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Regulation of Myoblast Motility and Fusion by the CXCR4-associated Sialomucin, CD164*[S]

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Gyu-Un Bae[‡1], Ursula Gaio[‡2], Youn-Joo Yang[‡], Hye-Jin Lee[§], Jong-Sun Kang[§3], and Robert S. Krauss[‡4]

From the [‡]Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, New York 10029 and the [§]Samsung Biomedical Research Institute, SungKyunKwan University School of Medicine, Suwon 440-746, South Korea

Myoblast fusion is fundamental to the development and regeneration of skeletal muscle. To fuse, myoblasts undergo cell-cell recognition and adhesion and merger of membranes between apposing cells. Cell migration must occur in advance of these events to bring myoblasts into proximity, but the factors that regulate myoblast motility are not fully understood. CD164 is a cell surface sialomucin that is targeted to endosomes and lysosomes via its intracellular region. In hematopoietic progenitor cells, CD164 forms complexes with the motility-stimulating chemokine receptor, CXCR4, in response to the CXCR4 ligand, CXCL12/SDF-1 (Forde, S., Tye, B. J., Newey, S. E., Roubelakis, M., Smythe, J., McGuckin, C. P., Pettengell, R., and Watt, S. M. (2007) *Blood* 109, 1825–1833). We have previously shown that CD164 stimulates myotube formation *in vitro*. We report here that CD164 is associated with CXCR4 in C2C12 myoblasts. Cells in which CD164 levels are increased or decreased via overexpression or RNA interference-mediated knockdown, respectively, show enhanced or reduced myotube formation and cell migration, the latter both basally and in response to CXCL12/SDF-1. Furthermore, expression of CD164 cytoplasmic tail mutants that alter the endosome/lysosome targeting sequence and, consequently, the subcellular localization in myoblasts, reveals a similar correlation between cell motility and myotube formation. Finally, *Cd164* mRNA is expressed in the dorsal somite (the early myogenic compartment of the mouse embryo) and in premuscle masses. Taken together, these results suggest that CD164 is a regulator of myoblast motility and that this property contributes to its ability to promote myoblast fusion into myotubes.

During vertebrate embryogenesis, skeletal muscle precursor cells originate in somites and undergo a multistep process of lineage commitment, migration, and differentiation into myofibers (1). Differentiation is itself a complex process, in which committed myoblasts exit the cell cycle, express muscle-specific genes, alter their morphology, and fuse to form elongated, multinucleated syncytia (2–4). Individual steps in myogenesis, including cell migration and differentiation, are promoted by cell surface proteins, including specific cadherins, integrins, and Ig superfamily members (5–7). CXCR4, a chemokine receptor that regulates migration and survival of hematopoietic and neuronal precursor cells, has recently been shown to play a role in skeletal muscle development (8–11). CXCR4 is expressed in migrating muscle progenitor cells of the vertebrate embryo, whereas the CXCR4 ligand, CXCL12 (aka SDF-1), is expressed in the mesenchyme of the limb, functioning as an attractant for $CXCR4^+$ muscle precursors (9, 10). Consistent with this, $CXCR4^{-/-}$ mouse embryos show defects in migration of these cells and have reduced musculature (9, 10).

Another cell surface protein, the mannose receptor, is also important for myofiber growth *in vivo* and functions *in vitro* to promote fusion with nascent myotubes (12). Mannose receptor stimulates motility and directed migration of myogenic cells, and this was suggested to be a mechanism by which it promoted cell-cell fusion and myotube growth. It is logical that muscle precursor cells must "find" each other while in a motile state and then adhere to one another for differentiation, including fusion, to ensue. Regulation of cell motility might therefore be an important general mechanism in the formation of myofibers *in vivo* and myotubes *in vitro*.

In a screen for cell surface proteins that promote myogenesis, we identified CD164 (aka endolyn), a widely expressed sialomucin implicated in adhesion, proliferation, and differentiation of hematopoietic stem and progenitor cells (13, 14). *Cd164* mRNA is expressed in proliferating C2C12 myoblasts and increases during differentiation of these cells. Stable overexpression of CD164 in myoblasts enhanced myogenesis, whereas expression of a soluble CD164 ectodomain reduced it. Interestingly, alteration of CD164 level or function had a greater effect on formation of multinucleated myotubes than on expression of muscle-specific proteins, suggesting that it might be more involved with regulating myoblast fusion than in coordinating the entire myogenic program (13).

CD164 has an extracellular region with two heavily glycosylated mucin domains, a transmembrane domain, and a 13-amino-acid cytoplasmic tail that terminates with the sequence YHTL (15, 16). This sequence conforms to the $YXX\Phi$ motif (X being any amino acid and Φ being a bulky hydrophobic amino acid) that targets proteins to endosomes and lysosomes via the *trans*-Golgi network (15, 17). A glycine residue preced-

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[1] Present address: Samsung Biomedical Research Institute, SungKyunKwan University School of Medicine, Suwon 440-746, South Korea.

[2] Present address: National Research Center for Environment and Health, Institute of Stem Cell Research, Neuherberg 85764, Germany.

[3] Supported by the T. J. Martell Foundation.

[4] To whom correspondence should be addressed: Box 1020, Mount Sinai School of Medicine, New York, NY 10029. Fax: 212-860-9274; E-mail: Robert.Krauss@mssm.edu.

ing the tyrosine in Y*XX*Φ motifs is necessary for efficient "direct" transport from the Golgi to endosomes, bypassing the cell surface (18, 43). Proteins with Y*XX*Φ motifs that lack glycine at the "Y − 1" position are transported to the plasma membrane prior to entering the endosomal and lysosomal compartments, defining an "indirect" route (18, 43). CD164 has an asparagine residue at the Y − 1 position, and consistent with lack of glycine at this position, in epithelial and hematopoietic progenitor cell lines, CD164 is located primarily in endosomes and lysosomes. However, in a minority of cells, CD164 is prominently expressed at the cell surface (19), suggesting that its subcellular localization might be governed by a regulated mechanism. As specific lysosomal proteins are required for efficient myoblast fusion (20), it is not clear whether CD164 exerts its effects on myotube formation from a vesicular or plasma membrane location. Furthermore, it was recently shown that CD164 associates with CXCR4 in hematopoietic progenitor cells, and RNAi-mediated[5] knockdown of CD164 inhibited migration of these cells toward the CXCR4 ligand, CXCL12 (21). We report here that: 1) CD164 associates with CXCR4 in C2C12 cells; 2) RNAi-mediated knockdown of CD164 inhibits both migration of C2C12 cells toward CXCL12 and myotube formation; and 3) the ability of CD164 cytoplasmic tail mutants to promote myotube formation correlates with cell surface location and ability to promote motility in response to CXCL12. Taken together, these results suggest that CD164 promotes myogenesis by enhancing CXCR4-dependent cell motility.

EXPERIMENTAL PROCEDURES

Expression Vectors—All expression vectors were constructed with a combination of conventional cloning and PCR techniques; complete details are available on request. To construct a GFP-tagged CD164 expression vector, a sequence encoding enhanced green fluorescent protein (GFP) from pEGFP-N1 (Clontech) was inserted between codons 24 and 25 of a complete mouse *Cd164* open reading frame in the expression vector pMV7 (13). This *GFP-Cd164* cDNA was then inserted into the pcDNA3.1 expression vector, and cytoplasmic tail mutants were constructed from this. The N193G, Y194A, and ΔC mutants were constructed by site-directed mutagenesis as described by Makarova *et al.* (22). For RNAi studies, oligonucleotides corresponding to five different mouse *Cd164* sequences were cloned into the pSilencer 2.0-U6 vector (Ambion). Two effective sequences were chosen for further use and correspond to nucleotides 205–223 (RNAi-3) and 473–491 (RNAi-4) of murine CD164 (GenBank™/EMBL/DDBJ accession no. NM_016898); the sequences are: RNAi-3, 5′-CCTGTGCGAGCTTCAACAG-3′ and RNAi-4, 5′-TACCACACTGACTCCAACC-3′. For rescue experiments with RNAi-4 and GFP-CD164 and cytoplasmic tail mutants, seven silent mutations were introduced into each GFP-CD164 construct at the RNAi-sequence (mutations indicated in bold: 5′-TAC**G**AC**TT**T**A**ACA**C**C**G**AC**G**-3′), again by the technique of Makarova *et al.* (22), so as to render them impervious to RNAi-mediated knockdown. All site-directed mutants were confirmed by sequencing.

Cell Culture—C2C12 and 293T cells were cultured as described previously (23). To induce differentiation, C2C12 cells were transferred from medium containing 15% fetal bovine serum into medium containing 2% horse serum (differentiation medium, DM). Quantification of myotube formation, by counting the number of nuclei in myosin heavy chain $(MHC)^+$ cells, was performed as described previously (23). For transient and stable expression of CD164 variants, C2C12 cells were transfected with the appropriate pcDNA3.1-based vector and FuGENE 6 reagent (Roche Applied Science); stable expressors were selected as G418-resistant colonies and pooled for analysis.

For stable RNAi expression studies, C2C12 cells were transfected with pSilencer 2.0-U6 vector containing the RNAi sequences described above or, as a control, an irrelevant sequence, and FuGENE 6, and hygromycin-resistant colonies were selected and pooled for analysis. For experiments in which CD164 variants were assessed for their ability to rescue the effects of *Cd164* RNAi, a transient co-expression approach was employed as described in Kang *et al.* (23). Briefly, C2C12 cells were cotransfected with pSilencer 2.0 U6/RNAi-4, the appropriate pcDNA3.1/GFP-CD164 vectors, and pQ-lacZ, a vector driving expression of nuclear-localized β-galactosidase. Forty-eight h after transfection, the cells were transferred into medium plus 2% horse serum, and 48 h after that, fixed and stained for both MHC and β-galactosidase activity. Transfection efficiencies for these experiments were ~10% to minimize fusion of independent β-galactosidase$^+$ transfectants (23, 24).

Western Blot, Immunoprecipitation, Immunofluorescence, and RT-PCR analyses—Western blot and immunoprecipitation analyses were done as described previously (25). Primary antibodies used were anti-CD164 (R&D Systems), anti-MHC (MF20; Developmental Studies Hybridoma Bank), anti-cadherin (AbCam), anti-GFP (Upstate Biotechnology), anti-CXCR4 (AbCam), and anti-EEA1 (AbCam). For immunofluorescence, C2C12 cells cultured in six-well plates were transfected with 500 ng of control pcDNA3.1 vector or pcDNA3.1/GFP-CD164 constructs. Thirty-six to 48 h later, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with phosphate-buffered saline, 0.5% Triton X-100, blocked with phosphate-buffered saline, 5% goat serum, and probed with anti-cadherin antibodies (1:200; visualized with Alexa Fluor 568 goat anti-mouse antibody (Molecular Probes)) and also visualized for direct GFP fluorescence on a ZEISS LSM-510 Meta confocal microscope. For RT-PCR analyses of *CXCL12* and *Gapdh* mRNA expression, the following primers were used with total RNA and 30 cycles of amplification: *CXCL12* forward primer, 5′-TGCATCAGTGACGGTAAAC-3′, *CXCL12* reverse primer, 5′-TATGCTATGGCGGAGTGTC-3′; *Gapdh* forward primer, 5′-ACCACAGTCCATGCCATCAC-3′; and *Gapdh* reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′.

Subcellular Fractionation—Subcellular fractionation was performed as described previously (26, 27). Briefly, cells were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM

[5] The abbreviations used are: RNAi, RNA interference; DM, differentiation medium; GFP, green fluorescent protein; MHC, myosin heavy chain; RT-PCR, reverse transcription-PCR; E, embryonic day.

NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitor mixture (Roche Applied Science) and fractionated by sequential centrifugation steps. The cell homogenate was centrifuged at 1000 × *g* for 10 min, and the pellet was discarded. The supernatant was centrifuged at 20,000 × *g* for 20 min at 4 °C to separate a vesicle-containing soluble fraction and membrane-containing pellet fraction. The membrane-containing pellet was resuspended in lysis buffer containing 1% Triton X-100, incubated on ice for 30 min, and cleared by centrifugation at 10,000 × *g* for 10 min at 4 °C. The vesicle-containing soluble fraction was centrifuged at 180,000 × *g* for 60 min at 4 °C. The resulting vesicle-containing pellet was resuspended in lysis buffer containing 1% Triton X-100, incubated on ice for 30 min, and cleared by centrifugation at 10,000 × *g* for 10 min at 4 °C. The vesicle and membrane fractions were analyzed by Western blotting.

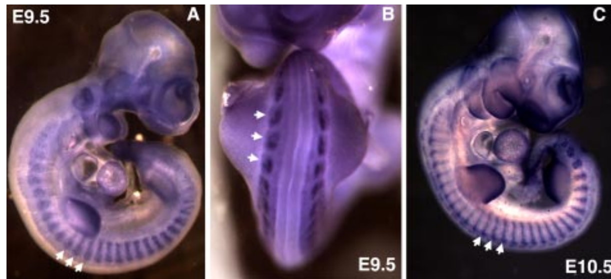

FIGURE 1. **Whole mount RNA *in situ* hybridization analysis of *Cd164* expression in mouse embryos.** *A*, side view of E9.5 embryo. *B*, axial view of E9.5 embryo. *C*, side view of E10.5 embryo. The *white arrows* indicate dorsal somites.

Cell Motility Assays—C2C12 cells expressing GFP-CD164 constructs, *Cd164* RNAi contructs, or appropriate control vectors were analyzed using a 96-well Boyden chamber assay with a polycarbonate filter (8-μm pores; NeuroProbe, K.U. Leuven, Belgium). Cell migration is measured as the number of cells that migrate from the top of the filter through the pores to the bottom in response to medium in the lower chamber. Briefly, the lower wells of each chamber were loaded with 395 μl of control medium (to evaluate basal migration) or medium containing 200 ng/ml CXCL12. Cells (7.5 × 10^4) in 200 μl of control medium were loaded into each upper chamber and incubated at 37 °C. After 5 h, the filter was wiped clean of cells that had not migrated, fixed in 100% methanol for 5 min, and incubated in Gill-2 hematoxylin (Thermo Electron Corp., Pittsburgh, PA) for 6–12 h to stain cells that had migrated. The total number of cells in 20 fields (on a phase contrast microscope) per well was determined. All conditions were tested in triplicate wells, and the experiment was repeated three times.

Whole Mount RNA in Situ Hybridization—CD1 mice were allowed to mate, and noon of the plug date was designated embryonic day (E) 0.5. Embryos collected at E9.5 and E10.5 were prepared for whole mount RNA *in situ* hybridization essentially as described (28). A mouse *Cd164* cDNA (13) was subcloned into the Bluescript vector pSK+, and digoxigenin-labeled antisense riboprobe was synthesized with T7 RNA polymerase using the Roche Applied Science DIG RNA labeling kit according to the manufacturer's instructions. Embryos were allowed to hybridize with the riboprobe as described in detail in Mulieri *et al.* (28); they were subsequently washed, blocked, and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody and developed with color was BM purple AP substrate, precipitating (Roche Applied Science) (28).

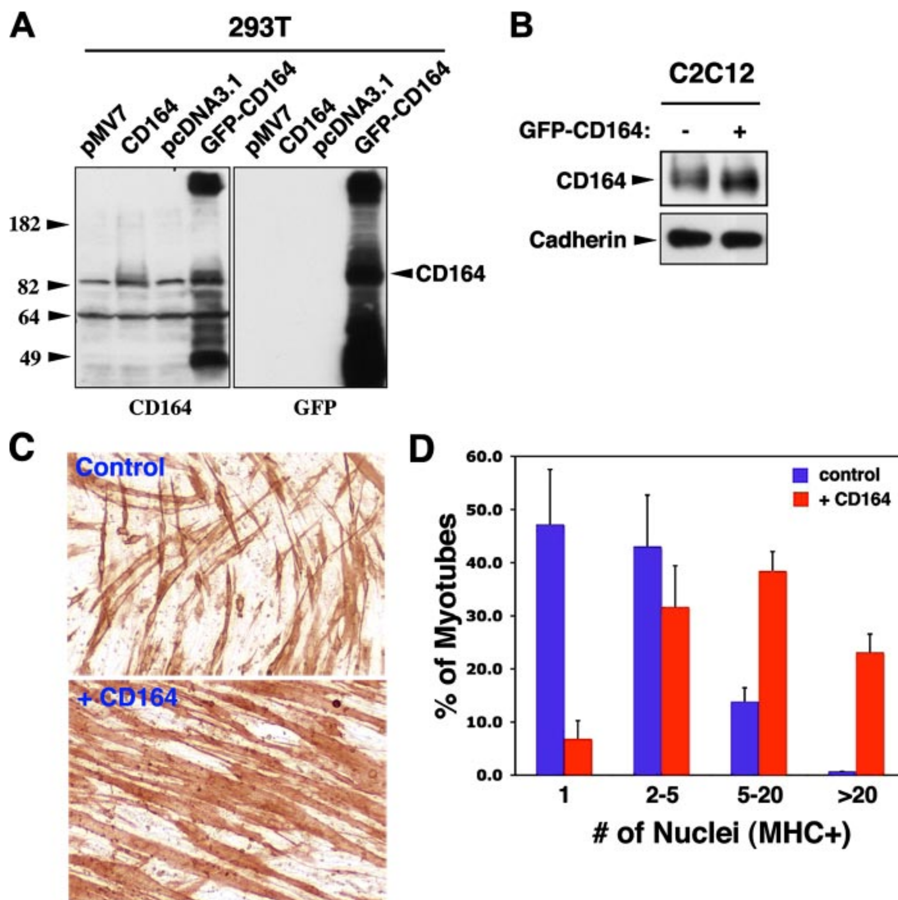

FIGURE 2. **Expression of GFP-CD164 enhances myotube formation by C2C12 cells.** *A*, 293T cells were transiently transfected with the indicated expression vectors encoding wild-type or GFP-tagged CD164 or the appropriate control vectors, and cell lysates were analyzed by Western blotting techniques with antibodies to CD164 (*left panel*) or GFP (*right panel*). *B*, C2C12 cells were stably transfected with pcDNA3.1 encoding GFP-CD164 (+) or, as a control, pcDNA3.1 itself (−), and cell lysates were analyzed by Western blotting techniques with antibodies to CD164 or, as a loading control, cadherin. *C* and *D*, C2C12 cell lines indicated in *B* (designated + CD164 and control, respectively) were cultured in DM, fixed and stained with an antibody to MHC (*C*), and then quantified for the percentage of MHC^+ nuclei present in myotubes that contained the indicated number of nuclei (*D*). Values represent means of triplicate determinations ± S.D.

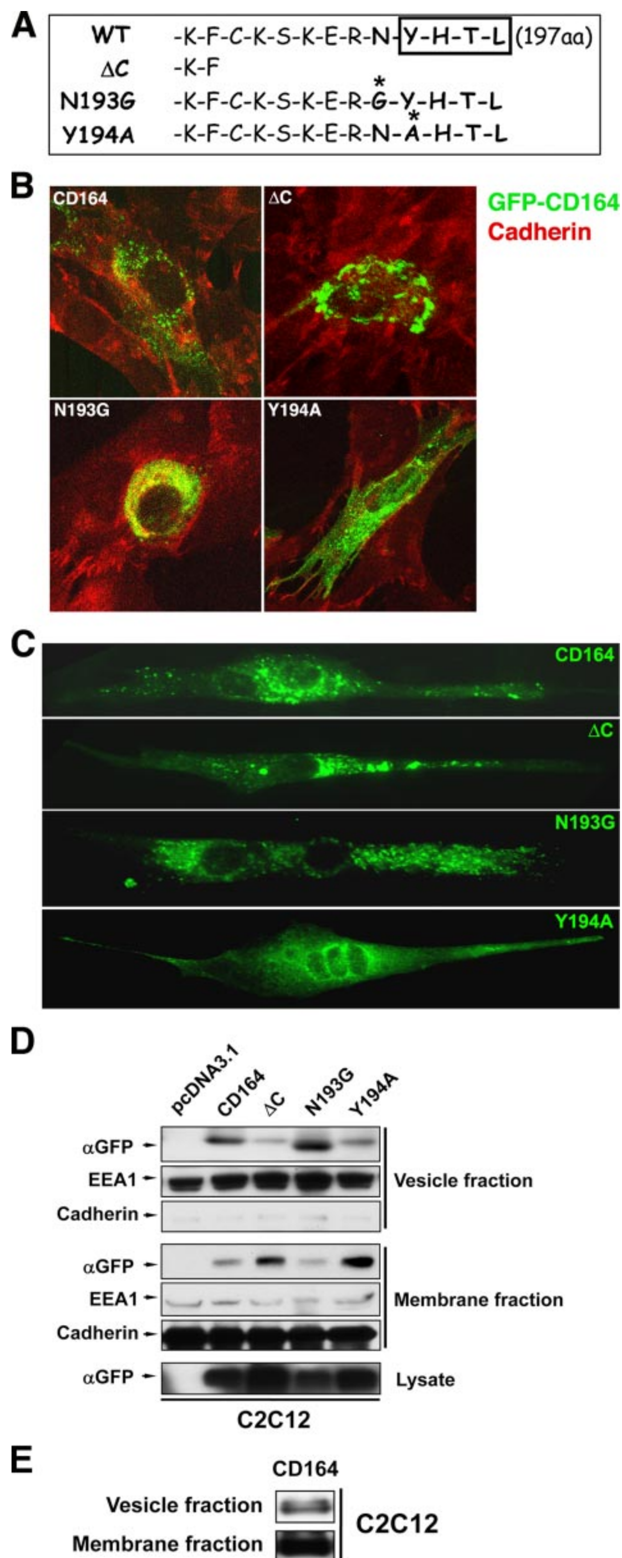

FIGURE 3. **GFP-CD164 cytoplasmic tail mutants have distinct subcellular locations in C2C12 myoblasts.** *A*, sequence of the intracellular region of CD164 and of three mutants. *WT*, wild type. *B* and *C*, confocal micrographs of C2C12 myoblasts (*B*) and nascent myotubes (*C*) expressing GFP-CD164 (CD164) or the indicated GFP-CD164 mutants. The myoblast cultures were also stained with an antibody that recognizes classical cadherins, including two that are expressed in C2C12 cells, N- and M-cadherin. *D*, biochemical fractionation of C2C12 myoblasts expressing GFP-CD164 (CD164) or the indicated GFP-CD164 mutants into vesicle and membrane preparations. The vesicle and membrane fractions were analyzed by Western blotting with antibodies to the indicated proteins. Antibodies against the early endosomal marker EEA1 and classical cadherins were used to demonstrate the purity of the vesicle and membrane fractions, respectively. A portion of the total cell lysate was also probed with antibodies to GFP to document approximately equivalent expression levels of each CD164 derivative. *E*, the pcDNA3.1 control vector lanes from *D* were probed with antibody against CD164 to reveal relative vesicle and membrane distribution of endogenous CD164 in C2C12 cells. Note that the EEA1 and cadherin signals in *D* are therefore the appropriate controls (*E*).

RESULTS

Cd164 Is Expressed in Somites during Mouse Development—CD164 is expressed in myoblast cell lines (13), but little is known of its expression pattern during myogenesis *in vivo*. To determine whether *Cd164* is expressed during early stages of skeletal muscle development, whole mount *in situ* hybridization was performed on mouse embryos at E9.5 and E10.5. At both stages, *Cd164* was expressed strongly in the dorsal somite, the structure of origin for skeletal muscle precursors (Fig. 1). Cd164 is also expressed at later stages of muscle development; thin section *in situ* hybridization analysis of E14.5 embryos performed by GenePaint revealed strong expression of *Cd164* in premuscle masses of the limb and trunk (29).

Expression of CD164 Mutants Modulates Myotube Formation—In epithelial and hematopoietic precursor cell lines, CD164 is present in both vesicular and cell surface locations, with targeting regulated by glycosylation and by its C-terminal YHTL sequence (15, 17, 30). To investigate the role of the YHTL sequence in the promyogenic activity of CD164, we initially constructed a GFP-tagged version of CD164. A GFP-encoding sequence was inserted in-frame between residues Gln-24 and Pro-25, placing it downstream of the signal sequence but upstream of the first predicted oligosaccharide attachment site (19). The resulting construct, GFP-CD164, was transiently expressed in 293T cells, and lysates were immunoblotted with antibodies to CD164 or GFP; wild-type CD164 was also expressed as a control. 293 cells express CD164 endogenously (31) as an ~85-kDa species, and overexpression of wild-type CD164 increased the signal intensity of this band (Fig. 2*A*). Expression of GFP-CD164 resulted in a band of similar size that was immunoreactive with antibodies to both CD164 and GFP (Fig. 2*A*). GFP-CD164 therefore migrates with an apparent molecular mass that is smaller than expected by about the amount contributed by GFP itself (~26 kDa). Because GFP is in the extreme N terminus of GFP-CD164, GFP-CD164 is cell-associated (see below), and the CD164 cytoplasmic tail is only 13 amino acids long; the most likely explanation for this aberrant migration is that the presence of GFP in the ectodomain resulted in an altered glycosylation pattern, likely producing a protein with lower oligosaccharide content than wild-type CD164. Transient overexpression of GFP-CD164 in 293T cells also resulted in variable amounts of a much higher molecular weight immunoreactive band (Fig. 2*A*). This band was not seen in C2C12 cell transfectants (supplemental Fig. S1A), and inclusion of 0.1% SDS in the lysis buffer converted this high molecular mass band to the ~85-kDa band (supplemental Fig. S1B). This high molecular mass band is therefore very likely to represent protein aggregates formed by high level overexpression in 293T cells.

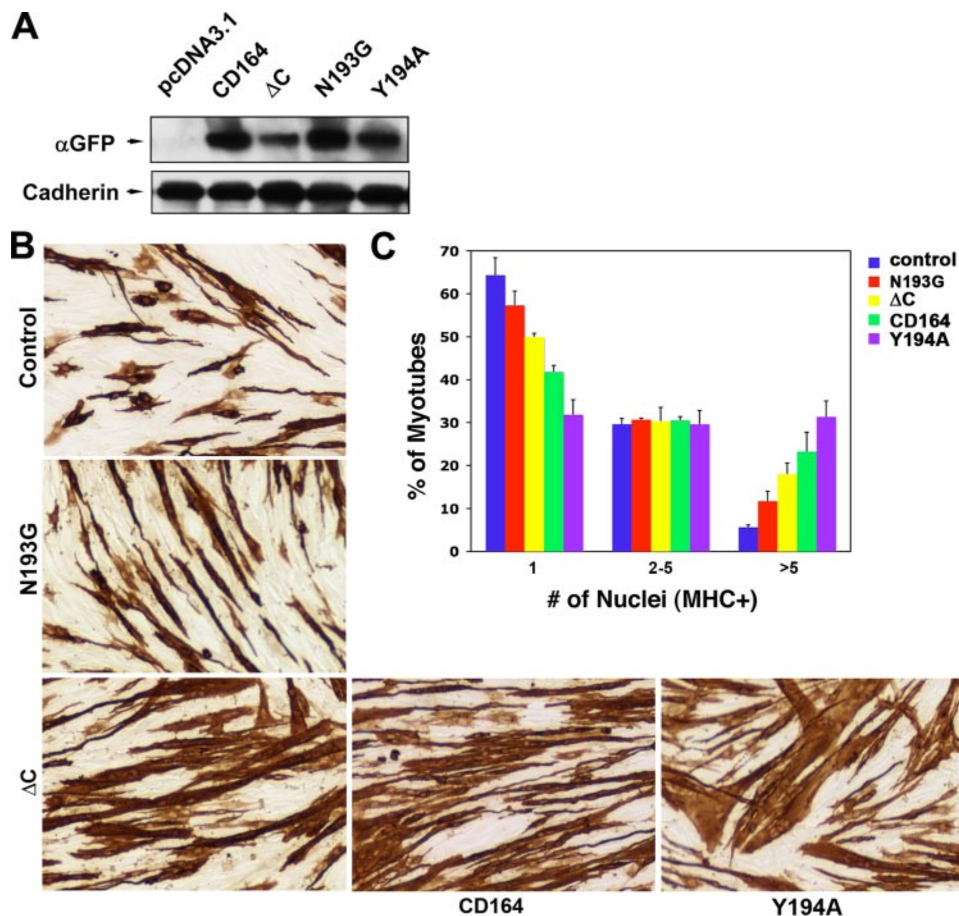

FIGURE 4. **Myotube formation by C2C12 cells that stably express GFP-CD164 cytoplasmic tail mutants.** *A*, Western blot analysis of expression of GFP-CD164 and the indicated mutants. Cell lysates were analyzed by Western blotting techniques with antibodies to GFP or, as a loading control, cadherin. *B* and *C*, C2C12 cell lines indicated in *A* were cultured in DM, fixed and stained with an antibody to MHC (*B*), and then quantified for the percentage of MHC^+ nuclei present in myotubes that contained the indicated number of nuclei (*C*). Values represent means of triplicate determinations ± S.D.

To assess the activity of GFP-CD164, C2C12 cells were stably transfected with a vector designed to drive expression of this protein and a puromycin resistance gene. Transfected cultures were selected and analyzed for total immunoreactive CD164 levels and production of muscle-specific proteins and myotubes when stimulated to differentiate. GFP-CD164 transfectants produced approximately twice the amount of immunoreactive CD164 protein as control cultures transfected with an expression vector lacking a cDNA insert (Fig. 2*B*; the GFP-CD164 protein was also detectable with antibodies to GFP (see Fig. 4*A*)). Similar to our published findings with wild-type CD164 (13), stable expression of GFP-CD164 significantly enhanced myotube formation (as assessed by the size of MHC^+ myotubes produced and by the increased percentage of myotubes with more than five nuclei; Fig. 2, *C* and *D*) but had little or no effect on expression of MyoD, myogenin, and MHC (supplemental Fig. S2A). These results suggest that the putative altered glycosylation pattern present in GFP-CD164 did not significantly change its function in this assay and that GFP-CD164 is therefore a useful construct for further analysis of the C-terminal YHTL sequence.

Functional analyses of the Y*XX*Φ motif of several different proteins allows construction of site-directed mutants that are expected to have a greater or lesser fraction at the cell surface (18, 43). Mutation of the tyrosine in the terminal YHTL sequence of CD164 to alanine should result in production of a protein (designated Y194A) that is inefficiently targeted to the endosomal/lysosomal (vesicular) compartment and thus would be predicted to have a greater fraction of the total residing at the cell surface. Conversely, mutation of the Y − 1 position from asparagine to glycine should produce a protein (designated N193G) that is directly transported to endosomes/lysosomes and would be predicted to have a smaller fraction at the cell surface. These mutants were constructed in the context of GFP-CD164, as was a mutant that deleted all but two amino acids of the cytoplasmic tail (designated ΔC; Fig. 3*A*). Vectors encoding each mutant, as well as GFP-CD164 itself, were transiently expressed in C2C12 myoblasts, and their localization was examined by confocal fluorescence microscopy in proliferating mononucleated cells and in nascent myotubes produced by incubation of the cells in DM for 2 days. In mononucleated myoblasts, GFP-CD164 was expressed largely in a vesicular pattern that was distributed throughout the cytoplasm. In contrast, the N193G mutant was strongly concentrated in a perinuclear location, and the Y194A mutant was much less punctate than wild type, with substantial cell surface expression (Fig. 3*B*). The ΔC mutant displayed a localization that appeared intermediate between wild type and N193G, with the punctate signal in larger structures (Fig. 3*B*). In nascent myotubes, these differences were not as pronounced, with GFP-CD164 and each mutant showing mainly localization to a vesicular compartment (Fig. 3*C*).

The distribution of these proteins in mononucleated C2C12 myoblasts was also analyzed by fractionation of cell lysates into vesicle and membrane preparations. Approximately twice the amount of GFP-CD164 was present in the vesicular fraction as in the membrane fraction (Fig. 3*D*). This ratio is reversed from that observed with endogenous CD164 (Fig. 3*E*), likely because of overexpression. However, the N193G and Y194A mutants were expressed at the same overall level as GFP-CD164 (Fig. 3*D*), and their distribution was altered as predicted. As compared with GFP-CD164, a greater percentage of N193G was found in the vesicle fraction, and a lower percentage was found in the membrane fraction (Fig. 3*D*). Conversely, a much higher percentage of Y194A was present in the membrane fraction than seen with GFP-CD164, with correspondingly less in the

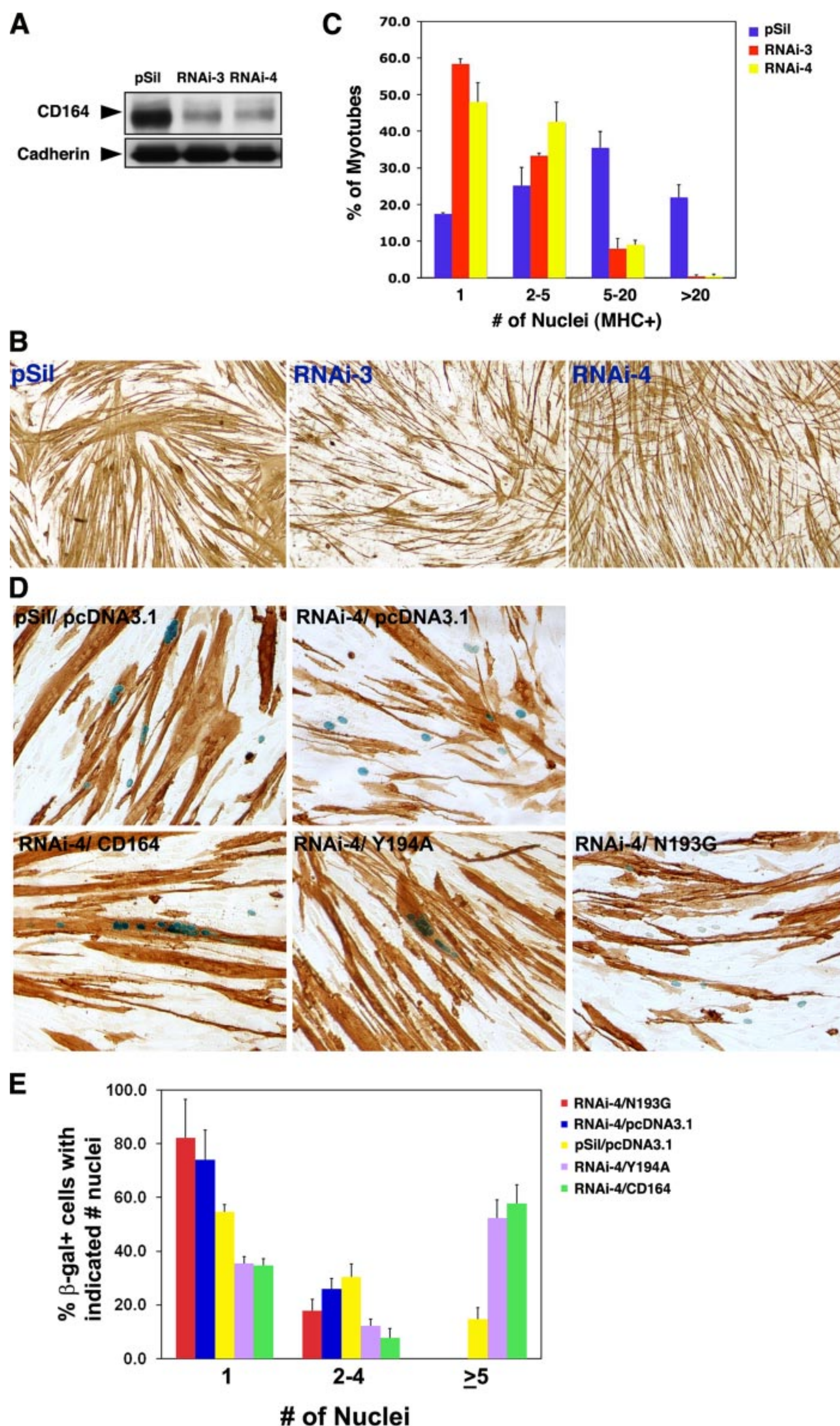

FIGURE 5. **Reduction of C2C12 myotube formation by RNAi against *Cd164* and rescue by GFP-CD164 constructs.** *A*, Western blot analysis of CD164 levels by two independent RNAi sequences. Cell lysates were analyzed by Western blotting techniques with antibodies to CD164 or, as a loading control, cadherin. *pSil*, pSilencer. *B* and *C*, C2C12 cell lines indicated in *A* were cultured in DM, fixed and stained with an antibody to MHC (*B*), and then quantified for the percentage of MHC^+ nuclei present in myotubes that contained the indicated number of nuclei (*C*). Values represent means of triplicate determinations ± S.D. *D* and *E*, C2C12 cells were transiently transfected with pSilencer or pSilencer driving expression of Cd164 RNAi-4 plus pcDNA3.1 or pcDNA3.1 driving expression of GFP-CD164 or the indicated cytoplasmic tail mutants in each case plus an expression vector for nuclear localized β-galactosidase (*β-gal*). Cultures were fixed and double-stained for MHC and β-galactosidase activity (*D*) and myotube formation by transfectants, which was quantified as the percentage of β-galactosidase$^+$ cells with the indicated number of nuclei (*E*). Values represent means of triplicate determinations ± S.D. Note that the level of myotube formation by the control cultures in panels *B* and *C* and panels *D* and *E* (pSil cells and pSil/pcDNA3.1 cells, respectively) is different (~18% mononucleate MHC^+ pSil cells in *B* and *C* and ~55% mononucleate MHC^+ pSil/pcDNA3.1 cells in *D* and *E*). These conditions were selected so as to permit easy visualization of diminished myotube formation by *Cd164* RNAi in (*B*) and (*C*) and either enhanced or diminished myotube formation by the various CD164 mutants in (*D*) and (*E*). See "Results" for further details.

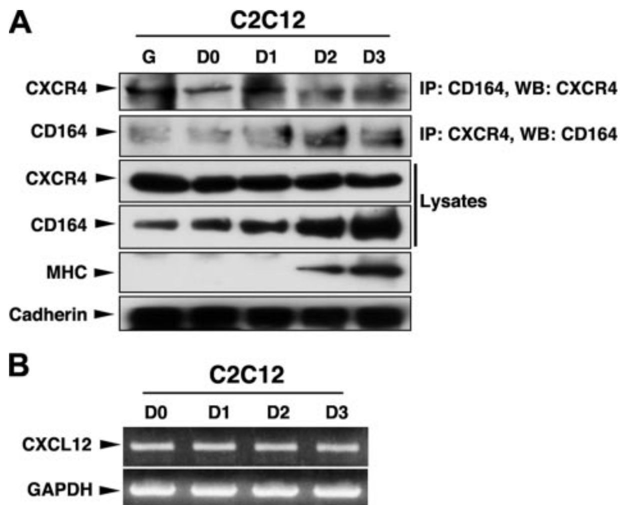

FIGURE 6. **Co-immunoprecipitation of CD164 and CXCR4.** *A*, C2C12 cells were analyzed for association of CD164 and CXCR4 through a time course of differentiation. Cells were harvested from proliferating cultures in growth medium (*G*), from cultures at ~80% confluence at the time of transfer to DM (*D0*), and from cultures in DM for 1, 2, or 3 days (*D1*, *D2*, and *D3*, respectively). Cell lysates were immunoprecipitated (*IP*) with the indicated antibodies and then analyzed by Western blotting (*WB*) techniques with the reciprocal antibody. Straight cell lysates were also analyzed by Western blotting with the indicated antibodies. MHC reveals the progression of differentiation, and cadherin serves as a loading control. *B*, RT-PCR analysis of *CXCL12* mRNA expression and, as a control, *GAPDH* mRNA expression in C2C12 cells through a time course of differentiation.

vesicle fraction (Fig. 3*D*). Despite the large, punctate signal seen with ΔC by immunofluorescence, the majority of this mutant was found in the membrane fraction (Fig. 3*D*); the intracellular membrane compartment this may correspond to is not clear.

Each individual mutant was then stably expressed in C2C12 cells, and the cells were assessed for their ability to form myotubes. Cells that stably expressed GFP-CD164 and cells transfected with an expression vector that lacked a cDNA insert were used as controls. GFP-CD164, N193G, and Y194A were expressed at very similar levels, whereas ΔC was expressed at a somewhat lower level than these three proteins (Fig. 4*A*). Interestingly, the promyogenic activities of the GFP-CD164 proteins correlated with their apparent ability to reside on the cell surface. Each of the different constructs enhanced myotube formation, relative to vector control cells. However, the N193G and ΔC mutants were less effective than wild-type GFP-CD164, whereas the Y194A mutant was more effective (Fig. 4, *B* and *C*).

CD164 forms homodimers (16), and it is possible that dimers are formed between endogenous CD164 and exogenously expressed mutants in the transfectants. To assess the activity of the cytoplasmic tail mutants in a background with reduced endogenous CD164, an RNAi rescue approach was taken. Stable expression of vectors encoding either of two independent RNAi sequences against *Cd164* resulted in substantial reduction of steady state CD164 protein levels (Fig. 5*A*). Cells expressing *Cd164* RNAi produced thinner myotubes with fewer nuclei than those formed by cells transfected with a vector encoding a control RNAi sequence (Fig. 5, *B* and *C*). In contrast, the *Cd164* RNAi-expressing cells had similar levels of myogenin and only a small reduction in MHC levels relative to control cells (supplemental Fig. S2B); therefore, enhanced or diminished levels of CD164 affected myotube formation without substantially affecting biochemical measures of differentiation.

A transient myogenesis assay was used next to test the ability of CD164 mutants to rescue the defective myotube formation induced by RNAi against *Cd164* (23,24) (Fig. 5, *D* and *E*). C2C12 cells were cotransfected with control or *Cd164* RNAi expression vectors; control vectors or vectors encoding wild-type or mutant CD164 proteins; and a vector that drives expression of nuclear-localized β-galactosidase to mark transfectants. Two days later, the cultures were transferred to DM for 48 h and then fixed and double-stained for MHC and β-galactosidase activity. When double control vector transfectants fused with non-transfected cells, most of the nuclei in the myotube became positive for β-galactosidase activity because the cytoplasmically translated protein diffused within the myotube (23). When the number of nuclei in β-galactosidase$^+$ cells was scored, ~30% had 2–4 nuclei, and ~18% had 5 or more nuclei. In contrast, the distribution of β-galactosidase$^+$ cells that received the *Cd164* RNAi vector was skewed toward single nucleus-containing cells, with ~25% displaying 2–4 nuclei and none having 5 or more nuclei. Cotransfection of expression vectors for wild-type CD164 and for the Y194A mutant both resulted in enhanced myotube formation, relative to control cells, with >50% of β-galactosidase$^+$ cells displaying 5 or more nuclei. However, cotransfection of the vector encoding the N193G mutant failed to rescue the reduction in myotube formation imposed by *Cd164* RNAi.

CD164 Interacts with CXCR4 in Myoblasts and Is Important for CXCL12-induced C2C12 Cell Migration—Association of endogenous CD164 with CXCR4 in C2C12 cells was assessed by reciprocal co-immunoprecipitation of cell lysates over a time course of differentiation. CXCR4 co-immunoprecipitated with CD164 in proliferating myoblasts, at the time of transfer to DM and throughout 3 days of culture in DM (Fig. 6*A*). This result was somewhat surprising as the CD164-CXCR4 interaction was previously shown to be induced by the CXCR4 ligand, CXCL12, which is not expressed in muscle precursors during developmental myogenesis (9, 10). To determine whether C2C12 cells express CXCL12, RT-PCR analysis for *CXCL12* mRNA was performed and, indeed, was easily detected in cells prior to and after culture in DM (Fig. 6*B*).

To assess the functional relevance of the CD164-CXCR4 interaction in C2C12 cells, cells stably expressing RNAi against *Cd164* were examined for their migratory ability across an 8-μm membrane. Although control cells displayed a basal level of migration that could be stimulated by CXCL12, cells expressing *Cd164* RNAi had reduced basal migratory ability and were refractory to CXCL12-mediated migration (Fig. 7, *A* and *B*). The reduced myogenicity of cells knocked down for CD164 therefore correlated with a reduced ability to migrate. The ability of CD164 cytoplasmic tail mutants to stimulate cell migration was tested next. The ability of these mutants to enhance basal and CXCL12-mediated migration was similar to their ability to promote myotube formation, with the rank order of potency being Y194A > wild type > ΔC > N193G, although the quantitative effect was stronger in myotube formation than on migration (Fig. 7, *C* and *D*). Taken together, these gain- and loss-of-function approaches suggest that CD164 regulates

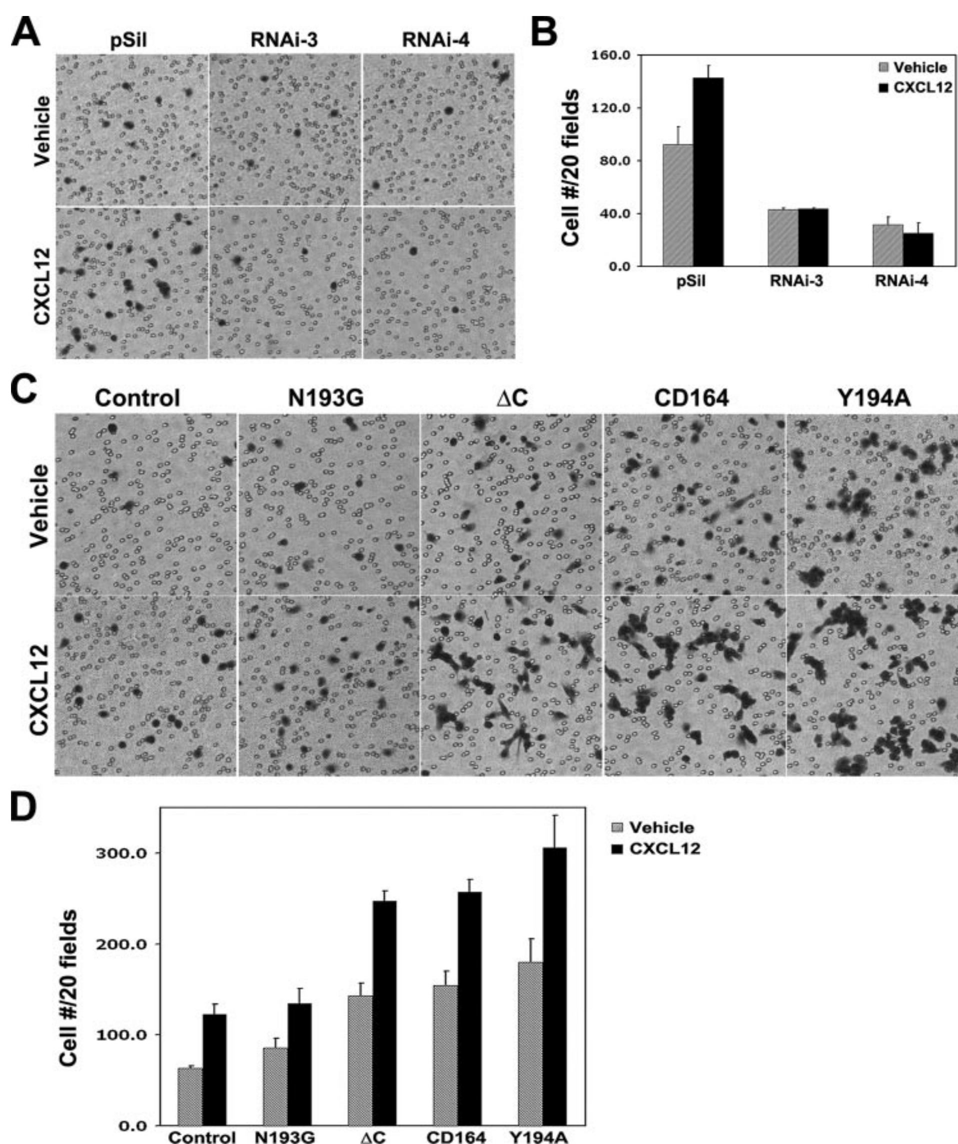

FIGURE 7. **Motility of C2C12 cells expressing RNAi against *Cd164*, GFP-CD164, or GFP-CD164 cytoplasmic tail mutants.** *A* and *B*, C2C12 cells expressing the indicated RNAi sequences against *Cd164* or control vector (pSilencer (*pSil*)) were analyzed for migration in Boyden chambers in response to control medium or medium containing CXCL12. Cells that had migrated were photographed (*A*) and quantified (*B*). Values represent means of triplicate determinations ± S.D. *C* and *D*, C2C12 cells expressing the indicated GFP-CD164 variant or control vector (control) were analyzed for migration in Boyden chambers in response to control medium or medium containing CXCL12. Cells that had migrated were photographed (*C*) and quantified (*D*). Values represent means of triplicate determinations ± S.D.

myoblast motility and that this is a likely mechanism by which it promotes myotube formation.

DISCUSSION

Myoblast fusion is fundamental to the development and regeneration of skeletal muscle and is dependent on a sequence of events that include migration, differentiation, cell-cell recognition and adhesion, and merger of membranes between apposing cells (2–4). The role of cell migration in muscle development has mostly focused on the migration of muscle progenitor cells from somites to sites of future musculature, such as the limbs (7). However, recently published results have highlighted the importance of myoblast migration in the growth of myofibers *in vivo* and myotubes *in vitro* (12, 32, 33). This is a complex process involving numerous factors that stimulate or inhibit myoblast motility, presumably in a coordinated fashion, so as to permit appropriate cell-cell interactions that occur in preparation for fusion (12, 32, 33). Among the secreted factors that may be involved in this process are the promigratory peptides hepatocyte growth factor, basic fibroblast growth factor, transforming growth factor-β, IGF-1, and CXCL12 and the antimigratory factors prostacyclin and sphingosine-1-phosphate (32, 34–38). Here we report that the sialomucin CD164 positively regulates myoblast migration and fusion.

Expression of CD164 cytoplasmic tail mutants revealed a correlation between their cell surface location, the ability to promote cell migration, and the ability to promote myotube formation. Furthermore, RNAi-mediated knockdown of CD164 resulted in both reduced migration and reduced myotube formation. The recent discovery that CD164 associates with the chemokine receptor CXCR4 (21), possibly as a co-receptor for the CXCR4 ligand CXCL12, offers a likely mechanism by which CD164 influences migration and myotube formation. CXCR4 is well established as a motility factor for muscle precursors and other cell types (8–11), and CD164 knockdown resulted in reduced migration of C2C12 cells toward a source of CXCL12.

An unanticipated finding from this study was that C2C12 cells express CXCL12. Migrating muscle progenitor cells in the mouse embryo express CXCR4, but not CXCL12, and migrate toward sources of CXCL12 along the routes and at the destinations of such cells (*e.g.* limb mesenchyme) (9, 10). C2C12 cells are a line derived from satellite cells (39, 40), adult muscle stem cells, and are therefore a distinct cell type from embryonic migrating muscle progenitors. However, it is not known whether satellite cells express both CXCR4 and CXCL12 *in vivo* during muscle regeneration or whether expression of CXCL12 in C2C12 cells reflects a property acquired during adaptation to long term culture. Zhu *et al.* (41) also reported that C2C12 cells express CXCL12 and that inhibition of CXCL12/CXCR4 signaling blocked BMP-2-mediated osteogenic differentiation of these cells.

Identification of the mechanisms that underlie motility and directed migration of muscle precursors is important because

such factors, including CXCL12, may be useful in promoting the efficacy of cell-based therapies for muscular dystrophies and other myopathies (42). Our results suggest that CD164-mediated migration enhances myotube formation of C2C12 cells. The observation that alteration of CD164 level or function had a much stronger effect on formation of multinucleated myotubes than on expression of muscle-specific proteins is consistent with this notion. However, it is possible that CD164 stimulates myotube formation by more than one mechanism. CD164 regulates cell adhesion of hematopoietic precursors (14), and this property might contribute to myotube formation; however, C2C12 cells that expressed RNAi against *Cd164* did not show obvious changes in adhesive state[6] and still expressed classical cadherins, major adhesion molecules in these cells. Nevertheless, CD164 may bind ligands independently of CXCR4, perhaps lectin-type counter-receptors (31); such factors have not yet been identified but could contribute to additional potential CD164 functions. As shown in this study, *Cd164* is expressed in the early myogenic compartment of the somite and also later during skeletal muscle development, in premuscle masses (29). This expression pattern is consistent with the possibility that CD164 might be involved in multiple aspects of myogenesis. Future work, including construction and analysis of targeted mutations of *Cd164*, should shed light on these issues.

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