Complex biosynthesis of the muscle-enriched iron regulator RGMc

David Kuninger, Robin Kuns-Hashimoto, Ryan Kuzmickas and Peter Rotwein*
Department of Biochemistry and Molecular Biology, Mail code L224, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239-3098, USA
*Author for correspondence (e-mail: rotweinp@ohsu.edu)

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Summary

The recently discovered repulsive guidance molecule c (RGMc or hemojuvelin) gene encodes a putative glycosylphosphatidylinositol (GPI)-anchored protein that is expressed in striated muscle and in liver. Mutations in this gene have been linked to the severe iron storage disease, juvenile hemochromatosis, although the mechanisms of action of RGMc in iron metabolism are unknown. As a first step toward understanding the molecular physiology of this protein, we studied its biosynthesis, processing and maturation. Production of RGMc occurs as an early and sustained event during skeletal muscle differentiation in culture and is secondary to RGMc gene activation. As assessed by pulse-chase studies and cell-surface labeling experiments, two classes of GPI-anchored and glycosylated RGMc molecules are targeted to the membrane and undergo distinct fates. Full-length RGMc is released from the cell surface and accumulates in extracellular fluid, where its half-life exceeds 24 hours. By contrast, the predominant membrane-associated isoform, a disulfide-linked heterodimer composed of N- and C-terminal fragments, is not found in the extracellular fluid, and is short-lived, as it disappears from the cell surface with a half-life of <3 hours after interruption of protein synthesis. A natural disease-associated RGMc mutant, with valine substituted for glycine at residue 320 (313 in mouse RGMc), does not undergo processing to generate the heterodimeric membrane-linked isoform of RGMc, and is found on the cell surface only as larger protein species. Our results define a series of biosynthetic steps leading to the normal production of different RGMc isoforms in cells, and provide a framework for understanding the biochemical basis of defects in the maturation of RGMc in juvenile hemochromatosis.

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Key words: RGMc, Hemojuvelin, Muscle, Juvenile hemochromatosis

Introduction

Iron balance in humans and other mammals depends on the regulated absorption of dietary iron by duodenal enterocytes and the recycling of iron recovered from macrophages by macrophages (Hentze et al., 2004). In hemochromatosis, a heterogeneous hereditary disorder of iron overload, these homeostatic processes are perturbed, causing the abnormal accumulation of iron in many cell types, which, if untreated, leads to tissue damage, organ dysfunction and death (Beutler, 2006). Juvenile hemochromatosis is an uncommon and severe form of this disease that is characterized by a rapid rate of organ damage (Beutler, 2006). Recent studies have identified mutations in a novel gene termed HJV, encoding the protein hemojuvelin, in patients with juvenile hemochromatosis (Lanzara et al., 2004; Papanikolaou et al., 2004), and a targeted knockout of the mouse homologue has yielded a similar disease phenotype (Huang et al., 2005; Niederkofler et al., 2005). The normal function of hemojuvelin in iron metabolism is unknown. In patients with juvenile hemochromatosis and in mice lacking hemojuvelin, levels of gene expression of the liver-derived iron-regulating hormone, hepcidin, are reduced (Papanikolaou et al., 2004; Huang et al., 2005; Niederkofler et al., 2005). Hepcidin limits both iron absorption from the duodenum and release of recovered iron by macrophages through binding to the iron export protein, ferroportin, leading to its internalization and degradation (Nemeth et al., 2004), and thus appears to function as a key nodal point in a final common pathway of iron homeostasis (Verga Falzacappa and Muckenthaler, 2005).

Hemojuvelin is identical to a gene termed repulsive guidance molecule c (RGMc) and represents the third characterized member of a small gene family related to chick RGM, which was originally identified as a protein involved in the correct geographical patterning of retinal axons during brain development (Monnier et al., 2002). Recent studies have demonstrated that chick RGM and its mammalian homologue, RGMa, also may play a role in maintaining neuronal viability in the developing nervous system (Matsunaga et al., 2004; Rajagopalan et al., 2004). RGMb was initially characterized as a novel gene expressed in the dorsal root ganglion of the sympathetic nervous system (Samad et al., 2004), and RGMc was cloned based on its similarity to RGMa and b (Niederkofler et al., 2004; Samad et al., 2004; Schmidtmer and Engelkamp, 2004). The RGM genes encode proteins of similar length and predicted structure; each contains an N-terminal signal peptide, a RGD integrin binding motif, a partial von...
Willebrand type D domain and a C-terminal GPI anchor sequence. We independently identified mouse RGMc as a novel gene that was induced during skeletal muscle differentiation, and showed by in situ hybridization that RGMc mRNA was expressed during early embryonic development in committed muscle precursor cells, and later in fetal muscle, heart and liver (Kuninger et al., 2004). This muscle-enriched pattern of gene expression differs radically from that of RGMa and b, which are found primarily in the central nervous system (Niederkofler et al., 2004; Samad et al., 2004; Schmidtmer and Engelkamp, 2004), although RGMb also has been localized to the rodent reproductive tract (Xia et al., 2005). In this report, we have defined the complex pathway of RGMc biosynthesis, processing and maturation. Our results provide a framework to study the regulation of RGMc or hemojuvelin as a new component of the systemic iron regulatory network, and for assessing the biochemical mechanisms underlying the genetic defects in juvenile hemochromatosis.

Results
Rapid induction of RGMc mRNA and protein expression during muscle differentiation
Little is known about the regulation of RGMc beyond expression of its mRNA in several mouse and human tissues including skeletal muscle, heart, and liver (Niederkofler et al., 2004; Papanikolaou et al., 2004; Samad et al., 2004; Schmidtmer and Engelkamp, 2004), and in cultured muscle cells (Kuninger et al., 2004). As seen in Fig. 1, we now find that RGMc mRNA and protein are induced as early events in muscle differentiation. As shown by RT-PCR, RGMc mRNA levels are upregulated in C2 myoblasts within 12 hours of addition of differentiation medium (Fig. 1A). Induction of RGMc mRNA paralleled the rapid increase in gene expression of the muscle-specific transcription factor myogenin and preceded the accumulation of transcripts for the muscle structural protein, myosin heavy chain. RGMc protein production also was initiated early in muscle differentiation, with multiple bands ranging in size from ~20 to 50 kDa being detected by immunoblotting of whole-cell protein extracts by ~24 hours after addition of differentiation medium (DM), with the rate of increase of different isoforms being similar to the rise in myogenin protein levels (Fig. 1B). RGMc proteins also were produced during the differentiation of other cultured muscle cells. As depicted in Fig. 1C, several RGMc isoforms were detected in extracts from C3H10T1/2 fibroblasts undergoing muscle differentiation after conversion to myoblasts by infection with a recombinant adenovirus encoding the myogenic transcription factor, MyoD. Thus, production of RGMc occurs during muscle differentiation.

To address potential mechanisms accounting for the multiple RGMc protein species detected in differentiating myoblasts, we first tested the hypothesis that the four-exon mouse RGMc gene was transcribed and processed in muscle cells into several distinct mRNAs that contained alternative coding sequences. It has been suggested based on searches of EST databases that differentially spliced RGMc transcripts lacking exon 2 and/or 3 are produced by human tissues, and that these mRNAs potentially encode proteins of different lengths (Papanikolaou et al., 2004). As seen in Fig. 1D, analysis of RNA from differentiating muscle cells by RT-PCR with exon-specific primer pairs was consistent with only a single full-length RGMc transcript being present, thus arguing that alternative RNA splicing could not account for the multiple RGMc protein species observed. As the pattern of immunoreactive RGMc polypeptides is consistent with differentially spliced RGMc transcripts, RGMc mRNA species are proposed to be synthesized in differentiating mouse muscle cells. The upper panel shows a schematic of the mouse RGMc gene. Exons are depicted as boxes, with predicted coding regions in black and introns are indicated by dotted lines. The relative positions of primers and predicted product sizes are displayed below the gene. The lower panel shows results of RT-PCR experiments, using exon-specific primers and RNA from differentiating muscle cells (C2AS12 and C2 myoblasts, 72 hours in DM, Ad-MyoD infected C3H10T1/2 cells, 48 hours). Positions of molecular mass markers are indicated to the left of blots.

Fig. 1. Induction of RGMc mRNA and protein expression during myogenic differentiation. (A) Time course of accumulation of RGMc mRNA and mRNAs for myogenin, myosin heavy chain (MHC) and S17 over 96 hours of differentiation of C2 myoblasts, as measured by RT-PCR. Relative increases in mRNA levels compared with T0 are listed below each time point. (B) Time course of accumulation of RGMc, myogenin and α-tubulin over 96 hours of differentiation of C2 myoblasts, as measured by immunoblotting using whole-cell protein extracts. The arrows indicate different immunoreactive RGMc polypeptides. (C) Detection of RGMc in whole-cell protein extracts from C3H10T1/2 cells infected with Ad-β-gal or Ad-MyoD, and incubated in differentiation medium (DM) for 48 hours. Arrows indicate the immunoreactive RGMc proteins. (D)
proteins detected in myoblasts was also observed in Cos-7 cells expressing recombinant adenoviral-derived RGMc (see later), our results suggest that the multiple RGMc protein isoforms seen in muscle cells derive from a single protein precursor.

Muscle-cell-associated RGMc is a GPI-anchored membrane protein

As seen with RGMa and b, RGMc encodes a recognition sequence at its C-terminus for addition of a GPI-anchor during its biosynthesis, which would result in protein localization to the outer layer of the plasma membrane (Hooper, 2001). We thus performed a series of experiments to determine if in muscle cells, RGMc was found as a GPI-linked cell surface protein. As seen by immunocytochemistry of non-permeabilized differentiated myoblasts, RGMc was located on the surface of multinucleated myofibers (Fig. 2A). Immunostaining was eliminated by pre-absorption of affinity-purified polyclonal anti-RGMc antibodies with an immobilized RGMc-MalE fusion protein, thus indicating specificity of detection. Immunoblotting of isolated muscle membranes with the same antisemur detected three immuno-reactive species of ~50, 35 and 20 kDa (Fig. 2B, left panel). Identical results were observed in streptavidin pull-down experiments after labeling intact muscle cells with a cell-impermeable biotin-containing crosslinking reagent (Fig. 2C). If muscle RGMc is a GPI-anchored protein it should be cleaved from the cell membrane by a PI-specific phospholipase (PI-PLC) (Hooper, 2001). Incubation of differentiated myoblasts with bacterial PI-PLC led to release of the same 50, 35 and 20 kDa RGMc proteins into the culture medium (Fig. 2D). Taken together, the results in Fig. 2A-D demonstrate that differentiated skeletal muscle cells synthesize and process distinct RGMc polypeptides that are targeted to the outer part of the cell membrane. These observations also suggest that the other RGMc isoforms detected in muscle cell protein extracts may represent intracellular precursors. These results also are consistent with the idea that a major portion of muscle-derived RGMc undergoes proteolytic cleavage to yield N- and C-terminal polypeptides, and that these proteins are located on the outer face of the cell membrane.

Fig. 2. Muscle-cell-associated RGMc is a GPI-anchored membrane protein. (A) Immunocytochemistry for cell-surface associated RGMc (red) in differentiated C2 myotubes (left panel). After pre-absorption of polyclonal anti-RGMc anti-serum with bacterially expressed RGMc bound to CNBr-Sephadex, no binding is seen (right panel). Nuclei in both panels are stained with Hoechst 33258 dye (blue). Magnification, 200×. (B) Detection of RGMc by immunoblotting (arrows) using membranes isolated from differentiated (T72) but not undifferentiated C2 myoblasts (T0), after separation by SDS-PAGE under reducing (upper left panel) and non-reducing conditions (right panel). Equivalent amounts of membrane proteins were used, based on equal detection of cadherin (lower left panel). (C) Detection of cell-surface RGMc by immunoblotting (arrows) after labeling membrane proteins of differentiating C2 myoblasts (72 hours in DM) with a cell-impermeable biotin crosslinking reagent (EZ-link), followed by pull-down of protein extracts with streptavadin-agarose (+, cells exposed to EZ link; –, mock-treated cells). Immunostaining for cadherin is shown below. (D) Detection of muscle RGMc (arrows) released from the surface of differentiated C2 cells (T72) after incubation with (+) or without (–) PI-PLC.

Mature, full-length RGMc (lacking signal peptide and GPI-anchor sequences) contains 15 cysteine residues and previous studies using recombinant RGMc have suggested that the 35 and 20 kDa peptides may be joined together by disulfide bonds (Lin et al., 2005; Zhang et al., 2005). To determine if these two species comprise a disulfide-linked heterodimer, muscle proteins isolated from membrane fractions were examined by immunoblotting with anti-RGMc antibodies after SDS-PAGE under non-reducing conditions. As depicted in Fig. 2B (right panel), only a single protein band of ~50 kDa was observed, compared with the three species detected under reducing conditions (left panel).

Analogous results were seen with membrane-associated RGMc produced in undifferentiated C2 myoblasts infected with tetracycline-regulated Ad-HA-RGMc (supplementary material Fig. S1A) and also in whole-cell protein extracts from Cos-7 cells infected with Ad-HA-RGMc (supplementary material Fig. S1B). To ensure that complete protein reduction was being achieved, we evaluated the effects of different reducing and denaturing agents on the pattern of immunoreactive RGMc polypeptides. No differences were observed when addition of 100 mM DTT was supplemented with either β-mercaptoethanol (1%), β-mercaptoethanol plus 8 M urea, or β-mercaptoethanol, 8 M urea and the cysteine alkylating reagent, iodoacetamide (supplementary material Fig. S2). Together these observations indicate that GPI-linked RGMc found on the cell membrane consists of two classes of proteins, the full-length, intact molecule of ~50 kDa, and a disulfide-linked heterodimer composed of 20 kDa N- and 35 kDa C-terminal fragments. These results also indicate that in muscle cells, proteolytic cleavage of RGMc takes place during biosynthesis at or before the protein reaches the cell membrane.

Muscle-derived RGMc accumulates in the extracellular fluid

In addition to its expression on the cell surface, RGMc also...
could be detected in conditioned culture medium of differentiating muscle cells. As assessed by immunoblotting, two immunoreactive proteins of 50 and 40 kDa progressively appeared in the medium of differentiating C2 myoblasts beginning by 24 hours of incubation in DM (Fig. 3A). Identical results were observed in media from both differentiating C2AS12 myoblasts and C3H10T1/2 fetal fibroblasts converted to myoblasts by infection with Ad-MyoD (Fig. 3B).

Mature mouse RGMc is predicted to contain 362 amino acids, with a calculated molecular mass of 39.1 kDa. The largest protein band seen on the cell membrane and in conditioned culture medium is ~50 kDa, indicating that RGMc undergoes other post-translational modifications beyond addition of a GPI anchor. As mouse RGMc encodes three putative Asn-linked glycosylation sites at amino acids 111-113, 206-208, and 365-367, we addressed the question of whether muscle-derived RGMc is a glycoprotein by incubating it with PNGase F, which cleaves Asn-linked sugars at the residue adjacent to the amide bond. As pictured in Fig. 3C, incubation of culture medium from differentiating C2 myoblasts with PNGase F (+) or after mock treatment (−). White arrowheads indicate digestion products.

Production of recombinant RGMc in muscle and non-muscle cells

To extend our analysis of muscle-derived RGMc we examined its processing and localization using tetracycline-inhibited recombinant adenoviruses expressing the full-length, HA-tagged protein (Ad-HA-RGMc) and a derivative lacking the GPI-anchor sequence (Ad-HA-RGMc\(\Delta\)GPI). As shown in Fig. 4A, the pattern of RGMc polypeptides produced in undifferentiated myoblasts infected with Ad-HA-RGMc is similar to that of RGMc synthesized during muscle differentiation (compare with Fig. 1C,D). Surface labeling of cells infected with Ad-HA-RGMc with a biotin crosslinking reagent followed by streptavidin-pull down yielded results similar to those observed with endogenous muscle RGMc, although the relative proportions of the 50 and 35 kDa polypeptides differed slightly (compare Fig. 4A center and right panels with Fig. 2C). Immunoblotting with an antibody to the HA epitope reacted with only the 50 and 20 kDa bands, indicating that these proteins contained an intact N-terminus, whereas the 35 kDa band did not. By contrast, recombinant RGMc engineered without the GPI attachment sequence (Ad-HA-RGMc\(\Delta\)GPI) was not found on the cell surface (Fig. 4A).
HA-RGMc also could be released from the cell membrane by incubation with PI-PLC, leading to the accumulation of 50, 35 and 20 kDa RGMc polypeptides in the medium. Again, the 50 and 20 kDa species were found to contain an intact N-terminus, as they were detected with anti-HA antibodies (Fig. 4B).

Incubation of membrane-associated HA-RGMc with PNGase F after its release from the cell surface by treatment with PI-PLC demonstrated that all three cell-surface proteins are glycosylated (Fig. 4C). Based on the banding pattern seen after exposure to PNGase F, and the location of Asn-linked glycosylation sites within RGMc, we infer that the 50 kDa protein contains three carbohydrate side chains, the 35 kDa C-terminal RGMc polypeptide, two, and the 20 kDa N-terminal fragment, one. Infection of both myoblasts and Cos-7 cells with Ad-HA-RGMc also led to accumulation of ~50 and ~40 kDa immunoreactive proteins in conditioned culture medium (Fig. 4D, Fig. 6A and supplementary material Fig. S1B and Fig. S2), as was seen with muscle-derived RGMc. Both protein bands could be detected by anti-HA antibodies (Fig. 4D, right panel). As with endogenous muscle RGMc, incubation with PNGase F resulted in the appearance of several faster-migrating protein species of ~34 and ~30 kDa that were detected with either anti-RGMc or anti-HA (Fig. 4D).

**Forced expression of RGMc or RGMcΔGPI does not alter muscle differentiation**

In order to determine if the levels of cell-associated or soluble RGMc influenced muscle development, C2 myoblasts were infected with tetracycline-inhibited recombinant adenoviruses, Ad-HA-RGMc or Ad-HA-RGMcΔGPI (see below), and induced to differentiate. As shown in Fig. 5, precocious overexpression of either form of RGMc had no visual impact on the rate or extent of myogenic differentiation, as assessed by myotube formation, when compared with virus-infected cells incubated in differentiation medium plus doxycycline or to non-infected myoblasts (data not shown). Notably, both HA-RGMc and HA-RGMcΔGPI proteins were detected both in multinucleated myotubes and in mononuclear myoblasts. As seen in Fig. 2A, endogenous RGMc also accumulated on the surface of myotubes. Thus, an increase in the amount of RGMc neither enhances nor retards muscle differentiation.

**Biosynthesis and processing of RGMc**

To study the earliest events in the biosynthesis of RGMc, Cos-7 cells were infected sequentially with Ad-tTA followed 12 hours later with either Ad-HA-RGMc or Ad-HA-RGMcΔGPI. Ad-tTA encodes a tetracycline-repressible transcriptional activator that stimulates the promoter regulating expression of the gene encoding HA-RGMc and HA-RGMcΔGPI. Under these conditions, HA-RGMc or HA-RGMcΔGPI was detected by immunoblotting of whole-cell protein extracts as early as 4 hours after viral infection, and accumulated to similarly high levels by 12 hours (Fig. 6A,C). In cells producing HA-RGMc, the 50 and 40 kDa immunoreactive proteins were detected in conditioned culture medium starting at 8 hours, and as demonstrated by cell-surface biotin crosslinking experiments, full-length and proteolytically cleaved RGMc were found on the cell surface from 4 hours after viral infection (Fig. 6B). Based on these results, we conclude that the processing, cell-surface expression, and accumulation in the extracellular fluid of newly synthesized RGMc are all rapid events.

Because it lacks the GPI-anchor, HA-RGMcΔGPI is not found at the cell membrane (Fig. 4A). Rather, as shown in Fig. 6C, the cleaved 35 and 20 kDa RGMc protein fragments appeared rapidly in the conditioned culture medium with kinetics identical to the 50 and 40 kDa immunoreactive proteins. Immunoblotting with anti-HA antibodies confirmed that the 20 kDa band is the N-terminal fragment (Fig. 6D). These results demonstrate that the GPI-anchor sequence is required for membrane retention of the internally cleaved, disulfide-linked 35 kDa C- and 20 kDa N-terminal fragments of RGMc, and indicate that proteolytic cleavage occurs intracellularly during the biosynthesis of RGMc by mechanisms that do not require prior addition of the C-terminal GPI moiety.

We next used pulse-chase experiments to assess the turnover of newly synthesized RGMc and to establish the origin of the immunoreactive RGMc proteins found in extracellular fluid. Cos-7 cells, infected 12 hours earlier with Ad-HA-RGMc, were treated with doxycycline (to block RGMc RNA production) and cycloheximide (to inhibit protein synthesis). To establish that protein biosynthesis was effectively impaired, we examined levels of cadherin, using a pan cadherin antibody.
Fig. 6. Biosynthesis and processing of RGMc and RGMcΔGPI in Cos-7 cells. (A) Time course of accumulation of RGMc in whole-cell protein extracts (WCE) and conditioned culture medium (CM) after infection with Ad-HA-RGMc. Full-length and internally cleaved RGMc proteins are indicated by black or gray arrows, respectively. Immunoblots for tubulin and albumin protein staining (lower panels) indicate equivalent sample loading. (B) RGMc rapidly accumulates at the cell membrane following its biosynthesis. RGMc was analyzed by immunoblotting at the indicated times following infection with Ad-HA-RGMc after labeling of cell membranes with the EZ-link reagent and streptavidin-agarose pull-down. Immunostaining for tubulin and cadherin (lower panels) is included for loading and cell-surface specificity controls, respectively. (C) Time course of accumulation of RGMc in WCE and CM after infection with Ad-HA-RGMcΔGPI. Full-length and internally cleaved RGMc proteins are indicated by black or gray arrows, respectively. Immunoblots for tubulin and albumin protein staining (lower panels) are included to demonstrate equivalent sample loading. (D) Immunostaining with anti-HA antibody of CM samples from C.

As seen in Fig. 7A,B, the abundance of cadherins in whole-cell protein extracts and on the cell surface was reduced by ~50% within 12 hours of addition of cycloheximide, whereas levels of tubulin did not change. Under the same experimental conditions, the amount of RGMc in whole-cell protein extracts declined to 14% of starting values within 4 hours and to 6% by 24 hours. By contrast, after inhibition of protein synthesis RGMc accumulated in conditioned medium by 4 hours, and then remained relatively constant for up to 36 hours. Biotin crosslinking experiments were used to assess the stability of cell-surface-bound RGMc. As depicted in Fig. 7B, there was a rapid decline in the abundance of all membrane-associated RGMc species after protein synthesis was prevented, with a decrease to ~25% of starting values by 4 hours, and a nearly complete disappearance by 24 hours. These results indicate that cell-membrane-bound RGMc turns over rapidly.

We next addressed the origins of the 50 and 40 kDa RGMc polypeptides found in the extracellular fluid. Cos-7 cells infected for 12 hours with Ad-HA-RGMc were incubated for 30 minutes with a cell-impermeable biotin crosslinker followed immediately by addition of cycloheximide and doxycycline. As pictured in the left panel of Fig. 7C, RGMc was detected in conditioned medium within 4 hours after new protein synthesis was blocked, and then remained constant for up to 24 hours, demonstrating as in Fig. 7A that production of RGMc was impaired but that the protein was relatively long-lived in the extracellular fluid. By contrast, the amount of biotin-labeled RGMc in the medium increased modestly over the same interval, as assessed by streptavidin pull-down assay (Fig. 7C). Taken together, this series of observations implies that the 50 and 40 kDa immunoreactive RGMc proteins found in the extracellular fluid are released from the cell surface. As the kinetics of disappearance of membrane-associated RGMc are very rapid after protein biosynthesis is blocked, our results also indicate that the full-length and cleaved isoforms undergo distinct fates. The full-length 50 and 40 kDa proteins, which may be differentially glycosylated, are released into the extracellular environment, where they remain for many hours. By contrast, the 35 and 20 kDa disulfide-linked heterodimer is not found in the extracellular fluid, and presumably is internalized and rapidly degraded intracellularly.

Altered processing of an RGMc mis-sense mutation
To test the hypothesis that at least some of the mutations in RGMc linked to juvenile hemochromatosis represent defects in protein biosynthesis or processing, we developed an expression vector encoding the murine equivalent of the most common disease-associated missense mutation, involving alteration of Gly320 to Val (G313V in mouse RGMc). Upon transfection into Cos-7 cells, the expression plasmid encoding the mutant protein directed the production of immunoreactive RGMc and its apparently normal accumulation in the extracellular fluid (Fig. 8A). However, the pattern of biosynthesis was dramatically different from wild-type RGMc. The cleaved 35 and 20 kDa RGMc fragments were absent in cells expressing RGMc G313V, as assessed in both whole-cell protein extracts and after cell-surface biotin crosslinking studies (Fig. 8B). By contrast, a novel protein of ~40 kDa appeared on the surface of these cells. Immunoblotting with anti-HA antibodies confirmed that the new protein species contained an intact N-terminus (Fig. 8C). Thus, a single amino acid substitution of Gly313 to Val causes radically different maturation of RGMc.

Discussion
Our observations show that RGMc is a secreted glycoprotein produced by skeletal muscle cells beginning early in myoblast differentiation by a pathway that is secondary to induction of
Fig. 7. Differential stability of cell-surface-associated and secreted RGMc proteins in Cos-7 cells. (A) Time course of the decrease in RGMc protein expression in whole-cell protein extracts (WCE) and conditioned culture medium (CM) from Cos-7 cells infected 12 hours earlier with Ad-HA-RGMc and treated with cycloheximide and doxycycline for the times indicated. Immunoblotting for cadherin demonstrates effective inhibition of new cellular protein synthesis and immunoblotting for tubulin and albumin staining show equivalent sample loading (lower panels). Graphical analysis of average band intensity from at least two independent experiments is displayed to the right. (B) Time course of decline in cell-surface RGMc after inhibition of protein synthesis with cycloheximide. Membrane-associated RGMc was labeled by treating cells infected 12 hours earlier with Ad-HA-RGMc with the EZ-link reagent for 30 minutes, followed by addition of cycloheximide and doxycycline for up to 24 hours. Biotin-labeled RGMc or cadherin was detected by immunoblotting following streptavidin-agarose pull-down from WCE. An immunoblot for tubulin (lower panels) is included as a loading control. Average cell-surface expression for two independent experiments of full-length RGMc (fl), RGMc N- and C-terminal fragments (N/C), and cadherin proteins is shown in the graph to the right. (C) Time course of accumulation of biotin-labeled RGMc in conditioned culture medium following treatment of cells with the EZ-link reagent and incubation with cycloheximide and doxycycline for the times indicated. Total and biotin-labeled RGMc in CM was determined by immunoblotting as outlined in B. Average levels for two independent experiments of total and biotin-labeled RGMc are plotted to the right. Black and gray arrows in blots indicate full-length and internally cleaved RGMc proteins, respectively.

Fig. 8. Altered processing of the juvenile hemochromatosis-linked RGMc G313V mutation. (A) Detection of HA-RGMc (WT) and HA-RGMc G313V in whole-cell protein extracts (WCE) and conditioned culture medium (CM) in transiently transfected in Cos-7 cells. Full-length and internally cleaved RGMc proteins are indicated by black or gray arrows, respectively. (B) Detection of RGMc WT and G313V proteins after labeling of cell membranes with the EZ-link reagent and streptavidin-agarose pull down. Immunostaining for tubulin and cadherin (lower panels) is included for loading and cell-surface specificity controls, respectively. (C) Immunostaining with anti-HA antibody of samples from B. In B and C, the full-length, internally cleaved and anomalously processed RGMc proteins are indicated by black, gray or open circles respectively.
RGMc gene expression. We find that myoblasts synthesize several isoforms of RGMc from a single predominant mRNA species, and show that two classes of RGMc molecules are targeted to the outer face of the cell membrane through addition of a GPI anchor during protein biosynthesis and processing. One isoform consists of an intact full-length polypeptide, and the other, N- and C-terminal fragments, held together by interchain disulfide bonds, although the pattern of disulfide bonding among the 15 cysteines present in mature RGMc has not been established (Fig. 9A). Both classes of RGMc molecules appear to be glycosylated at up to three distinct sites. The nature and location of the protease responsible for intramolecular cleavage of RGMc to generate N- and C-terminal fragments is unknown, although our results with a modified RGMc lacking the GPI-recognition sequence support the idea that proteolytic cleavage occurs within the cell during biosynthesis. It has been suggested that cleavage of RGMc occurs within a Gly-Asp-Pro-His sequence (amino acids 164-167) that is conserved in RGMa and RGMb. The mechanism is postulated to be an acid-stimulated catalytic process that is similar to the steps responsible for cleavage of human mucin (Zhang et al., 2005), although our preliminary results do not confirm this pathway (see supplementary material Fig. S3). Whatever the mechanism, the cleavage enzyme must be present in a wide range of cell types, as we observed identical processing for endogenous RGMc in differentiating myoblasts and for adenoviral-expressed RGMc in Cos-7 cells and liver-derived Hep3b and HepG2 cells (not shown).

Full-length RGMc accumulates in the extracellular fluid of cultured muscle cells and in Cos-7 cells expressing adenovirus-derived HA-RGMc, and is represented by two isoforms, each containing an intact N-terminus, and potentially varying in the number of asparagine-linked glycosylation sites. It should be noted however that the mobility of the smallest deglycosylated molecule after reducing SDS-PAGE (~30 kDa) is faster than expected based on the predicted molecular mass of full-length mature RGMc (39.1 kDa). At present, we do not know if migration of deglycosylated RGMc during gel electrophoresis is anomalous or if the results suggest other modifications (see Fig. 9). For example, release of RGMc from the cell surface may reflect the actions of a phospholipase or a protease that differentially targets full-length RGMc and not the disulfide-linked heterodimer of N- and C-terminal fragments. An outline of potential steps leading to production of different RGMc polypeptides is depicted in Fig. 9B.

The role of RGMc in muscle biology is unknown. Expression of RGMc or hemojuvelin does not appear to be required for muscle development, as both juvenile hemochromatosis patients and RGMc knockout mice display grossly normal muscle, although it is possible that subtle defects remain unrecognized. Previous studies by Kang et al. demonstrated that overexpression of the transmembrane protein neogenin or addition of one of its ligands, netrin-2, could enhance myotube formation, and that reduction of endogenous levels of neogenin reduced the size and number of multi-nucleated myofibers (Kang et al., 2004). We also find neogenin to be expressed in cultured muscle cells (data not shown), and because it has been suggested that neogenin may be a receptor for RGMc (Zhang et al., 2005), we addressed the question of whether RGMc influenced muscle cell function or fate. As depicted in Fig. 5, we find that overexpression of RGMc or RGMcΔGPI had no discernible impact on the rate or extent of differentiation of cultured C2 myoblasts, at least as assessed by myotube formation. In related observations, RGMc neither enhanced myoblast survival nor promoted cell death during differentiation (data not shown). Thus the role of RGMc in skeletal muscle remains undefined.

Multiple distinct mutations in the human RGMc gene have been linked to juvenile hemochromatosis (Papanikolaou et al., 2004; Beutler, 2006). These mutations predict the existence of truncated RGMc molecules as well as modified proteins containing single amino acid substitutions that presumably lead to loss of RGMc function in affected individuals (Papanikolaou et al., 2004; Beutler, 2006). In agreement with observations in humans, mice lacking RGMc by targeted gene knockout were shown to have a phenotype that resembled

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Fig. 9. Biosynthesis and processing of muscle RGMc. (A) Diagram of RGMc proteins found on the cell membrane of differentiating skeletal muscle cells and released into the culture medium. Green box, RGD motif; yellow box, partial von Willebrand factor type D domain; gray stars, asparagine-linked sugars; jagged line, GPI-anchor; S-S, disulfide bonds. (B) Model for biosynthesis and processing of RGMc. The arrows designate different processing paths: (1) intracellular proteolytic processing of RGMc (indicated by red scissors) targeted to the cell membrane by a GPI moiety; (2) full-length, glycosylated RGMc is targeted to the cell membrane by a GPI sequence; release of full-length, glycosylated RGMc from the membrane by a phospholipase (3) or protease (4) (black scissors).
juvenile hemochromatosis (Huang et al., 2005; Niederkofler et al., 2005). In these mice, expression of hepcidin in the liver was reduced (Huang et al., 2005; Niederkofler et al., 2005). Hepcidin is a secreted hepatic protein responsible for regulating absorption of iron from the duodenum and its release from macrophages through modulation of cellular levels of the iron transporter, ferroportin (Nemeth et al., 2004). Deficiency of hepcidin leads to an increase in ferroportin and enhanced iron absorption (Ganz, 2005). The mechanisms by which RGMc might influence hepcidin production or secretion are largely unknown, although one report points to regulation of hepcidin gene expression (Lin et al., 2005). Very recent work suggests that RGMc may function as a co-receptor for bone morphogenic proteins (BMPs), enhancing BMP-mediated signaling and potentially leading to hepcidin gene activation in the liver through BMP-activated Smads (Babitt et al., 2006). These authors also reported that two juvenile hemochromatosis-associated mutations (G99V and G313V in the mouse protein) were impaired in their ability to stimulate hepcidin gene expression in conjunction with BMPs. RGMc also has been shown to associate with neogenin in co-expression studies in HEK293 cells, and the combination of neogenin and RGMc led to increased iron uptake via an unknown, apparently hepcidin-independent, process (Zhang et al., 2005). In the same studies, the human RGMc G320V mutation (equivalent to mouse G313V) failed to interact with neogenin and was functionally inert (Zhang et al., 2005).

The results detailed in this manuscript show that a complex series of steps are involved in the normal maturation of RGMc in skeletal muscle and in other cell types. Based on our observations, it seems likely that at least some of the many mutations found in juvenile hemochromatosis would undergo defective biosynthesis or processing. We find that the most common amino acid substitution mutation associated with juvenile hemochromatosis, RGMc G320V (mouse G313V), is impaired in its maturation, and does not form the cell-surface GPI-linked 35 and 20 kDa heterodimer seen in cells expressing wild-type RGMc. We do not know how a single amino acid alteration prevents proteolytic cleavage at a site located over 140 residues proximal to the mutation, nor how the mutant protein, which appears to accumulate normally in the extracellular fluid, is biologically non-functional. Answers to these questions and others will require detailed biochemical and structural studies of wild-type and mutant RGMc molecules, and the identification and characterization of the enzymes involved in their biosynthesis and processing. RGMc mRNA is produced in skeletal muscle, in the heart and in the liver (Brinks et al., 2004; Kuninger et al., 2004; Niederkofler et al., 2004; Papanikolaou et al., 2004; Samad et al., 2004; Schmidtmr and Engelkamp, 2004), and RGMc proteins have been detected in human serum (Lin et al., 2005). It has not been established which of these tissues synthesize the RGMc that regulates hepcidin, and it is not known whether changes in systemic iron metabolism can modulate RGMc biosynthesis, protein processing or secretion in any of these organs or tissues. Moreover, it is possible that RGMc is processed differently in the liver than in muscle, resulting in polypeptides with different functional properties. It is also conceivable that muscle may control hepatic hepcidin expression through its contributions to the circulating pool of RGMc. If true, RGMc may be considered a hormone of iron homeostasis and skeletal muscle, a new component in a systemic iron regulatory network.

Materials and Methods

Expression of RGMc in skeletal muscle

Fetal calf serum (FCS), newborn calf serum (NCS), and horse serum were from Hyclone (Logan, UT). Dulbecco’s modified Eagle’s medium (DMEM) and phosphate-buffered saline were purchased from Mediatech-Cellogrow (Herndon, VA). Doxycycline (Clontech, Palo Alto, CA) was dissolved in distilled water at 1000× concentration (1 mg/ml) and stored at −20°C until use. Protease inhibitor tablets were from Roche Applied Sciences (Indianapolis, IN), okadaic acid was from Alexis Biochemicals (San Diego, CA), and sodium orthovanadate and phosphatidylinositol-specific phospholipase C (PI-PLC) were from Sigma (St Louis, MO). The BCA protein assay kit, EZ-link sulfo-slc-NHS-biotin and streptavidin-agarose (UltraLink) were obtained from Pierce (Rockford, IL). P.NaseF was from New England Biolabs (Beverly, MA). CNBr-Sephadex was purchased from Amersham-Pharmacia (Piscataway, NJ) and cycloheximide from US Biologicals (Swampscott, MA). The nickel affinity reagent, Ni-NTA agarose, was from Qiagen (Valencia, CA). TRIZol Reagent, RT-PCR kit, and trypsin/EDTA solution were purchased from Invitrogen (Carlsbad, CA); TransFET-LT-1 was from Mirus Corp., (Madison, WI), Immobilon-FL was from Millipore Corporation (Billerica, MA) and AquaBlock tm/EIA/WIB solution from East Coast Biologicals (North Berwick, ME). The IGF-I analogue, R3-IGF-I, was purchased from GroPep (Adelaide, Australia) and PDGF-BB was from Invitrogen. The Advantage2 GC PCR kit was from BD Biosciences-Clontech, and restriction enzymes, ligases and polymerases were obtained from BD Biosciences-Clontech and Fermentas (Hanover, MD). Primary antibodies were from the following sources and were used at the indicated dilutions: anti-pan cadherin (Cell Signaling, 1:1000), anti-HA (Covance, Denver, PA, 1:1000), anti-α-tubulin (Sigma, 1:4000), and anti-mycogenin (FSD, 1:500) and anti-mycogenin heavy chain (MF20, 1:200), both from Developmental Studies Hybridoma Bank (Iowa City, IA). Polyclonal antibodies to mouse RGMc were generated as described below. Secondary antibodies were purchased from the following companies, and used at the indicated dilutions: Alexa Fluor 680-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, 1:4000), IRD 800-conjugated anti-rabbit IgG (Rockland, Gilbertsville, PA, 1:4000), Alexa Fluor 594-conjugated goat anti-mouse IgG2a, (Molecular Probes, 1:1000). All other chemicals were reagent grade and were purchased from commercial suppliers.

Generation of polyclonal antibodies to mouse RGMc

A chimeric gene consisting of codons 31-350 of mouse RGMc followed by a 6x His tag was generated by PCR, subcloned into the pET-23d bacterial expression vector (Novagen, San Diego, CA), using 5′ Neo and 3′ HindIII restriction sites, and verified by DNA sequencing. The fusion protein was purified by nickel affinity chromatography after being produced in the BL-21 strain of E. coli, and was used to immunize two New Zealand white rabbits. Antibodies were purified from serum by affinity chromatography with a chimeric protein coupled to CNBr-Sephadex that contained amino acids 31-350 of RGMc fused to the C-terminus of maltose binding protein. The RGMc-MalE fusion protein was produced in the E. coli BL-21 strain from a recombinant gene expressed in the pMAL-c2 vector (New England Biolabs) and was purified by affinity chromatography using amylose resin as recommended by the supplier.

Cell culture

All cells were incubated at 37°C in humidified air with 5% CO2. Murine C2 myoblasts were used between passages 6-12, and were grown on gelatin-coated dishes in growth medium (DMEM with 10% FCS and 10% NCS). Upon reaching confluence, cells were washed twice with phosphate buffered saline (PBS), and incubated in differentiation medium (DMEM with 2% horse serum) for up to 6 days. IGF-II-deficient C2 myoblasts (C2A8S12 cells) (Stewart and Rotwein, 1996) were grown on gelatin-coated dishes in GM with 400 µg/ml G418, and after reaching confluent density were incubated in DM with either R3-IGF-I (2 nM) or PDGF-BB (0.4 nM) for up to 4 days. Murine C3H10T1/2 mouse embryonic fibroblasts (ATCC cat. no. CCL-226) were incubated on gelatin-coated dishes in DMEM plus 10% FCS, Cos-7 cells (ATCC cat. no. CRL-1651) and 293T cells (ATCC cat. no. CRL-11268) were incubated in DMEM with 10% FCS.

Preparation and use of recombinant adenoviruses

A HA-epitope tagged mouse RGMc cDNA in pShuttleTetR has been described (Kuninger et al., 2004). A mutant HA-RGMc, lacking the C-terminal GPI-anchor sequence (amino acids 392-420), was engineered by PCR and subcloned into pShuttleTetR using standard cloning methods. All modifications were verified by DNA sequencing. Recombinant adenoaviral plasmids were produced in E. coli by homologous recombination of PacI-linearized pShuttleTetR and pAdEasy-1 (Stratagene, La Jolla, CA) after electroporation into the E. coli BJ5183 strain. Recombinant adenoviruses were generated after transfection of PacI-linearized adenoaviral DNA into 293T cells, as outlined previously (Wilson et al., 2003).
Individual plaques were picked and screened by PCR, and recombinant viruses were amplified and purified by banding on discontinuous CsCl gradients (Wilson et al., 2003). Viral titers were determined by optical density (OD). Antiviruses encoding the tetracycline transactuator protein (Ad-TTA), mouse MyoD (Ad-MyoD), and bacterial β-galactosidase (Ad-β-gal) have been described (Wilson et al., 2003). For infections of C2 and C2AS12 myoblasts, recombinant adeno-viruses were diluted in DMEM containing 2% FCS (Ad-TTA) at an MOI of 1500 and other viruses at 3000), filtered through a Pall Acrodisk syringe filter (0.45 M), and added to cells at 37°C for 90-120 min. After addition of an equal volume of DMEM with 20% FCS, and incubation for 18 hours, cells were washed and incubated in DM for up to 4 days. For infections of C7-7 cells Ad-HA-RGMc and Ad-HA-RGMcΔGPI were used at MOIs of 250 and Ad-TTA at an MOI of 100. For infections of C3H10T1/2 cells, Ad-MyoD and Ad-β-gal were used at MOIs of 250, and incubated in DM for 3 to 5 days.

Generation and analysis of the RGMc G313V mutant

The cDNA encoding full-length HA-RGMc was blunt-end ligated into EcoRV-digested pcDNA3. The G313V mutation was made with a site-directed mutagenesis kit (Stratagene) using oligonucleotides that used codon wobble to create a PstI restriction site for screening, without altering the reading frame. The mutant cDNA was verified by DNA sequencing. Expression plasmids encoding wild type and mutant RGMc molecules were individually transfected into Cos-7 cells (2 μg plasmid per 35 mm dish) using LI-1 transfection reagent, and cellular protein fractions were evaluated 48 hours later as described below.

Preparation of conditioned culture medium, cellular protein lysates and immunoblotting

Conditioned culture medium (CM) was collected, centrifuged at 3000 g for 5 minutes to remove debris, aliquoted and stored at −20°C until analysis. Where indicated, proteins were concentrated tenfold from CM by precipitation after centrifugation at 14,000 g for 5 minutes, and resuspension of the pellet in SDS-PAGE loading buffer. Whole-cell protein extracts were prepared as described (Wilson et al., 2003), and aliquots stored at −80°C until use. Cytoplasmic and membrane proteins were prepared as described (VanStyke and Musil, 2001). Protein concentrations were determined using the BCA assay kit. For immunoblotting, protein samples were first separated by SDS-PAGE, transferred to nitrocellulose membranes (Unosportics, Westborough, MA), and incubated for 16 hours at 4°C with primary antibodies to RGMc (1:750 dilution), HA (1:1000), myogenin (1:500), and incubated in DM for 1 to 3 days. Immunoblotting of cell surface proteins was performed by incubating monolayer cultures with 1 mg/ml of EZ-link sulfo-NHS-biotin reagent for 30 minutes at 4°C as recommended by the supplier, followed by ‘pull-down’ with streptavidin-agarose of whole-cell protein extracts (1 mg for myoblasts and 80 μg for Ad-HA-RGMc infected cells) or conditioned medium (200 μl), and immunoblotting with antibodies to RGMc or HA. For analysis of proteins attached to the cell membrane by a GPI linkage, monolayer cultures were incubated with 1 U/ml of Pl-PLC for 1 hour at 37°C, and the resultant supernatants (50 μl) analyzed by immunoblotting with antibodies to RGMc or HA. The presence of Asn-linked sugars in RGMc was assessed by immunoblotting after incubation of CM from differentiating C2 myoblasts (500 μl) or from cells expressing Ad-HA-RGMc (5 μl) with PNGaseF (250 or 50 U, respectively) for 16 hours at 37°C, using a protocol from the supplier (Sigma).

Studies of RGMc biosynthesis, processing, and secretion

Cos-7 cells were infected with a recombinant adeno-virus expressing a tetracycline-repressible transcriptional activator (Tet-TA). After 12 hours, cells were infected with adeno-viruses encoding HA-RGMc or HA-RGMc lacking the C-terminal GPI anchor sequence (HA-RGMcΔGPI). Whole-cell protein extracts and conditioned culture media were collected starting 1 hour after infection with the RGMc adeno-viruses, and were assessed by immunoblotting with primary antibodies to RGMc or HA. Biotin crosslinking studies of cell surface proteins were performed as outlined above starting at 4 hours after infection with Ad-HA-RGMc. Analyses of turnover of RGMc were performed beginning 12 hours after infection with Ad-HA-RGMc after addition of cycloheximide (100 μg/ml) and doxycycline (1 μg/ml) to cells. Starting 4 hours later, whole-cell protein extracts and conditioned culture media were collected at intervals and biotin crosslinking experiments were performed.

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