Impaired Expression of Integrin α-4 Subunit in Cultured Mast Cells Derived From Mutant Mice of mi/mi Genotype

By Dae-Ki Kim, Eiichi Morii, Hideki Ogihara, Koji Hashimoto, Kenji Oritani, Young-Mi Lee, Tomoko J Ippo, Shiro Adachi, Yuzuru Kanakura, and Yukihiko Kitamura

The mi locus encodes a member of the basic-helix-loop-helix-leucine zipper protein family of transcription factors (hereafter called MITF). We have reported that expression of several genes was impaired in cultured mast cells (CMCs) of mi/mi mice due to a defective transactivation ability of mutant MITF (mi-MITF). Because attachment of mi/mi CMCs to fibroblasts is impaired, we examined the expression of integrin genes in mi/mi CMCs in the present study. Among the integrin genes examined, the expression of integrin α4 subunit was barely detectable in mi/mi CMCs, and the α4 protein was not detected by flow cytometry either. The specific adhesion to vascular cell adhesion molecule-1 (VCAM-1), the ligand for α4 subunit, was observed in +/+ CMCs but not in mi/mi CMCs, indicating that the expression of integrin α4 subunit at a functional level did not occur in mi/mi CMCs. In the promoter region of the α4 subunit gene, there was a CACCTG motif to which normal MITF (+/- MITF) bound. The coexpression of +/-MITF but not of mi-MITF transactivated the promoter of the α4 subunit gene. The deletion or mutation of the CACCTG motif abolished the transactivation by +/-MITF, suggesting that +/-MITF directly transactivated the gene encoding α4 subunit of integrin.

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The mi LOCUS OF MICE encodes a member of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) protein family of transcription factors (hereafter called mi-transcription factor [MITF]). The MITF encoded by the mutant mi allele (hereafter mi-MITF) deletes 1 of 4 consecutive arginines in the basic domain. The mi-MITF is defective in the DNA binding activity and the nuclear localization potential, and it does not transactivate target genes. The mi/mi mice show microphthalmia, depletion of pigment in both hair and eyes, osteopetrosis, and a decrease in the number of mast cells. In addition to the decrease in number, the phenotype of mast cells is abnormal in mi/mi mice. A motif in the promoter region of the mi-α4 subunit gene, and the protein was not detected by flow cytometry either. The specific adhesion to vascular cell adhesion molecule-1 (VCAM-1), the ligand for α4 subunit, was observed in +/+ CMCs but not in mi/mi CMCs, indicating that the expression of integrin α4 subunit at a functional level did not occur in mi/mi CMCs. In the promoter region of the α4 subunit gene, there was a CACCTG motif to which normal MITF (+/- MITF) bound. The coexpression of +/-MITF but not of mi-MITF transactivated the promoter of the α4 subunit gene. The deletion or mutation of the CACCTG motif abolished the transactivation by +/-MITF, suggesting that +/-MITF directly transactivated the gene encoding α4 subunit of integrin.

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Materials and Methods

Mice. The original stock of C57BL/6-mi/+ (mi/+ mice was purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained in our laboratory by consecutive backcrosses to our own inbred C57BL/6 colony (more than 15 generations at the time of the present experiment). Female and male mi/+ mice were crossed together, and the resulting mi/mi mice were selected by their white coat color. Mast cell-deficient (WB × C57BL/6) F1-W/W" (W/W") mice were purchased from the Japan SLC (Hamamatsu, Japan). Cells. Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al. Mice of mi/mi, W/W", and control C57BL/6-/+ mouse genotypes were used. The mice were killed by decapitation after ether anesthesia and the spleens were removed. Spleen cells were cultured in α-minimal essential medium (α-MEM; ICN Biomedicals, Costa Mesa, CA) supplemented with 10% PWM-SCM and 10% fetal calf serum (FCS; Nippon Bio-supp Center, Tokyo, Japan). Half the medium was replaced every 3 days. Cells were harvested at various times after the initiation of the culture. Cytospin preparations of cells were fixed with Carnoy’s solution and stained with alcian blue and nuclear fast red. The proportions of alcin blue-positive cells were determined under the microscope. IC-2 cells were provided by Dr I. Yahara (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and maintained in α-MEM supplemented with 10% PWM-SCM and 10% FCS. CHO cells (American Type Culture Collection, Manassas, VA) were maintained in α-MEM supplemented with 10% FCS. In one experiment, recombinant mouse SCF (rmSCF; a generous gift of Kirin Brewery Co Ltd, Tokyo, Japan) was added to the α-MEM containing 10% PWM-SCM and 10% FCS. Semiquantitative reverse transcriptase modification of polymerase chain reaction (RT-PCR). Total RNA (5.0, 0.5, and 0.05 µg) obtained from +/+ or mi/mi CMCs was reverse transcribed in 20 µL of the reaction mixture containing 20 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and random hexamer. One microliter of each reaction...
product was amplified in 25 µL of PCR mixture containing 0.125 U of Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) and 12.5 pmol each of sense and antisense primers by 30 cycles of 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C, and 2 minutes of synthesis at 72°C. Ten microliters of the PCR products was electrophoresed in 2% agarose gel containing ethidium bromide. The following oligonucleotide primers were used: 3′ sense primer, 5′-TACTTGGGCA TAA TCCGGTAGTAG (3849 through 3872); antisense primer, 5′-TTGGAGGCTTGAGCTGAGCTT (2045 through 2064), antisense primer, 5′-CCATTCAACCATCAGCCT (1202 through 1221); 3′ sense primer, 5′-CATTCCAGTCTGGGGCAA (2836 through 2855), antisense primer, 5′-GGGAGCTTGAGCTGAGCTT (3136 through 3155); α sense primer, 5′-GTTGGGAGATTAGACAGAGGA (2787 through 2810), antisense primer, 5′-CAAAACAGCCAGTAGCAACAA (3031 through 3054); β1: sense primer, 5′-TTGTCAGTGCATACCCCAGCA (2045 through 2064), antisense primer, 5′-CTCTATACTGGCTGTGAC (33 through 53), antisense primer, 5′-TGGTCAGTGCATACCCCAGCA (153 through 173); β2: sense primer, 5′-TAAAGACCTCTATGCCAACAC (950 through 970), antisense primer, 5′-CTCTTGCTCTGTGCCCATC (1143 through 1163). The numbers represent the nucleotide numbers on the complementary strands of each cDNA sequence. 

Flow cytometry. CMCs were harvested and washed once with cold phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.1% sodium azide. The cells were incubated with PS/2 anti-integrin α4 rat monoclonal antibody (MoAb; Chemicon, Temecula, CA) at 4°C for 30 minutes, rinsed, developed with fluorescein isothiocyanate-conjugated mouse antirat IgG, and then analyzed on a FACScan (Becton Dickinson, Los Angeles, CA).

Construction of expression plasmids. Bluescript KS(−) plasmid (pBS; Stratagene, La Jolla, CA) containing the whole coding region of GST-MITF or GST-α4-MITF (hereafter called pBS-α4-MITF and pBS-mi-MITF, respectively) had been constructed in our laboratory.5 The vascular cell adhesion molecule-1 (VCAM-1) cDNA was obtained by PCR according to the sequence reported by Araki et al.39 and was verified by sequencing. The VCAM-1 cDNA was subcloned into pBS (pBS-VCAM-1). The pEF-BOS expression vector was kindly provided by Dr K. Nakajima (Osaka University Medical School). To construct reporter plasmids, a DNA fragment containing the promoter region and the first exon (noncoding region of the integrin α4 subunit gene (−949 to +221) was cloned into the upstream of the luciferase gene in pSPLuc. The deletion of the integrin α4 promoter was produced by PCR using the appropriate primers. The mutation was introduced by PCR with mismatch primers. Deleted or mutated products were verified by sequencing.

Transfection of reporter plasmids and luciferase assay. Reporter plasmids (40 µg) were transfected into LC-2 cells by electroporation (380 V, 975 µF). The cells were harvested 18 hours after the transfection and lysed with 0.1 mol/L potassium phosphate buffer (pH 7.4) containing 1% Triton X-100. Soluble extracts were then assayed for luciferase activity with a luminometer LB96P (Berthold GmbH, Wildbad, Germany). The cotransfection of the reporter (40 µg) and expression (5 µg) plasmids was also performed.

EGMSA. The production and purification of glutathione-S-transferase (GST)-α4-MITF or GST-α4-MITF fusion protein was described previously.5 Oligonucleotides were labeled with α-[32P]-dCTP by filling 5′-overhangs and used as probes of EGMSA. DNA-binding assays were performed in a 20 µL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 75 mmol/L KCl, 1 mmol/L dithiothreitol, 4% Ficoll type 400, 50 ng of poly (dl-dC), 25 ng of labeled DNA probe, and 1.0 µg of GST-α4-MITF fusion protein. In some experiments, 1.0, 2.0, or 4.0 µg of GST-α4-MITF was added to the reaction mixture containing 1.0 µg of GST-α4-MITF. After incubation at room temperature for 15 minutes, the reaction mixture was subjected to electrophoresis at 14 V/cm at 4°C on a 5% polyacrylamide gel in 0.25× TBE buffer (1× TBE is 90 mmol/L Tris-HCl, 64.6 mmol/L boric acid, and 2.5 mmol/L EDTA, pH 8.3). The polyacrylamide gels were dried on Whatman 3MM chromatography paper (Whatman, Maidstone, UK) and subjected to autoradiography.

RESULTS

Impaired expression of integrin α4 subunit in mi/mi CMCs. We compared the expression of integrin genes between +/+ and mi/mi CMCs. CMCs were used 4 weeks after the initiation of the culture, because the mRNA expression of integrins reached maximum levels at this time.23,24 RNAs were extracted from +/+ and mi/mi CMCs, and semiquantitative RT-PCR was performed to estimate the expression levels of integrin subunits. We first examined the expression of integrins of α subunit family (α3, α4, α5, and αv) that were expressed by CMCs.25 Expression levels of α3, α5, and αv genes were comparable between +/+ and mi/mi CMCs. In contrast, the expression of the α4 gene was significantly lower in mi/mi CMCs than in +/+ CMCs (Fig 1). We next examined the expression of integrin β1 and β7 subunits, because only these can associate with the α4 subunit.27 Comparing +/+ and mi/mi CMCs, no significant difference in the expression of β1 and β7 genes was detectable (Fig 1).

The expression of the α4 subunit gene was investigated at various times after the initiation of the culture. More than 95%
of cells were alcian blue-positive throughout the experimental period and were considered to be mast cells (Table 1). The α4 expression was slight 2 weeks after culturing +/+ spleen cells and was clearly observed at 4 weeks (Fig 2). The α4 gene expression started to decrease after 6 weeks of culture and was not detectable after 8 weeks of culture. The expression of α4 subunit was barely detectable in mi/mi CMCs throughout the observation period (Fig 2).

We compared the surface expression of integrin α4 protein between +/+ and mi/mi CMCs by flow cytometry. The expression of α4 subunit was detected in +/+ CMCs 4 weeks after the initiation of culture, and the expression level started to decrease after 6 weeks of culture and was not detectable after 8 weeks (Fig 3). In mi/mi CMCs, the surface expression of integrin α4 subunit was not detectable throughout the observation period (Fig 3).

Because the addition of rmSCF normalized the deficient mRNA expression of MMCP-5 by mi/mi CMCs,⁹ we examined the effect of SCF-c-kit signals on mRNA expression of the α4 subunit. First, we examined the expression of α4 protein on the surface of W/Wv CMCs that lack normal SCF-c-kit signals.²⁹,⁴¹,⁴² Despite the defect of the c-kit receptor tyrosine kinase, W/Wv CMCs expressed the α4 protein normally (Fig 4). Second, rmSCF was added to the culture medium of CMCs. The α4 expression was not influenced by the addition of rmSCF in all +/+, mi/mi, and W/Wv CMCs (Fig 4).

VCAM-1 was the ligand for the integrin α4β1.²⁷ The expression vector containing the sense VCAM-1 cDNA was

Table 1. Proportion of Mast Cells in Suspension Culture of Spleen Cells at Various Times After the Initiation of Culture

<table>
<thead>
<tr>
<th>Time After Initiation of the Culture (wk)</th>
<th>Proportion of Alcian Blue-Positive Cells (%)*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
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<tr>
<td>2</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>4</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>6</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>8</td>
<td>98 ± 1</td>
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*Mean ± SE of three experiments.
transfected into CHO cells (hereafter called CHO-VCAM-1). The expression vector for an antisense VCAM-1 cDNA was also transfected into CHO cells as a negative control (hereafter called CHO-control). The proportion of 1/1 CMCs adhering to CHO-VCAM-1 cells was significantly higher than that of 1/1 CMCs adhering to CHO-control cells (Fig 5). The adhesion of 1/1 CMCs to CHO-VCAM-1 cells was inhibited by the treatment of 1/1 CMCs with anti-α4 MoAb (Fig 5). No adhesion of mi/mi CMCs to CHO-VCAM-1 cells was detectable. The mi/mi CMCs did not adhere to CHO-control cells, either (Fig 5).

Transactivation of integrin α4 subunit gene by +MITF.

We cloned 949 bases of the 5′-upstream region of the integrin α4 gene to examine the regulation mechanism by +MITF. The reporter plasmid that contained the luciferase gene under the control of the integrin α4 promoter starting from nt −949 (hereafter called −949 reporter plasmid) was constructed. We also constructed the deleted reporter plasmid that contained the α4 promoter starting from nt −819, −516, −434, or −199 to examine the elements that mediate the transactivation (hereafter called −819, −516, −434, or −199 reporter plasmid, respectively). The reporter plasmids were transfected into the mast cell line, IC-2 cells, which expressed both +MITF and integrin α4 subunit mRNAs. When the −949 or −819 reporter plasmid was introduced, the IC-2 cells showed only a low level of luciferase activity (Fig 6A). The luciferase activity increased eightfold when the −516 or −434 reporter plasmid was transfected. The deletion of the promoter to −199 reduced the luciferase activity to one fourth the value obtained with the −434 reporter plasmid (Fig 6A). The bHLH-Zip proteins recognize the CANNTG motif (any nucleotides are compatible with the position N).43 There was only one CANNTG motif in the region between nt −434 and −199, ie, CACTTG between nt

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**Fig 4.** Effect of the addition of rmSCF on the expression of α4 protein on the surface of +/+, mi/mi, or W/W v CMCs. Three weeks after initiation of the culture, various CMCs were incubated with 10% PWM-SCM and 50 ng/mL rmSCF for 0, 1, or 2 weeks. The surface expression of integrin α4 subunit was examined by flow cytometry. Cells were incubated with either rat anti-α4 MoAb (solid line) or control rat IgG (dotted line).

**Fig 5.** Adhesion of CMCs to VCAM-1. CMCs were used 4 weeks after the initiation of culture, because the expression of α4 subunit reached the maximum level at that time. The adhesion of CMCs to CHO cells transfected with either sense (CHO-VCAM-1) or antisense VCAM-1 cDNA (CHO-control) was determined. The adhesion of CMCs pretreated with anti-α4 MoAb was also determined. Bars indicate the standard error of three assays.

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**Fig 6.** (A) Luciferase activity under the control of normal, deleted, or mutated α4 promoter in IC-2 cells. (B) The effect of overexpression of + or mi-MITF on the luciferase activity in IC-2 cells. The luciferase gene under the control of the normal, deleted, or mutated α4 promoter was cotransfected with + or mi-MITF or with the expression vector. The data represent the mean ± SE of five experiments. In some cases, the standard error was too small to be shown by bars.
−294 and nt −289. We mutated the CACTTG motif to CTCTAG in the reporter plasmid starting from nt −434. The mutation decreased the luciferase activity to a level comparable to that obtained by the −199 reporter plasmid.

We next transfected the −434 reporter plasmid into IC-2 cells overexpressing + or mi-MITF cDNA. IC-2 cells overexpressing expression vector alone were used as a control. The luciferase activity in IC-2 cells overexpressing +MIF was comparable to that of IC-2 cells overexpressing the vector alone (Fig 6B). The luciferase activity was significantly decreased by the overexpression of mi-MITF (Fig 6B). The intact CACTTG motif was necessary for such a negative effect of mi-MITF, because either its deletion or mutation abolished the effect of overexpressing mi-MITF.

The binding of +MIF to the oligonucleotide containing the CACTTG motif was examined. We performed EGMSA by using the nuclear extract of +/+ CMCS, but a specific DNA/protein complex was barely detectable. We then used the purified GST+MIF fusion protein. When the oligonucleotide containing the CACTTG motif was used as a probe, the specific binding of +MIF was observed (Fig 7). The excess amount of the nonlabeled oligonucleotide containing the CACTTG motif abolished the binding of +MIF to the CACTTG motif, whereas the excess amount of unlabeled oligonucleotide mutated in the CACTTG motif (ie, CTCTAG) did not affect the binding (Fig 7).

The effect of mi-MITF on the binding of +MIF was examined (Fig 8). The amount of DNA/protein complex was slightly reduced by the addition of an equivalent amount of GST-mi-MITF to the GST+MIF. The addition of GST-mi-MITF at twice the amount of GST+MIF completely inhibited the DNA binding of +MIF. The addition of GST-mi-MITF at four times the amount of GST+MIF completely abolished the binding of +MIF. The GST-mi-MITF itself did not bind to DNA, as reported previously.

**DISCUSSION**

The expression of the integrin α4 subunit gene was deficient in mi/mi CMCS. The adhesion to CHO-VCAM-1 was detectable in +/+ CMCS but not in mi/mi CMCS, indicating that the expression of integrin α4 subunit at a functional level did not occur in mi/mi CMCS. We surmised that the mi/mi mouse was a mutant with deficient expression of integrin α4 subunit in mast cells. The overexpression of +MIF but not mi-MITF transactivated the α4 promoter containing the CACTTG motif. The mutation or deletion of the CACTTG motif significantly decreased the transactivation ability of +MIF. These results indicated that the CACTTG motif played an important role for the transactivation by +MIF. The specific binding of +MIF to the CACTTG motif suggested that the +MIF directly transactivated the integrin α4 subunit gene.

The overexpression of mi-MITF reduced the luciferase activity in IC-2 cells. Hemesath et al reported the mechanism of dominant negative behavior of mi-MITF; the mi-MITF did not bind DNA, but it dimerized with proteins such as TFE3 and thereby inhibited the DNA binding of its normal partners. This was consistent with the present result. Apparently the overexpression of mi-MITF in IC-2 cells decreased the luciferase activity by inhibiting the binding of endogenous +MIF to the CACTTG motif.

Meirsman et al analyzed the mouse integrin α4 promoter and 5′-untranslated region in a lymphocytic leukemia cell line. The region around the CACTTG motif was not examined, but they demonstrated the presence of a silencer between nt −936 and −735 and an enhancer between nt +33 and +221. This finding is consistent with our present result. The transfection of the −949 or −819 reporter plasmid but not of the −516 reporter plasmid showed a low level luciferase activity, suggesting that some negative factors for α4 gene transcription bound upstream from nt −517. All reporter constructs used in the present study contained the region between nt +33 and +221. Even when the CACTTG motif was deleted or mutated, the reporter activity was not completely abolished. Endogenous transcription factors in IC-2 cells may associate the region between nt +33 and +221 and may transactivate the α4 gene in collaboration with +MIF.

We previously demonstrated that the addition of rmSCF changed the gene expression in mi/mi CMCS, although their
suggested the involvement of α4 subunit-VCAM-1 interaction in the augmentation of mast cells at inflammatory sites. In the present study, we demonstrated that +/+ CMCs but not mi/mi CMCs bound VCAM-1. Further analysis using mi/mi mice will clarify the role of α4 subunit-VCAM-1 interaction in the increase of mast cells at inflammatory sites.

The integrin α4 subunit was important not only for the migration to inflammatory sites but also for the differentiation of lymphocytes. Arroyo et al. demonstrated that mast cells expressed the α4 subunit, indicating that the α4 subunit was essential for differentiation of mast cells in tissues of the chimeric mice. Although mast cell number in tissues of the chimeric mice was not reported, there is a possibility that the development of mast cells may also be impaired in the chimeric mice. Smith and Weis demonstrated that mast cells expressed the α4 subunit. They suggested that the α4 subunit was important for the migration of mast cells from blood to tissues. The present result is consistent with their idea. Mast cell progenitors of mi/mi mice may show some difficulties in invading tissues due to the lack of the α4 subunit. Mast cell progenitors have not been identified in the peripheral blood of adult mice. Such identification would promote further analysis of the migration of mast cell progenitors. The mi/mi mice should be useful in evaluating the physiological roles of the integrin α4 subunit.

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REFERENCES

5. Morii E, Takebayashi K, Motohashi H, Yamamoto M, Nomura S, Kitamura Y: Loss of DNA binding ability of the transcription factor
encoded by the mutant \textit{mi} locus. Biochem Biophys Res Commun 205:1299, 1994


42. Reith AD, Rottapel R, Giddens E, Brady C, Forrester L, Bernstein A: W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor. Genes Dev 4:390, 1990


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