Characterization of Chicken Kit Tyrosine Kinase Receptor in Cos Cell Transfectants and in Chicken Brain

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ABSTRACT. The Kit tyrosine kinase (Kit) encoded by the c-*kit* proto-oncogene is a receptor for stem cell factor (SCF). Kit proteins of mice and humans are expressed in various kinds of hematopoietic progenitor cells and are essential for the growth of these cells. Wild-type chKit (chKit+) and a mutant chKit (chKit42) that contained an amino acid change from Asp^{777} to Asn corresponding to that in Kit of the W^{42} mutant mice were produced in Cos-1 cells transfected with expression plasmids containing the chicken c-*kit* cDNA, and characterized using two kinds of anti-chKit antisera. The W^{42} mutant Kit has previously been shown to be defective for kinase activity. The chKit+ of 145 kilodalton (kDa) and 130 kDa with varying degrees of N-linked glycosylation were detected. Western blot analysis using an anti-phosphotyrosine monoclonal antibody showed that autophosphorylation of chKit+ was greatly enhanced upon chicken SCF induction. The chKit+ did not respond to mouse SCF. The kinase activity of chKit42 was abolished by the amino acid substitution, indicating the Asp^{777} residue was essential for the activity. In addition, 145 kDa chKit conjugated with sialic acid residue(s) was detected in chicken brain by immunoprecipitation using the antisera. An *in vitro* kinase assay showed the kinase activity of this protein. These structural and functional similarities of chKit to mammalian Kit proteins shown in this study implicate a possible role of chKit in chicken hematopoietic system.—KEY WORDS: autophosphorylation, c-*kit*, Cos cell, stem cell factor, tyrosine kinase.

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The Kit protein encoded by the c-*kit* proto-oncogene [13, 21] is a transmembrane tyrosine kinase that is a receptor for stem cell factor (SCF) [9]. The importance of Kit for hematopoiesis became apparent from the *dominant white spotting* (*W*) mutant mice which show various lesions in the c-*kit* gene locus and are characterized by anemia, mast cell deficiency and a reduction of progenitors for various hematopoietic lineages [2, 4]. Mammalian Kit proteins from various sources including mouse mast cells [10, 11, 19], cat brain [8] and a human glioblastoma cell line [21] have been detected as proteins with apparent sizes ranging from 160 kilodalton (kDa) to 124 kDa, and shown to have a tyrosine kinase activity *in vitro*. The apparent sizes of the Kit proteins are larger than those deduced from cDNA data [13, 21], suggesting the glycosylation of the proteins. In fact, Majumder *et al.* [8] have shown that Kit protein in cat brain is conjugated with sialic acid residues and N-linked sugar chains. Mammalian Kit proteins have been shown to respond to stimulation with their cognate ligands. Upon binding with SCF, Kit forms a dimer, autophosphorylates on tyrosine, and then associates with a distinct set of proteins that generate intracellular second messengers [1, 7, 15]. These steps are essential for signal transductions mediated by Kit.

Many independent *W* mutations varying in their effects on various cell lineages have been characterized [3, 16]. The tyrosine kinase activity of the Kit proteins encoded by several of the *W* mutant alleles have been shown to be impaired at varying degrees [10, 11, 19]. Among them, the W^{42} mutant Kit completely lacks its kinase activity due to an amino acid (aa) substitution Asp^{780} to Asn within its kinase domain, but its size and cell surface expression are not affected by the mutation [19]. The Asp^{780} resides within an aa sequence DLAARN that is completely conserved among protein kinases and is implicated in the binding of the kinases with ATP [5].

Cloning of chicken c-*kit* cDNA has suggested that chicken Kit (chKit) has structural features similar to mammalian Kit proteins [17]. In this study, two kinds of antisera that specifically reacted with chKit were prepared. Then, wild-type chKit and a mutant chKit with an aa substitution that corresponded to that in the W^{42} mutant Kit were produced in Cos-1 cells, and characterized using the antisera. In addition, 145 kDa chKit in chicken brain was detected.

MATERIALS AND METHODS

Construction of prokaryotic gene fusion plasmids: The chicken c-*kit* cDNA [nucleotide (nt) 1–5111] [17] was inserted to the *Not*I site of the pUC118 vector in which the *Sma*I site had been changed into a *Not*I site by linker ligation and the *Sal*I site had been erased by *Sal*I digestion followed by filling-in and religation. The resultant plasmid pKitN was cut with *Hpa*I, ligated with *Sal*I linker DNA, and recut with *Sph*I and *Sal*I. Then the fragment containing the cDNA (nt 1–3088) was inserted between the *Sph*I and *Sal*I sites of the pUC18 vector to make pKitP. A part of the cDNA (nt 89–669) was amplified by polymerase chain reaction (PCR) using pKitN as a template and oligonucleotide primers (5')-CCGCATGC-AGTACTTCAGTGCCTCATGAA-(3') and (5')-TGCT-

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ATCCACATCCGTGATT-(3'). The amplified DNA was digested with *Sph*I and *Eco*T22I, and then inserted between the *Sph*I and *Eco*T22I sites of pKitP to make plasmid pKitC. The *Sca*I-*Sal*I fragment of pKitC was inserted between the *Xmn*I and *Sal*I sites of pMal-c2 (New England Biolabs, Beverly, MA, U.S.A.) to make a gene fusion plasmid pMalchKit, which was supposed to encode the chKit sequence from aa 25 to aa 960 fused to the maltose binding protein (MBP) and an extra Thr residue between these proteins. For making another gene fusion plasmid pGSTchKit, pKitC was cut with *Sca*I and *Sal*I, filled-in, and inserted to the *Sma*I site of pGEX-3X (Pharmacia, Uppsala, Sweden). The resultant plasmid was cut with *Kpn*I and *Sma*I. The fragment containing the vector sequence was purified, filled-in, and then self-ligated to make pGSTchKit, which was supposed to encode the chKit sequence from aa 25 to 477 and from aa 920 to aa 960 fused to the glutathione-S-transferase (GST).

Construction of eukaryotic expression plasmids: One region of the c-*kit* cDNA (nt 17–353) was amplified by PCR using pKitN as a template and primers (5')-GGCTC-GAGCCATGGAGGGCGCGCAC-(3') and (5')-CAGC-AGGATGTCACTGTCTT-(3'). The amplified DNA was cut with *Xho*I and *Hin*cII, and inserted between the *Xho*I and *Hin*cII sites of pBluescript SK(−) (Stratagene, La Jolla, CA, U.S.A.) to make pKitH. The *Sal*I site of pKitP was changed into a *Not*I site by linker ligation. The *Not*I - *Hin*cII fragment of the resultant plasmid containing the cDNA (nt 283–3088) was inserted between the *Not*I and *Hin*cII sites of pKitH to make pCCKit. The pCCKit was cut with *Not*I, filled-in, and recut with *Xho*I. The resultant fragment containing the cDNA was inserted between the *Xho*I and *Sma*I sites of pSVL (Pharmacia, Uppsala, Sweden) to make an expression plasmid pchKit+. One region of the cDNA (nt 2191–2973) with a G to A base change at nt 2330 was prepared by the two-step PCR method [6] using pKitN as a template and two sets of primers [(5')-AAAAGGCCGGTGAAATCTGG-(3') and (5')-TTGCTGCCAGATTCCTATGAATGCA-(3'), and (5')-TGCATTCATAGGAAATCTGGCAGCAA-(3') and (5')-ACAGTGGTAGTAGTGAGTAA-(3')] (underlined bases correspond to the mutation site at nt 2330). The amplified DNA was cut with *Bgl*II and *Sma*I, and the *Bgl*II-*Sma*I portion of pchKit+ was replaced with this fragment to make pchKit42.

Preparation of anti-chKit antibodies: The MBP-chKit fusion protein was produced in *Eschericia coli (E. coli)* JM109 cells transformed with pMalchKit, and purified from the cell lysate by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%). A rabbit anti-chKit antiserum was raised by using the purified protein (4 mg) as an antigen. The GST-chKit fusion protein was produced in the bacterial cells transformed with pGSTchKit and purified as above. The coupling of the purified protein (1 mg) to a HiTrap NHS-activated column (Pharmacia, Uppsala, Sweden) and the affinity purification of the antiserum on this column were performed following the manufacturer's instructions. An anti-chKit peptide antiserum was raised in a rabbit using a decapeptide TQPLLVREDV that corresponded to the C-terminus of chKit as an antigen.

Transfection and labeling of Cos-1 cells: Cos-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS) and antibiotics. The cells (1×10^6) were transfected with 10 μg DNA using 20 μg Lipofectamine (Gibco-BRL, Gaithersberg, MD, U.S.A.). Seventy two hour after transfection, cells were labeled with 118.5 μCi [^{35}S]methionine (New England Nuclear, Boston, MA, U.S.A.) for 5 hr in 5 m*l* of methionine-free DMEM plus 10% dialyzed FCS. Cell lysates were prepared and immunoprecipitated with affinity purified anti-chKit antibody essentially as described by Reith *et al.* [14]. Then, immunoprecipitates were analyzed by SDS-PAGE (7.5%) and autoradiography. In some experiments, 10 μg/m*l* tunicamycin (Sigma, St. Louis, MO, U.S.A.) was added to the labeling medium.

Tyrosine phosphorylation analysis: Cos-1 cells were transfected as above. On the following day, cells were refed with 10 m*l* DMEM plus 0.5% FCS and grown for 16 hr. Cells were stimulated with 100 ng/m*l* recombinant soluble chicken SCF (chSCF) or 200 ng/m*l* recombinant soluble mouse SCF (mSCF) (R & D Systems, Minneapolis, MN, U.S.A.) for 2 min at 37°C. Recombinant chSCF was produced in insect cells infected with a recombinant baculovirus containing the chSCF cDNA [22] and purified by ion exchange column chromatography (unpublished result). Immunoprecipitation of lysates of the cells using affinity purified anti-chKit antibody and detection of tyrosine phosphorylated chKit using anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Lake Placid, NY, U.S.A.) as a primary antibody and horse radish peroxidase-conjugated rabbit anti-mouse IgG (Jackson Immune Research Laboratories, West Grove, PA, U.S.A.) as a secondary antibody were performed as described by Reith *et al.*[14] with slight modifications.

Detection and glycosidase digestion of chKit in chicken brain: The whole brain of a 2 month-old chicken (White Leghorn) was homogenized in PCL lysis buffer [14] (1 m*l* per 200 mg tissue weight). Immunoprecipitation of the lysate (1 m*l*) using 10 μ*l* anti-chKit peptide antiserum was performed as described above. One batch of the immunoprecipitate was digested with neuraminidase from *Streptococcus* (E.C.3.2.1.18) (Genzyme, Boston, MA, U.S.A.), another with neuraminidase and endo-α-N-acetylgalactosaminidase from *Diplococcus pneumoniae* (E.C.3.2.1.97) (Takara, Kyoto, Japan) under the conditions described previously [18]. The chKit proteins in the digests were detected by Western blot analysis using anti-chKit peptide antiserum as a primary antibody and horse radish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Buckinghamshire, England) as a secondary antibody.

In vitro kinase assay of chKit in chicken brain: The chicken brain lysate (1 m*l*) was immunoprecipitated with 100 μ*l* affinity purified anti-chKit antibody. *In vitro* kinase

reaction using the immunoprecipitate was carried out as described by Majumder *et al.* [8].

RESULTS

Preparation of anti-chKit antisera: Based on the comparison of deduced aa sequences between chKit and mammalian Kit proteins, a putative signal peptide (aa 1–24), an extracellular domain (aa 25–505), a transmembrane domain (aa 506–530), and an intracellular domain (aa 531–960) of chKit have been identified [17]. To make an antiserum against the putative mature chKit protein, a fusion protein containing the chKit polypeptide from aa 25 to 960 which was fused with MBP was produced in *E. coli* cells transformed with pMalchKit (Fig. 1A). This fusion protein was purified by preparative SDS-PAGE and used for raising a rabbit antiserum as an antigen. Another fusion protein containing the chKit polypeptide from aa 25 to 477 and aa 920 to 960 which was fused with GST was produced in bacterial cells and purified as above. An affinity column coupled to this purified protein was used to purify the anti-chKit antiserum.

Characterization of chKit proteins in Cos cell transfectants: To characterize chKit encoded by the cDNA [16], two expression plasmids were constructed for use in transient expression assays in Cos-1 cells (Fig. 1B). A plasmid pchKit+ encodes wild-type chKit (chKit+). The other plasmid pchKit42 encodes a mutant chKit containing an aa change Asp^{777} to Asn (chKit42). This plasmid was used in order to examine the effect of the aa substitution which corresponded to that in the W^{42} mutant Kit [19] on the properties of chKit. Cos-1 cells transfected with the expression plasmids were labeled with [^{35}S] methionine. And the chKit proteins were detected by immunoprecipitation with anti-chKit antiserum. As shown in Fig. 2, chKit+ species of 145 kDa and 130 kDa were detected in Cos-1 cells transfected with pchKit+. The chKit42 species with the same apparent sizes were observed in Cos cell transfectants, and its expression level was similar to that of the wild type protein. Upon tunicamycin treatment, a single 110 kDa protein band was observed in cells transfected with either plasmid, suggesting that the 145 kDa and 130 kDa bands represented chKit with different degrees of N-linked glycosylation.

Autophosphorylation at a tyrosine residue(s) is the immediate response to ligand stimulation of receptor-type tyrosine kinases [20]. To examine whether chKit+ could respond to stimulation with chSCF, Cos cell transfectants were incubated with or without chSCF, and the cell lysates were immunoprecipitated with anti-chKit antiserum. Then, tyrosine phosphorylated protein(s) was detected by Western blot analysis. As shown in Fig. 3, chKit+ species of 145 kDa and 130 kDa exhibited a constitutive level of tyrosine phosphorylation in the absence of ligand stimulation. The phosphorylation of chKit+ was greatly enhanced upon chSCF induction, indicating the response of

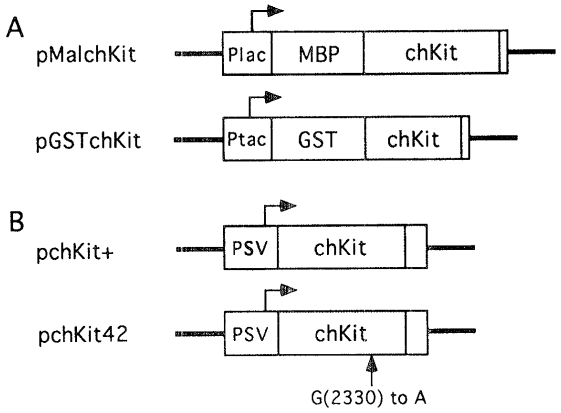

Fig. 1. Expression plasmids used in this study. (A) Prokaryotic gene fusion plasmids. In a plasmid pMalchKit, the sequence encoding a chKit polypeptide (aa 25–960) (chKit) is connected with the MBP gene (MBP). In pGSTchKit, the sequence encoding a chKit polypeptide (aa 25–477 and aa 920–960) (chKit) is connected with the GST gene (GST). Plac and Ptac indicate the prokaryotic *lac* and *tac* promoters, respectively. The transcription start sites are indicated by arrows. Horizontal bold lines depict the vector sequences. (B) Eukaryotic expression plasmids. In pchKit+and pochKit42, the entire coding region for chKit (chKit) is inserted to pSVL. PSV indicates the SV40 late gene promoter. Position of the single base change introduced in pchKit42 [G(2330) to A] is indicated.

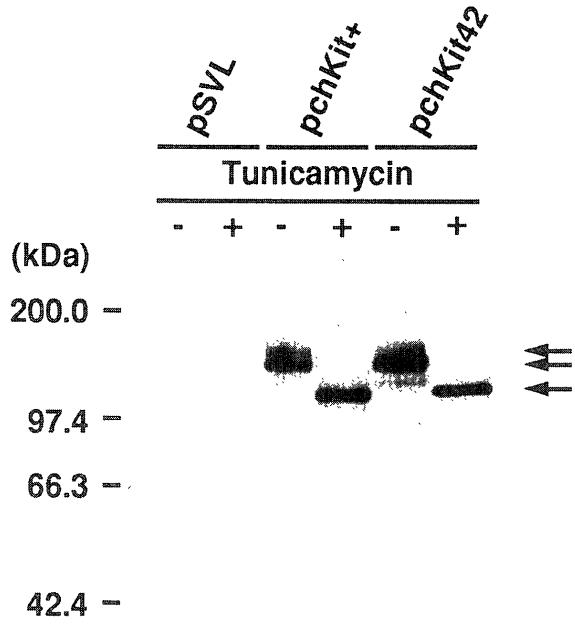

Fig. 2. Recombinant chKit proteins produced in Cos-1 cells. Cos cells were transfected with indicated plasmids, labeled with [^{35}S] methionine in the presence (+) or absence (-) of tunicamycin. Cell lysates (equivalent to approximately 3×10^5 cells) were immunoprecipitated with anti-chKit antiserum and subjected to SDS-PAGE prior to autoradiography. The chKit protein species are indicated by arrows. Sizes of molecular weight markers are indicated.

chKit+ to chSCF stimulation. Whereas stimulation with mSCF resulted in no increase in tyrosine phosphorylation of chKit+, indicating that the response by chKit+ was species specific. In contrast to chKit+, tyrosine phosphorylation of chKit42 was not detected even upon chSCF stimulation, suggesting that the kinase activity of chKit42 was abolished by the single aa substitution. In agreement with these results, *in vitro* kinase assays with these two proteins indicated a significant level of kinase activity of chKit+ and no activity of chKit42 (not shown).

Detection and characterization of chKit in chicken brain: A previous *in vitro* kinase assay indicated a high level of expression of mouse Kit in mouse brain and significantly lower levels in the spleen and testis [8]. Immunoprecipitation and subsequent Western blot analysis with anti-chKit peptide antiserum detected a 145 kDa protein in chicken brain (Fig. 4A). Immunoprecipitation with affinity purified anti-chKit antibody detected a protein with the same size (not shown). An *in vitro* kinase assay indicated

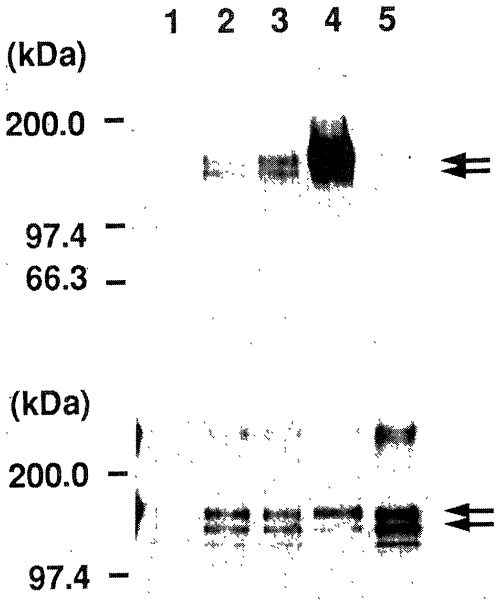

Fig. 3. Tyrosine phosphorylation of chKit+ and chKit42. Cos cells were transfected with pSVL (line 1), pchKit+ (lines 2, 3, 4) or pchKit42 (line 5). The cells were incubated with (lines 1, 4, 5) or without (line 2) chSCF, or with mSCF (line 3), as described in MATERIALS AND METHODS. Then, cell lysates were immunoprecipitated with affinity purified anti-chKit antibody, and subjected to SDS-PAGE (7.5%) and Western blot analysis using anti-phosphotyrosine monoclonal antibody as a primary antibody (upper panel). To monitor the amounts of chKit proteins, immunoprecipitates were subjected to Western blot analysis using anti-chKit antiserum as a primary antibody (lower panel). Positive signals were detected by using a chemiluminescence-based system ECL (Amersham, Buckinghamshire, England). The chKit protein species are indicated by arrows. Sizes of molecular weight markers are indicated.

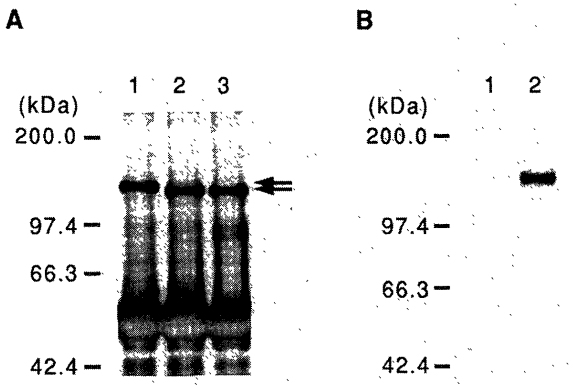

Fig. 4. chKit in chicken brain. (A) Chicken brain lysate was immunoprecipitated with anti-chKit peptide antiserum. Then, undigested immunoprecipitate (lane 1) and immunoprecipitate digested with neuraminidase (lane 2) or with neuraminidase and endo-α-N-acetylgalactosaminidase (lane 3) were subjected to SDS-PAGE (7.5%) and Western blot analysis. The chKit protein species are indicated by arrows. Sizes of molecular weight markers are indicated. (B) Immunoprecipitation of chicken brain lysate and *in vitro* kinase reaction were performed as described in MATERIALS AND METHODS. The reaction mixture was reimmunoprecipitated with preimmune serum (lane 1) or affinity purified anti-chKit antibody (lane 2), and subjected to SDS-PAGE (7.5%) and autoradiography.

the kinase activity of this protein (Fig. 4B). Treatment of the immunoprecipitate with glycosidases indicated that this protein was conjugated with sialic acid residue(s) and little, if any, of O-linked sugar chain (Fig. 4A). The apparent size of the protein after digestion with neuraminidase and endo-α-N-acetylgalactosaminidase, 135 kDa, was still much larger than the deduced molecular weight (105 kDa), suggesting the N-glycosylation of the protein. However, any discrete protein species was detected after digestion with N-glycanase, presumably due to residual proteinase activity in the immunoprecipitate (not shown).

DISCUSSION

In agreement with the prediction based on the nt sequence of the chicken c-*kit* cDNA [16], analysis of wild-type chKit (chKit+) produced in Cos-1 cells indicated the structural and functional features of chKit similar to mammalian Kit proteins in the following aspects: (1) its apparent sizes, (2) its possession of N-linked sugar chain(s), and (3) its autophosphorylation in response to stimulation with its ligand.

Mammalian Kit proteins have been shown to be expressed in progenitors for various hematopoietic lineages, and to be essential for the normal growth of these cells [10–12, 19]. In chickens, the expression of chKit mRNA was detected in hematopoietic organs including the spleen, as well as in the brain [17]. The biochemical similarities of chKit to mammalian Kit proteins shown in this study further suggest that chKit

would play a role that is analogous to that of mammalian Kit proteins. At variance with the mRNA data, however, Western blot analysis using the anti-chKit antibody failed to detect chKit in the spleen, presumably owing to low abundance of this protein (not shown). In fact, Majumder *et al.* [8] reported that the level of expression of mouse Kit in the spleen was significantly lower than that expected from the level of expression of the mRNA.

Two isoforms of mouse Kit differing by the presence or absence of a 4-aa insertion in the extracellular domain have been shown to be produced by alternative splicing [14]. Both isoforms recombinantly produced in Cos-1 cells respond to mSCF stimulation. However, one isoform without the insertion exhibits a significant level of tyrosine phosphorylation in the absence of ligand stimulation while the other isoform with the insertion does not. Sequence comparison between the chicken c-*kit* cDNA used for the expression of chKit proteins in this study [17] and those for the mouse isoforms [14] indicates that chKit+ corresponds to the isoform without the aa insertion. Consistent with this sequence similarity, chKit+ was shown to exhibit a detectable level of constitutive phosphorylation (Fig. 3). Characterization of chicken c-*kit* cDNA clones [17] has indicated that the isoform without the insert is the dominant species of chKit in chicken brain. The biological implication of the constitutive phosphorylation of this isoform remains unclear.

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