGF-I, an analog of IGF-I with greatly reduced affinity for IGFBPs, but normal affinity for the type-1 IGF receptor (14). These observations indicate that C2-pBP4 myoblasts were differentiation-competent, and that the reduced rate of differentiation in this sub-line is due to sequestration of IGFs by the overexpressed IGFBP-4.

Immunocytochemical assessment also indicated that C2-pBP4 myoblasts had rates of differentiation which were 3-4 times lower than those of C2-pLXSN myoblasts (figure 4, A). At all concentrations of IGF-I tested

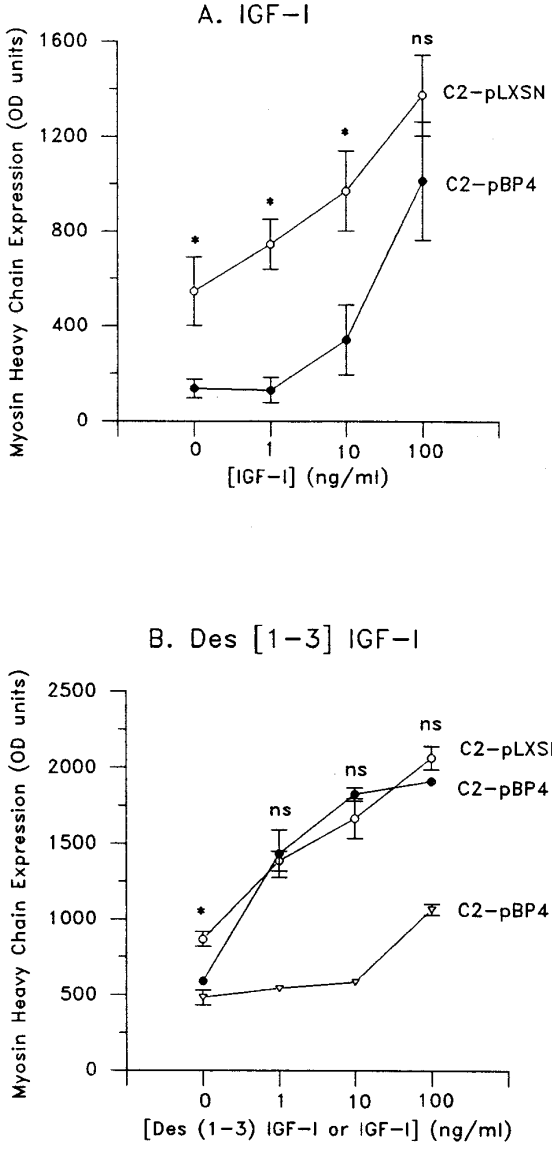

FIG. 3. Differentiation of C2-pLXSN and C2-pBP4 myoblasts in the presence of 0–100 ng/ml IGF-I (A) or 0–100 ng/ml des(1-3)IGF-I (B). Differentiation was assessed by Western blot analysis of MHC production. Asterisks denote significant difference at $p<0.05$ (t-test); ns denotes not significantly different. In panel B, "Des" refers to addition of des(1-3)IGF-I. Also in panel B, t-tests compared C2-pLXSN+Des to C2-pBP4+Des; curve of C2-pBP4+IGF is included for comparative purposes. Points represent the means (± SEM of three independent experiments.

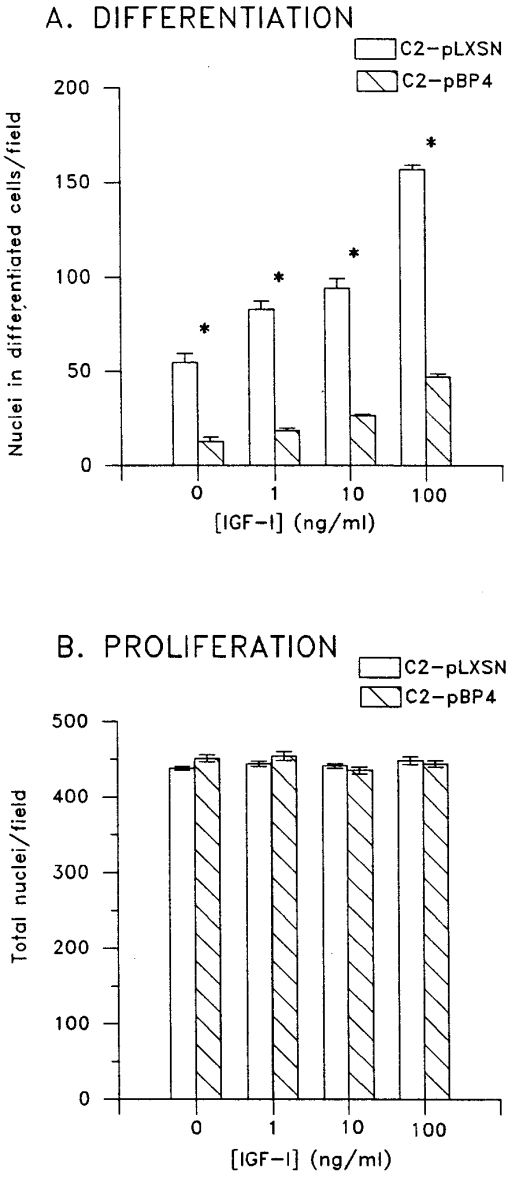

FIG. 4. Differentiation (A) and proliferation (B) of C2-pLXSN and C2-pBP4 myoblasts assessed immunocytochemically and by counts of nuclei. Asterisks denote significant differences ($p<0.05$, t-tests) between C2-pLXSN and C2-pBP4 at each concentration of IGF-I. Total numbers of nuclei (B) were not different at each concentration of IGF-I.

(0-100 ng/ml), the numbers of differentiated cells which arose were significantly different ($p<0.05$, t-test). Cell numbers were not significantly different in the two sets of cultures (t-tests). Thus, the percentages of differentiated cells which arose were 3-4 times lower in C2-pBP4 cultures.

Effects of IL-15 on Differentiation in C2-pBP4 and C2-pLXSN Myoblasts

C2-pBP4 and C2-pLXSN myoblasts were treated with 10 ng/ml IL-15 for 5 days following the switch to low serum medium. The cultures were fixed and stained immunocytochemically for MHC-positive cells to determine the effects of IL-15 on differentiation in the two cell types (figure 5). Addition of IL-15 to control C2-pLXSN cells increased the numbers of differentiated cells by less than 30%. In this case, the difference was significant at $p<0.05$ (t-test). In contrast, addition of IL-15 to C2-pBP4 myoblasts doubled the number of differentiated cells which arose ($p<0.05$, t-test). IL-15 did not, however, increase C2-pBP4 differentiation to the levels of those of C2-pLXSN cultures ($p<0.05$, t-test). These findings indicate that IL-15 can stimulate myogenic differentiation in conditions in which the effects of IGF on myoblasts were reduced.

DISCUSSION

Previous work from our laboratory indicated that IL-15 was anabolic for cultured skeletal myotubes (4). IL-15 stimulated accretion of MHC in cultured C2 myotubes and primary bovine muscle fibers. The mechanism of the anabolic effect of IL-15 on skeletal muscle fibers is at present unknown; one possibility is that IL-15 stimulates expression or action of one or more of the basic helix-loop helix muscle regulatory transcription factors (MRFs) such as MyoD1 or myogenin (15). These factors are also involved in skeletal muscle differentiation, but no significant effects of IL-15 on myoblast differentiation could be observed using parental C2 myoblasts or primary bovine myoblasts (4).

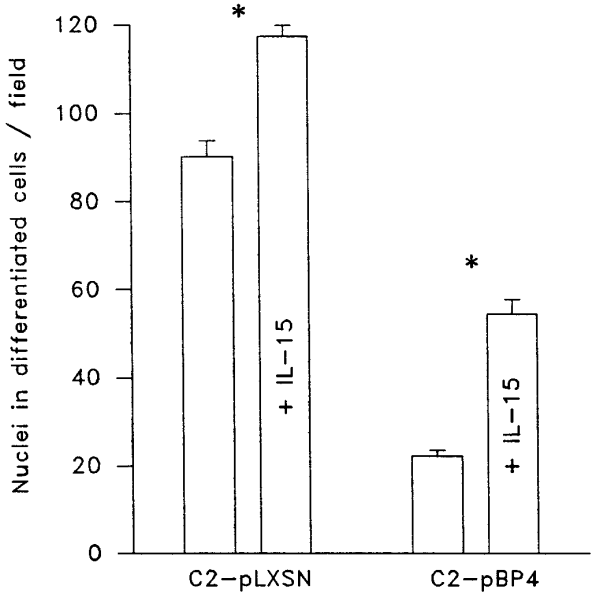

FIG. 5. Differentiation of C2-pLXSN and C2-pBP4 myoblasts with and without 10 ng/ml IL-15. Asterisks denote significant differences between the IL-15-treated group and control for each cell type ($p<0.05$, t-test). Data shown are the results of a single experiment, which was repeated three times with similar results.

The work reported here tested the hypothesis that in C2 myoblast cultures, the strong differentiation-stimulating effects of autocrine IGF expression (7) mask possible differentiative actions of IL-15. To do so, we developed a sub-line of C2 myoblasts (C2-pBP4) with reduced sensitivity to the IGFs due to increased expression of IGFBP-4. This binding protein sequesters IGF from IGF receptors, and in our study significantly reduced the rate of myogenic differentiation. However, by the use of des(1-3)IGF-I, an analog of IGF-I which binds to IGF receptors but exhibits greatly reduced affinity for IGFBPs (14), we showed that C2-pBP4 myoblasts remained fully competent to differentiate.

When the effects of IGF on myogenic differentiation were reduced, a differentiation-stimulating effect of IL-15 on skeletal myoblasts could be clearly discerned. IL-15 doubled the number of C2-pBP4 myoblasts which differentiated. Although IL-15 did not raise C2-pBP4 differentiation to levels equal to those of the control cells, differentiation in control myoblast cultures may have reflected the combined activities of autocrine IGF and IL-15. The differentiative activity of IL-15 may be of physiological significance in conditions in which IGF concentrations are low, or in which the IGFs are sequestered by binding proteins. Such situations may occur in catabolic states or in normal aging (16,17). IL-15 mRNA is expressed in adult human muscle (1), suggesting a physiological role for IL-15 in muscle regeneration and/or maintenance.

A number of growth factors which repress skeletal myoblast differentiation have been identified; these factors inhibit both transcription of the MRFs and the activity of the MRFs (15). Much less attention has been directed at factors which stimulate myogenic differentiation. The IGFs, which are similar to IL-15 in that they induce myoblast differentiation and muscle anabolism, may act by stimulating myogenin expression (13,18). These studies point to regulation of MRF expression or action as a key control point in myogenic differentiation. The present observation that IL-15 stimulates myoblast differentiation, coupled with previous observations indicating that IL-15 is anabolic for cultured muscle fibers, suggests that IL-15 may also act on MRF expression or action. Further work is needed to determine the mechanism of action of IL-15 on skeletal myoblasts and muscle fibers.

ACKNOWLEDGMENTS

We thank Dr. B. Osborne (University of Washington) for providing the pLXSN vector, Dr. S. Shimasaki (Whittier Institute for Diabetes and Endocrinology, Scripps Memorial Hospital) for the rat IGFBP-4 cDNA, Dr. D. Yaffe (Weizmann Institute) for the C2 cells, Dr. G. Merriam (VA Puget Sound) for use of the image analyzer, and Immunex Research and Development Corporation for the IL-15. We also thank Drs. R. Drivdahl and S. Plymate, VA Puget Sound, for advice and assistance. The MF-20 antibody (contributed by Dr. D. Fischman, Cornell University) was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Science, Johns Hopkins University School of Medcine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under Contract N01-HD-6-2915 from the NICHD. This work was supported by the Department of Veterans Affairs, USDA Grants 93-37206 and 96-35206-3858 to LSQ, and by a pilot grant to LSQ from the Nathan Shock Center of Excellence in Basic Biology of Aging (NIH/NIA AG 13280).

REFERENCES

1. Grabstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., Beers, C., Richardson, J., Schoenborn, M. A., Ahdieh, M., Johnson, I., Alderson, M. R., Watson, J. D., Anderson, D. M., and Giri, J. (1994) *Science* **264,** 965–968.
2. Giri, J., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. (1994) *EMBO J.* **13,** 2822–2830.
3. Giri, J. G., Kumaki, S., Ahdieh, M., Friend, D. J., Loomis, A., Shanebeck, K., DuBose, R., Cosman, D., Park, L. S., and Anderson, D. M. (1995) *EMBO J.* **14,** 3654–3663.
4. Quinn, L. S., Haugk, K. L., and Grabstein, K. H. (1995) *Endocrinol.* **136,** 3669–3672.
5. Tollefsen, S. E., Sadow, J. L., and Rotwein, P. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1543–1547.
6. Tollefsen, S. E., Lajara, R., McCuscker, R. H., Clemmons, D. R., and Rotwein, P. (1989) *J. Biol. Chem.* **264,** 13810–13817.
7. Florini, J. R., Magri, K. A., Ewton, D. Z., James, P. L., Grindstaff, K., and Rotwein, P. (1991) *J. Biol. Chem.* **266,** 15917–15923.
8. Cheung, P. T., Smith, E. P., Shimasaki, S., Ling, N., and Chernausek, S. D. (1991) *Endocrinol.* **129,** 1006–1015.
9. Kiefer, M. C., Schmid, C., Waldvogel, M., Schlapfer, I., Futo, E., Masiarz, F. R., Green, K., Barr, P. J., and Zapf, J. (1992) *J. Biol. Chem.* **267,** 12692–12699.
10. Miller, A. D., and Rosman, G. J. (1989) *Biotechniques* **7,** 980–990.
11. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
12. Bader, D., Masaki, T., and Fischman, D. A. (1982) *J. Cell Biol.* **95,** 763–770.
13. Quinn, L. S., Steinmetz, B., Maas, A., Ong, L., and Kaleko, M. (1994) *J. Cell. Physiol.* **159,** 387–398.
14. Francis, G. L., Upton, F. M., Ballard, F. J., McNeil, K. A., and Wallace, J. C. (1988) *Biochem. J.* **251,** 95–103.
15. Olson, E. N. (1992) *Devel. Biol.* **154,** 261–272.
16. Schwartz, R. S. (1995) *J. Gerontol.* **50A,** 151–156.
17. Orlowski, C. C., Brown, A. L., Ooi, G. T., Yang, Y. W., Tseng, L. Y., and Rechler, M. M. (1990) *Endocrinol.* **126,** 644–652.
18. Florini, J. R., Ewon, D. Z., and Roof, S. L. (1991) *Molec. Endocrinol.* **5,** 718–724.