The mammalian insulin-like growth factor II/cation-independent mannose 6-phosphate receptor (IGF-II/CIMPR) mediates both targeting and endocytosis of mannose 6-phosphate-containing proteins and binds insulin-like growth factor II (IGF-II). The cation-dependent mannose 6-phosphate receptor (CDMPR) lacks an IGF-II-binding site and participates only in the intracellular trafficking of lysosomal enzymes. During terminal differentiation of the myogenic C2 cell line, there is an increase in cell surface expression of the IGF-II/CIMPR in parallel with a rise in secretion of IGF-II (Tollefsen, S. E., Sadow, J. L., and Rotwein, P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1543–1547).

In this study we show that IGF-II/CIMPR mRNA increases by more than 10-fold during the initial 48 h of C2 muscle differentiation with kinetics similar to the rise in IGF-II mRNA. Comparable levels of both mRNAs are expressed in C2 myotubes and in primary cultures of fetal muscle. By contrast, no change is observed in CDMPR transcript abundance during differentiation, and only a small, transient increase is seen in the enzymatic activities and mRNA levels of several lysosomal enzymes. The differential regulation of the two mannose 6-phosphate receptors during muscle differentiation suggests that they may serve distinct functions in development.

The mammalian insulin-like growth factor II/mannose 6-phosphate receptor (IGF-II/CIMPR) is a multifunctional protein with distinct high affinity binding sites for two classes of ligands: mannose 6-phosphate containing lysosomal enzymes and growth factors, and IGF-II (1, 2). A critical role for the IGF-II/CIMPR in sorting newly synthesized lysosomal enzymes has been demonstrated (3, 4), although cells that lack this receptor still target lysosomal enzymes because of the presence of a second, cation-dependent mannose 6-phosphate receptor (CDMPR) (5). The reason for the existence of two functionally similar receptors is not known. The two mannose 6-phosphate receptors are localized to the same subcellular compartments but differ in their ligand specificity. Both receptors transport lysosomal enzymes from the trans-Golgi to a prelysosomal compartment and also cycle to the cell surface (2, 5, 6). Only the IGF-II/CIMPR, however, internalizes extracellular ligands and binds IGF-II (7, 8).

In contrast to the established role of the IGF-II/CIMPR in lysosomal enzyme targeting, its function with regard to IGF-II action is controversial (9), since not only are many of the effects of IGF-II in growth and development mediated through the IGF-I receptor (10–12), but in two nonmammalian vertebrates the CIMPR does not bind IGF-II (13, 14). In mammals, however, a role for the IGF-II/CIMPR in transmembrane signaling triggered by IGF-II is supported by the following observations. In human myoblasts, the metabolic effects of IGF-II are only partially inhibited by anti-IGF-I receptor antibodies (15), and in K562 human erythroleukemia cells, which lacks the IGF-I receptor, IGF-II promotes cell replication (16). In addition, there is evidence linking the IGF-II/CIMPR to several second messenger systems. Upon IGF-II binding, the receptor has been shown to activate calcium channels (17), to stimulate inositol phosphate turnover (18), and to interact with guanine nucleotide-binding proteins (19).

Muscle development has been a useful model system for examining the expression and actions of IGFs and their receptors (20, 21). We have previously reported (21) that there is a coordinate increase in IGF-II mRNA and protein and in the cell surface expression of the IGF-II/CIMPR during the terminal myogenic differentiation of C2 cells, a cell line originally derived from mouse skeletal muscle (22). We now show that the up-regulation of receptor number in these cells is preceded by a rise in IGF-II/CIMPR mRNA that temporally parallels the increase in IGF-II mRNA. During the same developmental period, we find no change in the abundance of CDMPR mRNA and only a small, transient increase in the enzymatic activities and mRNA levels of several lysosomal enzymes. Our results demonstrate that the two mannose 6-phosphate receptors are regulated by different mechanisms during muscle differentiation, suggesting that each may play a distinct role in developing muscle.

**MATERIALS AND METHODS**

**Cell Culture**—The mouse C2 muscle cell line (22) was grown on 0.2% (w/v) gelatin-coated 150-mm tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 10% fetal bovine serum (GIBCO), antibiotics, and antifungal...
agents at 37 °C in a humidified 5% CO2, 95% air atmosphere. Undifferentiated cells was harvested at about 60% confluency. Differentiated cells was induced at 70–80% confluency by changing the medium to Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum (GIBCO).

Primary muscle cell cultures were established from mouse limb buds of embryonic ages 17–19 days. The tissue was minced, and then cells were dissociated in 0.25% trypsin and 0.05% DNase I in Hank’s balanced salt solution (GIBCO) in the absence of Ca2+ and Mg2+. Cells were plated in Dulbecco’s modified Eagle’s medium supplemented with 5% newborn calf serum and 10% horse serum (GIBCO) on collagenized plates. Under these conditions, myotubes form within 4 days. Differentiated cells were maintained in medium plus 0.5 μM cytosine arabinoside for 4 additional days, until they were harvested and cellular RNA was isolated.

RNA Isolation—Cells were washed three times with cold Earle’s balanced salt solution, collected using a rubber policeman, pelleted, and stored frozen at −80 °C until all samples in a series were harvested. Total cellular RNA was extracted by differential precipitation after homogenizing the cells in guanidinium thiocyanate (23). The integrity of each RNA sample was assessed after electrophoresis in formaldehyde–agarose gels by staining with ethidium bromide. The quantity of RNA was determined spectrophotometrically.

**Molecular Cloning**—A fragment of the mouse IGF-II/CIMPR gene was isolated by plaque purification after screening a λ Charon 28 library with the bovine CIMPR cDNA (24, 25) by standard methods (26). The DNA contained in one hybridizing λ recombinant was digested with restriction enzymes, and a 371-nucleotide exon containing Real fragment was subcloned into plasmid Bluescript/KS (pBS/KS, Stratagene, La Jolla, CA) for use as a hybridization probe.

A 238-nucleotide portion of the mouse CDMPR cDNA was cloned from liver RNA, using the polymerase chain reaction (PCR) (27). First strand cDNA was prepared from total neonatal liver RNA with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Oligonucleotide primers for reverse transcription, for second strand cDNA synthesis, and for PCR amplification were synthesized using phosphoramidite chemistry. The DNA sequence of these primers matched the sequence of the mouse liver and bovine (29) CDMPR cDNAs. DNA amplification employing Tag polymerase and a Perkin-Elmer Cetus DNA thermal cycler (Perkin-Elmer Cetus Instruments) was performed on first strand CDNA using the following parameters: 30 cycles of 1-min denaturation at 94 °C, 2-min annealing at 58 °C, and 3-min extension at 72 °C, followed by a 10-min final extension at 72 °C. The product of this reaction was reamplified with nested primers using the same parameters as above, except that the annealing temperature was 55 °C. Amplified DNA of the expected size was cloned into the HincII site of pBS/KS.

Fragments of the mouse β-hexosaminidase β chain, cathepsins L and B cDNAs, were isolated from adult liver RNA by PCR amplification using specific oligonucleotides derived from published sequences (30–32).

**Nucleotide Sequence Analysis**—Double-stranded plasmid DNA was sequenced by the dideoxy chain termination method (33) using a modified T7 polymerase (Sequenase, U. S. Biochemical Corp.). All sequences were confirmed on both DNA strands.

**Probe Preparation**—Recombinant plasmids were linearized at convenient restriction sites within each inserted DNA fragment or in the polylinker region of pBS/KS 5’ to the cloned DNA (see Figs. 1, 2, and 7). RNA probes complementary to mRNA were synthesized by in vitro run-off transcription using T3 or T7 RNA polymerase and [32P]CTP, as previously described (34). The size and integrity of the probes were analyzed by autoradiography after electrophoresis through 8% urea, 6% acrylamide gels.

**RNA Analysis**—Hybridization–nuclease protection assays were performed as described previously (34). Total cellular RNA (12.5 μg) was incubated with individual RNA probes at 45 °C in a 15-μl reaction volume containing 80% formamide (v/v), 8 mM PIPES, pH 6.7, 80 mM NaCl, and 0.2 mM EDTA for 18–19 h. Samples then were digested with RNases A and T1, followed by treatment with proteinase K and phenol/chloroform extraction. Probe fragments resistant to nuclease digestion were visualized following electrophoresis through 8% urea, 6% acrylamide gels by autoradiography at −80 °C with Kodak XAR5 x-ray film and two Du Pont Laser Plus intensifying screens. Exposure times varied from 6 to 24 h. RNA abundance was calculated after scanning laser densitometry, using an LKB Ultrascan (Pharmacia LKB Biotechnology Inc.). Several exposures of each gel were scanned, and only those giving responses in the linear range were used for analysis.

RNA blots were prepared by standard methods (35). Total or polyadenylated RNA (isolated using the Stratagene poly(A) quick mRNA purification kit) was electrophoresed in formaldehyde–agarose gels, then transferred to nitrocellulose by blotting. The RNA was immobilized on the membrane by UV cross-linking using a Stratagene linker-2400 in the auto cross-link mode (1200 mJ, 30 s). Filters were prehybridized for a minimum of 2 h and then hybridized to RNA probes for 15 h at 60 °C in fresh hybridization solution. Both the prehybridization and hybridization solutions were composed of 50% freshly deionized formamide, 6× SSC, 100 μM NaPO4, pH 6.5, 0.2% SDS, 5 × Denhardt’s solution, and 2 μg EDTA. High-stringency washes were performed for 30 min at 68 °C with two changes of 0.1× SSC and 0.1% sodium dodecyl sulfate. Autoradiography was performed as described above.

**Assay for Lysosomal Enzymatic Activity**—Cells were harvested and stored as described above. Cell pellets were resuspended in 150–500 μl of buffer containing 50 mM Tris·HCl, pH 7.4, 1 mM MgCl2, 1 mM NaCl, and 0.75% Lubrol and were subjected to three 5-min cycles of freezing and thawing and 5 min of sonication in a Branson Ultrasonic Cleaner. Conditioned cell media were collected sequentially after 12 h of conditioning, centrifugation for 10 min at 5000 × g to remove cellular contaminants, and concentrated 20–25-fold using an Amicon P-10 Centrroprep.

Enzymatic activities of β-hexosaminidase and β-glucuronidase were measured in 50 μl of solubilized cell extract, and β-hexosaminidase activity was measured in 100 μl of concentrated medium, as described by Warren (36). Each culture medium was incubated in a total volume of 500 μl in 100 mM sodium acetate, pH 4.6, with 1 mM paranitrophenyl (PNP) glycoside as the substrate (PNP-β-glucosidase or PNP-β-glucuronidase, respectively). After an incubation period of 1–3 h at 37 °C, an equal volume of 1 M Na2CO3 was added, and the absorbance was measured at 400 nm. Experimental values were compared to a standard curve that was constructed using 1–100 nM solutions of PNP in 500 μl of reaction buffer. After subtracting values obtained for substrate blanks, specific activities were calculated as ng of PNP produced/60 min/mg of protein of cell extract.

Protein concentration was determined by using a modified Bradford assay (Bio-Rad) with lysozyme as a standard.

**RESULTS**

**Isolation of a Portion of the Mouse IGFII/CIMPR Gene and a Fragment of the Mouse CDMPR cDNA**—λ-clones containing fragments of the IGF-II/CIMPR gene were isolated from a mouse λ Charon 28 library after screening with bovine CIMPR cDNA (25). The cloned DNA was mapped with restriction enzymes; fragments which contained exons were isolated by high-performance liquid chromatography (HPLC) of cDNA, subcloned into pBS/KS, and sequenced. Fig. 1 illustrates the structure of a genomic DNA subclone that was transcribed in vitro to obtain mouse IGF-II/CIMPR probes. Fig. 1B shows the nucleotide and deduced amino acid sequence of this 165-nucleotide exon that encodes a portion of the extracellular domain of the IGF-II/CIMPR. As indicated in Fig. 1C, the deduced amino acid sequence of this exon is 81% identical with the bovine and 83% with the human IGF-II/CIMPR, confirming the authenticity of the cloned DNA as part of the IGF-II/CIMPR gene. RNA probes transcribed from this IGF-II/CIMPR template hybridized to a 9-kb mRNA species in mouse liver and C2 myotube polyadenylated RNA.2 Thus the length of the mouse receptor mRNA is similar to that seen in bovine and human tissues (24, 37).

Fig. 2A illustrates the structure and Fig. 2B shows the sequence of a 238-nucleotide fragment of the mouse CDMPR cDNA that was cloned from neonatal liver RNA by PCR, using oligonucleotide primers corresponding to conserved regions of the bovine (29) and human (28) CDMPRs. The deduced protein sequence, excluding the primers used in cDNA cloning, is 96% identical with the human and 94% with the bovine CDMPR, as indicated in Fig. 2C. The length of

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2. G. Szebenyi and P. Rotwein, unpublished experiments.
Mannose 6-Phosphate Receptors in Muscle Differentiation

The Accumulation of \( \beta \)-Hexosaminidase in C2 Cell-conditioned Medium Decreases during Muscle Differentiation—As a functional assay for the mannose 6-phosphate receptors, the accumulation of \( \beta \)-hexosaminidase activity in the cell media
**Mannose 6-Phosphate Receptors in Muscle Differentiation**

**Fig. 3. Expression of mannose 6-phosphate receptor mRNAs in differentiating C2 cells.** Total cellular RNA was isolated at the times indicated after exposure of cells to low serum differentiation medium. *Left panel,* RNA (12.5 µg) was hybridized in solution to (A) the IGF-II/CIMPR probe (see Fig. 1A) or (B) the CDMPR probe (see Fig. 2A), and a nuclease protection assay was performed as described under “Materials and Methods.” The sizes of protected fragments are indicated. Autoradiographic exposure times were (A) 20 h and (B) 8 h; C, 5-µg aliquots of the same RNA samples used in A and B were electrophoresed in a formaldehyde-agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. nt, nucleotide. *Right panel,* the bar graphs represent the relative levels ± S.E. of (A) IGF-II/CIMPR mRNA and (R) CDMPR mRNA observed during C2 cell differentiation (*n* = 9 experiments for A and *n* = 5 for B, using four independently isolated sets of RNA). Values were obtained using a scanning laser densitometer and have been normalized to mRNA levels measured in undifferentiated myoblasts that were arbitrarily set to 1.

**Fig. 4. Expression of IGF-II mRNA in differentiating C2 cells.** 12.5 µg of RNA used for the experiment in Fig. 3 was hybridized to a mouse IGF-II probe (21), and a nuclease protection assay was performed as described under “Materials and Methods.” Autoradiographic exposure time was 6 h. nt, nucleotide.

**Fig. 5. Detection of IGF-II and IGF-II/CIMPR mRNA in primary embryonic muscle cultures.** 10 µg of total cellular RNA isolated from two independent cultures (lanes 1 and 2) was hybridized to (A) a mouse IGF-II/CIMPR probe or (B) an IGF-II probe, and nuclease protection assays were performed as described under “Materials and Methods.” Autoradiographic exposure times were (A) 14 h and (B) 2 h. nt, nucleotide.

was measured during C2 myogenic development. Table I shows the results of an experiment in which differentiation medium was conditioned for 12-h periods by C2 cells. Parallelizing the increase in IGF-II/CIMPR mRNA and protein (21), there was a progressive decline in the percentage of lysosomal enzymes found in the medium. Similar results were obtained when the period of conditioning was extended to 24 h. Therefore, the increase in receptor number during differentiation results in more efficient intracellular targeting of lysosomal enzymes.

**DISCUSSION**

In this report, we demonstrate that mRNAs encoding the mouse IGF-II/CIMPR and the CDMPR are regulated differ-
was defined as the ratio of activity in the medium divided by the sum of enzymatic activity found in the cell extracts and in the corresponding medium. The percent activity in the medium during 96 h of differentiation. Both medium and cells were under "Materials and Methods." The percent activity in the medium was defined as the ratio of activity in the medium divided by the sum of enzymatic activity found in the cell extracts and in the corresponding medium.

| Time in differentiation medium | Period of conditioning | Specific activity in cell extracts | Activity in medium |
|-------------------------------|------------------------|-----------------------------------|--------------------|
| h                             | h                      | nM PNP/mg/h                       | % of total         |
| 12                            | 0–12                   | 112                               | 30.8               |
| 24                            | 12–24                  | 180                               | 28.8               |
| 36                            | 24–36                  | 336                               | 18.5               |
| 48                            | 36–48                  | 204                               | 13.9               |
| 60                            | 48–60                  | 156                               | 6.6                |
| 72                            | 60–72                  | 204                               | 8.9                |
| 84                            | 72–84                  | 180                               |                    |
| 96                            | 84–96                  | 156                               | 10.4               |

Fig. 7. Expression of β-hexosaminidase mRNA in differentiating C2 cells. 12.5 µg of total RNA from the RNA series shown in Fig. 3 was used in a solution-hybridization nuclease-protection assay, as described under "Materials and Methods," with a mouse β-hexosaminidase probe. The structure of the probe used in these experiments is illustrated under the autoradiograph. A portion of mouse β-hexosaminidase β chain cDNA was cloned by PCR (box) and was subcloned into pBS/KS (dotted line). The plasmid was linearized at the EcoRI site, and RNA probes were prepared using T3 RNA polymerase. nt, nucleotide.

Fig. 8. Expression of lysosomal thiol protease mRNAs in differentiating C2 cells. Total RNA (3 µg/lane) was blotted onto nitrocellulose and hybridized with RNA probes derived from mouse cDNAs for cathepsin B (CB) and cathepsin L (CL), which were isolated by PCR using primers based on published sequences (31, 32). Sizes of hybridizing mRNAs and the positions of 28S and 18S ribosomal RNAs are indicated.

The kinetics of accumulation of IGF-II/CIMPR mRNA (and protein (21)) in C2 cells correlate closely with those of one ligand, IGF-II, whereas levels of representative lysosomal acid hydrolases and proteases change only transiently during the same period. As a functional consequence of the increase in IGF-II/CIMPR receptor number, a larger percentage of β-hexosaminidase is found intracellularly during C2 cell differentiation, reflecting enhanced intracellular targeting and/or endocytosis.

After the initial discovery of the CIMPR as a lysosomal targeting protein, further observations showing that cells could direct newly synthesized mannose 6-phosphate containing proteins to lysosomes in the absence of this receptor led to the identification and characterization of the CDMPR (1, 2, 38). These two receptors are expressed in the same cells and are colocalized in intracellular compartments (6). To date, no unique roles have been defined for either of the two mannose 6-phosphate receptors with regard to lysosomal enzyme targeting. The receptors are functionally different, however, since the mammalian IGF-II/CIMPR also binds IGF-II (1, 2, 37) and can mediate endocytosis (1, 2, 4). Variation in relative levels of the IGF-II/CIMPR and the CDMPR has been observed in some cell types (39), suggesting that the expression of these receptors may be regulated by different mechanisms and possibly indicating functional differences as well. We now provide evidence that during myoblast differentiation the two mannose 6-phosphate receptors are differentially regulated at the level of their respective mRNAs, reflecting either divergent control of transcriptional activity or of mRNA turnover rates. Our results also suggest that the previously observed up-regulation of the IGF-II/CIMPR on the cell surface of differentiating C2 cells (21) reflects enhanced receptor biosynthesis, rather than receptor redistribution (40).

The kinetics of increase if IGF-II/CIMPR mRNA parallel the change in IGF-II mRNA and secretion (21) seen during differentiation of C2 cells. There are low levels of both mRNAs in dividing, mononucleated myoblasts. Each mRNA increases in abundance during the initial 48 h of differentiation, a period when multinucleated myotubes begin to form and muscle-specific enzymes and structural proteins are synthesized, and both remain elevated for the subsequent 48–72 h. In addition, differentiated embryonic primary muscle cultures express mRNAs for both ligand and receptor at steady-state levels approximating those found in C2 myotubes. Our observations are supported by other studies showing high levels of IGF-II/CIMPR and IGF-II in fetal rat muscle (41–43). The similar pattern of mRNA expression raises the possibility that the genes for IGF-II and the IGF-II/CIMPR are regulated coordinately by common factors during muscle formation.

The mRNA levels and enzymatic activities of several lysosomal enzymes of two different classes, glycosidases and thiol endoproteases, change only transiently and modestly during C2 myoblast differentiation. The mRNAs for the enzymes β-hexosaminidase, cathepsin B, and cathepsin L are expressed in dividing myoblasts and were increased 2–4-fold at only a single time point during differentiation. The enzymatic activities of β-hexosaminidase and β-glucuronidase rose maximally and transiently by 3-fold during the period of C2 cell myotube formation. Although it is possible that some lysosomal enzymes are selectively up-regulated during muscle formation, our data indicate that the increase in the IGF-II/CIMPR in C2 cells correlates best with the expression of IGF-II, rather than with its lysosomal ligands.

In summary, our results show that the IGF-II/CIMPR and
the CDMPR are differentially regulated at the level of their mRNAs during C2 cell myotube formation, suggesting that the two mammalian 6-phosphate receptors have distinct roles during mammalian muscle development. The rise of IGF-II/CIMPR mRNA precedes the previously observed enhancement in the cell surface expression of this receptor in C2 cells. The rate of increase in CIMPR mRNA precedes the previously observed enhancement in the cell surface expression of this receptor in C2 cells. The rise of IGF-II/CIMPR mRNA and protein (21) correlates best with the expression of IGF-II, rather than with any of four lysosomal enzymes. These results indicate a role for the IGF-II/CIMPR in cell muscle formation by mechanisms that could involve modulation of lysosomal enzyme activity and/or growth factor signaling.

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REFERENCES
1. Kornfeld, S., and Mellman, I. (1989) Annu. Rev. Cell Biol. 5, 483–525
2. Dahms, N. M., Lobel, P., and Kornfeld, S. (1989) J. Biol. Chem. 264, 12115–12118
3. Kyle, J. W., Nolan, C. M., Oshima, A., and Sly, W. S. (1988) J. Biol. Chem. 263, 16230–16235
4. Lobel, P., Fujimoto, K., Ye, R. D., Griffiths, G., and Kornfeld, S. (1989) Cell 57, 787–796
5. Stein, M., Zijderhand-Bleekemolen, J. E., Geuze, H., Hasilik, A., and von Figura, K. (1987) EMBO J. 6, 2677–2681
6. Duncan, J. R., and Kornfeld, S. (1988) J. Biol. Chem. 263, 16230–16235
7. Arai, H., and Nakamura, K. (1989) J. Biol. Chem. 264, 16230–16235
8. Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1989) Cell 57, 787–796
9. Roth, R. A. (1988) Science 239, 1269–1271
10. Ewton, D. Z., Falen, S. L., and Florini, J. R. (1987) Endocrinology 120, 115–123
11. Czech, M. P. (1989) Cell 59, 235–238
12. Gammeltoft, S., Haselmark, S. K., Hummel, M., Fehlmann, E., and van Obberghen, E. (1985) EMBO J. 4, 3407–3412
13. Canfield, W. M., and Kornfeld, S. (1989) J. Biol. Chem. 264, 7100–7103
14. Clairmont, K. B., and Czech, M. P. (1989) J. Biol. Chem. 264, 16295–16302
15. Shimizu, M., Webster, C., Morgan, D. O., Blau, H. M., and Roth, R. A. (1989) Am. J. Physiol. 251, E611–E615
16. Tally, M., Li, C. H., and Hall, K. (1987) Biochem. Biophys. Res. Commun. 148, 811–816