The Mutation $S^{511}N$ Leads to N-glycosylation and Increases the Cleavage of High Molecular Weight Kininogen In Rats Genetically Susceptible to Inflammation

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Running Title: A single mutation increases cleavage of HK by kallikrein.

Scientific Heading: Hemostasis, Thrombosis and Vascular Biology

Grants and Financial Support:
This work was supported by grants of the NIH RO1 DK 43735, HL60683 and P30 DK 34987 as well as a research grant from the Crohn’s and Colitis Foundation of America.

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Abstract Count: 199
Total Word Count: 5486
ABSTRACT

Crohn’s disease is immunologically mediated and characterized by intestinal and systemic chronic inflammation. In a rat model, injection of peptidoglycan-polysaccharide complexes into the intestinal wall induced chronic inflammation in Lewis but neither Fischer nor Buffalo rats, indicating a differential genetic susceptibility. Proteolysis of plasma high molecular weight kininogen (HK) yielding bradykinin and cleaved HK (HKa), was faster in Lewis than in Fischer or Buffalo rat plasma. A single point mutation at nucleotide 1586 was found translating respectively to Ser\(^{511}\) (Buffalo and Fisher) and Asn\(^{511}\) (Lewis). The latter defines a NXT consensus sequence for N-glycosylation. We expressed these domains in \textit{E.coli} and found no differences in the rate of cleavage by purified kallikrein in the three strains in the absence of N-glycosylation. We then expressed these domains in CHO cells, which are capable of glycosylation and found an increased rate of cleavage of Lewis HK. The Lewis mutation is associated with N-glycosylation as evidenced by a more rapid migration after treatment with N-glycosidase F. When CHO cells were cultured in the presence of tunicamycin the kallikrein- induced cleavage rate of Lewis HK was not increased. This molecular alteration might be one contributing factor resulting in chronic inflammation in Lewis rats.

Key Words: kininogen, N-glycosylation, rat, bradykinin, genetics
INTRODUCTION

Inflammatory bowel diseases, including Crohn’s disease and ulcerative colitis, are complex disorders characterized by intestinal and systemic chronic inflammation as well as unpredictable relapse and remissions. The etiology of Crohn’s disease is unknown. However, it appears to be immunologically mediated and have a genetic component. In 1996 Hugot et al reported that IBD1, a region on chromosome 16, was a locus for genetic susceptibility in Crohn’s disease. More recently the same group has identified variants of the gene NOD2 in chromosome 16, which encodes a protein important in the intracellular response to peptidoglycan and endotoxin, as a risk factor. Another laboratory simultaneously described that a frame shift mutant of NOD2 present in 8% of patients with Crohn’s disease. The hypothesis that this disorder is immunologically mediated is also supported by the findings that a cluster of cytokine regulatory genes on chromosome 5 promoted susceptibility to Crohn’s disease. In mice, a cluster of cytokine regulatory genes on chromosome 3 modified susceptibility to colitis in IL-10 deficient mice.

In animal models, the aggressiveness and chronicity of intestinal inflammatory processes are dependent on the genetic background of the host. The Lewis rat is clearly more susceptible to develop chronic intestinal and systemic inflammation than the Buffalo and the Fischer F344 rat. However, the genes involved in these differences have not yet been identified. We developed a rat model of chronic granulomatous enterocolitis by injecting purified bacterial cell wall polymers, peptidoglycan polysaccharide from group A streptococci (PG-APS), into the intestinal wall. Spontaneous reactivation of chronic granulomatous inflammation in the intestine, liver and peripheral joints were restricted to genetically susceptible Lewis and Sprague Dawley rats, and did not occur in Buffalo and Fischer rats. Female Lewis rats, the highest responders, develop biphasic
intestinal inflammation with an acute phase that peaks 2-4 days after PG-APS injection and gradually decreases over next 10 days\textsuperscript{9,13}. The enterocolitis spontaneously reactivates on days 12-14, accompanied by T lymphocyte-mediated peripheral erosive arthritis, granulomatous hepatitis, normochromic anemia, and leukocytosis.

The plasma contact system in humans and rats, which plays an important role in inflammatory processes, consists of four major proteins\textsuperscript{14,15}, factor XII, factor XI, prekallikrein (PK), and high molecular weight kininogen (HK). A negatively charged surface such as endotoxin is able to autoactivate zymogen factor XII, which converts PK to kallikrein and coagulation factor XI to factor XIa. Kallikrein, in the presence of HK, stimulates neutrophil chemotaxis\textsuperscript{16}, aggregation\textsuperscript{17} and induces neutrophil elastase release\textsuperscript{18}. Activated factor XII stimulates neutrophil aggregation\textsuperscript{19} and interleukin-1 expression in monocytes\textsuperscript{20}.

Human HK is a 110-120 kDa plasma glycoprotein. Approximately 30\% of the weight is due to N-linked or O-linked carbohydrates. The N-terminal heavy chain of HK (domains 1-3) contains 3 N-linked carbohydrate chains. The light chain of HK (domains 4-6) contains 9 O-linked carbohydrate moieties but lacks N-linked carbohydrates. The rat HK amino acid sequence based on nucleotide sequences has been reported (Table 1) and is illustrated in Isordia-Salas et al\textsuperscript{21}. The location of the carbohydrate moieties have not been reported for rat HK but are suggested by identity/homology with the human sequence. Thus the Fischer and Buffalo rat HK light chain would lack any N-linked carbohydrates.

Plasma kallikrein cleaves human and rat HK in a two-step pattern. The first and second cleavage yields a heavy chain (64kDa) joined by a single disulfide bond to a light chain (56kDa) and release of bradykinin. The third cleavage occurs later and proteolyses the 56 kDa light chain
to a 45 kDa light chain and a 9 kDa peptide. Bradykinin is pro-inflammatory\textsuperscript{22-25}.

We have documented that a specific plasma kallikrein inhibitor modulates chronic granulomatous enterocolitis\textsuperscript{26}, inflammatory arthritis\textsuperscript{27} and systemic inflammation in genetically susceptible Lewis rats. Previously we also examined the \textit{in vitro} activation of the contact system in Lewis and Buffalo rat plasma using a negatively charged surface, kaolin, as the contact activator and found that at 15 minutes, Lewis rat HK was cleaved 66\% while Buffalo rats showed only 23 \% cleavage\textsuperscript{9}. This result was not due to a difference in the amount of plasma kallikrein formed after exposure to kaolin as measured with an amidolytic substrate\textsuperscript{9}. We localized the abnormality to HK by showing that its cleavage by purified human plasma kallikrein in Lewis rat plasma was more rapid than that in Buffalo rat plasma\textsuperscript{9}. In this report we further examined the different plasmas to determine if differences in levels of other contact factors (factor XII and prekallikrein) may contribute to the observation.

The present report identifies the genetic structural defect and explains the molecular mechanism responsible for accelerated HK cleavage in susceptible Lewis rats.
METHODS

Materials. Purified human plasma kallikrein was purchased from Enzyme Research Laboratories (South Bend, IN). N-Glycosidase F from Calbiochem (San Diego, CA). Factor deficient plasmas were obtain from George King Biomedicals (Overland Park, KS).

Collection of rat plasma and assays of contact activation in vitro. Blood drawn from anesthetized Lewis, Fischer and Buffalo rats by cardiac puncture as collected into polypropylene test tubes to contain 3.8 % citrate for coagulant assays. Plasma was isolated by double centrifugation at room temperature and then stored at -70°C. Prekallikrein (PK) function levels were performed by a coagulant assay as described using plasma deficient in PK. HK coagulant activity was evaluated by our modification of an APTT test assay, using total kininogen deficient plasma. Factor XII coagulant activity was performed by a similar method using factor XII deficient plasma.

Release of bradykinin from rat plasma after activation of the contact system by incubation with kaolin. A suspension of kaolin (1mg/ml), phosphate buffered saline, pH 7.4 (PBS), was added to rat plasma (1:9; v:v) containing 25 mM of 1,10 phenanthroline. Aliquots were removed at 15,45,75,105, and 135 seconds. Bradykinin was determined by an enzymatic immunoassay method (Dainninppon, Pharm., Osaka, Japan).

Cleavage of HK in rat plasma after activation of the contact system by incubation with dextran sulfate. Citrated rat plasma was activated with dextran sulfate 500 kDa (40 µg/ml) and examined for HK cleavage by the Western blot technique. At the time points indicated, a 2 µl aliquot was removed from the reaction mixture and added to 200 µl LDS PAGE plasma sample buffer containing DTT and 1% additional LDS. The samples were run using 7% Tris-Acetate Gels
(Nupage-Invitrogen, Carlsbad, CA) and Western blotted onto a PVDF membrane. The blot was probed using an IgG- rabbit-anti-rat HK polyclonal antibody diluted 1:2000 (donated by Dr. Albert Adam, University de Montréal, Montréal, Canada), then probed with using a goat-anti-rabbit IgG alkaline phosphatase conjugate (Sigma). BCIP/NBT (Kirgegard/Perry Laboratories, Gaithersburg, MD) was added to visualize the protein.

**Generation of 531 bp (HK domains 4-5) and 492bp (HK5-6 domains) PCR products.**

Genomic DNA from rat kidneys was extracted (Nucleon BACC3, Belmont California). The primers used are listed in Table 2. The PCR reactions were carried out in pfu polymerase buffer (Pharmacia, Piscataway, New Jersey), 200 nM dNTPs (Stratagene, La Jolla California), 1 µM primers, 100 ng of rat genomic DNA template and 5U of pfu polymerase (Stratagene) for 30 cycles of 1 minute duration at 94°C, 1 minute annealing at 60°C, and 3 minutes extension at 72°C.

**Restriction enzyme analysis of PCR products from Lewis and Fischer rats.** The 492 bp PCR products (200 ng) corresponding to HK domains 5-6 from Lewis and Fischer rat were analyzed using the Sca I restriction enzyme (15 U) in 100 µl at 37°C for 16 hours. The products were analyzed using DNA 500 Assay LabChip (Agilent Technology) on an Agilent 2100 Bioanalyzer. Size was determined by the Bioanalyzer software using the DNA 500 ladder.

**Generation of HK 4-6 domains (819 bp) recombinant plasmid for the expression in E. coli.**

The primers used are described in Table 2. The sense primer used, SPK89G, contained a Bgl II site followed by the exact coding sequence of the N-terminal 7 amino acids of exon 10 (residues 358-364) of rat kininogen. The antisense primer SPK89F is located 31 nucleotides into the 3’ untranslated region sequence beyond the termination codon followed by an EcoRI site. The PCR reaction was carried out as described above. The 819 bp PCR product (of HK domains 4-6) was
cloned into pGEX2T vector (Pharmacia) according manufacturer instructions. This construction results in glutathione-S-transferase (GST) fused to N-terminal of HK cDNA containing rat HK (aa 360-621). The ligation reaction was used to transform competent E. coli, (Stratagene), and the transformants were selected by resistance to ampicillin (Gibco BRL, La Jolla, CA). The insert was analyzed by restriction analysis. Positive colonies were selected for the analysis of recombinant protein.

**Expression and Purification of recombinant proteins in E. coli.** Transformants containing the correct HK sequence of each of the three rats strains were grown overnight in LB broth containing 100 µg/ml ampicillin at 37°C. This culture was diluted (1:10) in fresh LB broth, grown for 4 hours, and then induced with 1mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2 hours. The cells were collected and resuspended in PBS containing 1mM phenylmethane sulfonl fluoride, 10 mM EDTA, and 0.5 µM pepstatin. The cells were disrupted by sonication adding 1% Triton X-100. Cell debris was removed by centrifugation at 12,000xg for 15 min at 4°C. The recombinant fusion protein was purified using GST-Sepharose column according to manufacture’s instructions (Amersham Biosciences, Piscataway, NJ). The protein was visualized on 10% SDS polyacrylamide gels (Biorad).

**Expression and Purification of recombinant proteins in CHO cells.** The same 819 bp PCR product which was used to create the bacterial expressed recombinant proteins was used to create CHO cell-expressed His-tag linked recombinant proteins. We cloned the PCR product into pcDNA 3.1 vector between the BamHI and EcoRI sites. (Invitrogen Carlsbad, CA). The ligation reaction was used to transform competent TOP 10F’ cells (Invitrogen), and gentamicin was used as a selectable marker. The integrity of the constructs was confirmed by sequencing. CHO cells
(ATCC, Rockville, M.D.) were grown in F12 Medium containing 10% fetal bovine serum at 37°C in 5% CO₂ atmosphere. In some experiments, CHO cells were grown in the presence of tunicamycin (800 ng/ml) in order to inhibit N-glycosylation. Cells were transfected with the target vector by the lipofectamine method according to the manufacturer’s instructions (Gibco Life Technologies, Gaithersburg, MD). The cells were grown in F12 medium and harvested for the assay. The cells were washed, and resuspended in PBS plus protease cocktail inhibitor (Sigma, St Louis, MO) to prevent proteolysis. Cell debris was removed by centrifugation. The purification of the expressed His-tagged HK domains 4-6 [(His)₆-HK4-6] was carried out under native conditions as per manufacturer instructions. The protein was eluted using an imidazole gradient 50-350 mM in an elution buffer pH 6.0. The protein was concentrated, dialyzed in 10 mM Tris pH 8.0 or at 4°C and quantified by a colorimetric assay.

Kallikrein cleavage of bacterial recombinant GST-HK4-6 and mammalian recombinant (His)₆-HK4-6. Recombinant GST-HK4-6 and (His)₆-HK4-6 (7.69 µM) were each activated by kallikrein (135 nM) at 0, 5, 15, 30, 60, 90, 120 minutes. Recombinant (His)₆-HK4-6 (5.00 µM) were each activated by kallikrein (50 nM) after produced in culture in the absence and presence of tunicamycin. Sample aliquots were transferred into a tube containing polyacrylamide sample buffer containing DTT. Electrophoresis was performed according to the method of Laemmli on 10% SDS-PAGE gel (Biorad laboratories, Hercules, CA) or 10% Bis-Tris LDS-PAGE gels using MOPS buffer (Invitrogen Life Technologies, Carlsbad, CA). The recombinants were stained with Gelcode Blue Stain (Pierce) or Simply Blue Safestain (Invitrogen). In the blotted experiments, recombinant CHO cell expressed (His)₆-HK4-6 were electro-transferred to a polyvinyl difluoride membrane (Immunlon-P) using a Mini Trans-Blot Transfer Cell for 1 hour. The membrane was
blocked for 60 minutes in TBST (Tris Buffered Saline with 0.1% Tween-20) and 3% BSA or 5% Irish Cream, washed 3 times with TBST, and incubated with anti-Xpress antibody, (Invitrogen, which recognizes N-terminal polyhistidine tag) diluted in TBST and 1% BSA or Irish Cream overnight at 4°C. After washing 3 times with TBST, the membrane was incubated with alkaline-phosphatase labeled anti-mouse IgG (whole molecule) (Sigma Chemical Co), for 1 hour. The protein was visualized by alkaline-phosphatase substrate (Kirgegard/Perry Laboratories, Gaithersburg, MD).

**N-Glycosidase F cleavage of CHO expressed (His)₆-HK4-6.** Purified CHO expressed Lewis, Fischer and Buffalo (His)₆-HK4-6 were diluted to 4.2-5.3 µM in 0.1% SDS, 50 mM β-mercaptoethanol and 50 mM NaHPO₄ in 50 µl and boiled for 5 minutes at 100°C. Then 10 µl of N-glycosidase F (10 Units) or H₂O were added to each tube and incubated for 72 hours at room temperature. The samples were subjected to LDS gel electrophoresis using a 10% Bis/Tris 1.5 mm gels using MOPS buffer (Invitrogen Life Technologies, Carlsbad, CA). Molecular weight of the samples were determined using SeeBlue Plus 2 (Invitrogen). Samples were also subjected to Western blot technique and the his-tagged protein was detected as described previously (see section on cleavage by kallikrein).

**Computer Analysis.** All cleavage patterns were analyzed using Quantity One software (Biorad Laboratories, Hercules, Ca).
RESULTS

Release of bradykinin from Lewis and Buffalo rat plasma. We have extended our previous findings\(^9\) using kaolin as activator by showing an accelerated rate of in-vitro bradykinin release and increased concentrations in Lewis rat plasma to compare to Buffalo rat plasma (Figure 1).

The rate was two times faster in Lewis rat plasma than in the Buffalo rat plasma (663 pg/ml/sec vs. 320 pg/ml/sec respectively). At 75, 105 and 135 seconds the concentrations of bradykinin in the plasma of Lewis rats were significantly higher than those in the Buffalo rat (p< 0.01, 0.01 and 0.05 respectively).

Figure 1. Plasma bradykinin release.

Bradykinin formation in Lewis rat plasma containing kinin inhibitor. The contact system of citrated Lewis (open circles) and Buffalo (closed circles) rat plasma containing 1,10-phenanthroline (2.5 mM) was activated by activation with kaolin. At the indicated time, an aliquot was removed and tested for the levels of bradykinin. Mean ±SD, n=3. * p < 0.05, ** p < 0.01 by Students t-test.

Cleavage of HK in rat plasma after activation of the contact system by incubation with dextran sulfate. We employed a soluble activator, dextran sulfate, to quantify the cleavage of HK in plasma. We also examined the plasma of a third strain of rat (Fischer rat) which behaves
similarly to resistant Buffalo rat in the chronic enterocolitis model. The HK band migrated at 110 kDa in all these plasmas prior to the addition of dextran sulfate (Figure 2).

Figure 2. Western Blot of Lewis (L), Fischer (F) and Buffalo (B) rat plasma HK. Blood was collected into sodium citrate (see methods) from each of the rat species. An aliquot was added to a customized LDS PAGE plasma sample buffer containing reducing agent (see methods). After electrophoresis, blotting and blocking, the blot was probed using an IgG- rabbit-anti-rat HK polyclonal antibody and detected using a goat-anti-rabbit IgG alkaline phosphatase conjugate and BCIP/NBT. A specific band at 110 kDa was observed representing rat HK in the plasma. A non-specific band at 68 kDa is an artifact of the assay dye reacting with blotted rat albumin.

That band disappeared almost completely by 2.5 minutes in Lewis rat plasma (Figure 3 top). In Buffalo and Fischer rat plasma, cleavage was markedly slower and the residual HK was greater at 30 minutes than the Lewis HK was at 2.5 minutes. Densitometry analysis demonstrates the marked increase in the mean rate of cleavage in the Lewis rat plasma compared to Buffalo and Fischer rat plasma (Figure 3 bottom). Buffalo and Fischer rat plasma, compared to Lewis rat plasma at points between 5-30 minutes, were highly significantly different (p< 0.001). No statistical difference was found at any time between Fischer and Buffalo rats.

Figure 3 (Top). Time dependent cleavage of HK in Lewis, Fischer and Buffalo Rat plasma. Citrated plasmas activated with dextran sulfate (40 µg/ml) and examined for HK cleavage by Western blot technique. At the time points indicated an aliquot was removed from the reaction
mixture and added to a customized LDS PAGE plasma sample buffer containing reducing agent and subjected to Western Blot as described in figure 2. A specific band at 110 kDa was observed representing rat HK in the plasma protein mix. Disappearance of the band represents cleavage of the rat HK to lower protein forms (not shown). (bottom). Quantitative densitometric analysis of the substrate stained bands above was performed to determine the rate of HK cleavage with time. The data is graphed indicating the % cleavage of original material (110 kDa) with time. Lewis rat plasma (circles), Fischer rat plasma, (squares), Buffalo rat plasma (triangles). The differences between Lewis and Buffalo rats as well as Lewis and Fischer rats at all points between 5-30 minutes, were highly significant (p< 0.001). No statistical difference was found at all points between Fischer and Buffalo rats.

**Contact system proteins in Lewis, Buffalo and Fischer rat plasma.** Levels of contact factors were determined in each rat plasma to determine if different levels of the contact factors could account for our previously observed accelerated cleavage of HK by kaolin. Coagulant assay for HK, prekallikrein (PK), and factor XII was examined in normal citrated plasma collected as a pool from a group of each rat strain. The results of each factor assay were normalized to 100% of the value obtained for Fisher plasma. The HK value for Fischer plasma was 100 ± 3.4%. The HK value of Lewis was 92.8±0.1% and Buffalo 91.4±1.4% (mean±SD, n=3), which did not significantly differ from each other. Factor XII levels were for Fischer, Lewis and Buffalo rat plasma respectively 100±1.7%, 96.1±0.5%, and 94.9±2.1%. Prekallikrein levels were respectively
100±1.4%, 90.6±3.1%, and 90.0±1.2% (n=4). This data indicates that the levels of these factors in plasma do not account for our observed changes in HK cleavage rates.

**Single point mutation in Lewis HK gene which distinguishes it from Buffalo and Fischer HK gene.** We amplified genomic DNA from the kidney of Lewis and Buffalo rat strains using sense primer SPK89A, and antisense primer 89D (Table 2). This rat HK sequence corresponds to a human plasma HK sequence containing the first two kallikrein cleavage sites Arg\(^{362}\)-Arg\(^{363}\) and Arg\(^{371}\)-Ser\(^{372}\) for release of bradykinin. The nucleotide sequence coding for the amino acid sequence Thr\(^{358}\)-Gly\(^{482}\) was identical in both Lewis and Buffalo rat HK. We therefore amplified the rest of the nucleotide sequences coding for the HK light chain from Buffalo, Fischer and Lewis rat genomic DNA using a sense primer, SPK89E, and antisense SPK89F, coding for amino acids 472-621 (C-terminal). Complete nucleotide analysis revealed a transition at nucleotide 1586 (AGT to AAT) converting Ser\(^{511}\) (as found in Buffalo and Fischer) to Asn\(^{511}\) (Lewis) (Figure 4).

**Figure 4. Nucleotide sequence of Lewis, Fischer and Buffalo rats.** The nucleotide 1586 AGT (Buffalo and Fischer) is mutated to AAT (Lewis). The codon change predicts that amino acid 511 will be Ser (wild type) in Buffalo and Fischer and Asn in Lewis.

This mutation results in the loss of a Sca I restriction enzyme site specific to the AGT/ACT site but not to the AAT/ACT site present in Lewis rats. Upon digestion with Sca I, the PCR product (492 bp) from Fischer rats (and Buffalo rats, not shown) containing the restriction site AGT/ACT resulted in fragments of the expected size, 369 bp and 112 bp, but the Lewis rat product was not
cleaved by *Sca I* confirming the presence of the mutation (Figure 5).

**Figure 5. Restriction enzyme analysis of 492 bp PCR products from Lewis and Fischer rats.** The 492 bp PCR product was subjected to analysis using *Sca I* restriction enzyme. The products were analyzed using an Agilent NA500 Labchip as described in Methods.

- **Line 1**, Lewis PCR product without *Sca I* restriction enzyme. **Line 2**, Lewis PCR product with *Sca I*. **Lane 3**, Fischer PCR product without *Sca I*. **Lane 4**, Fischer PCR product with *Sca I*. The 369 bp and 112 bp cleavage products are shown. The upper band in all the lanes is a high molecular weight internal marker.

**Kallikrein proteolysis of purified *E. coli* bacterial expressed recombinant HK.** The S\(^{511} \)N amino acid change is not in the rat HK region cleaved by kallikrein (amino acids 362-363 and 372-373) or in the putative rat binding sequence for prekallikrein of domain 6 (amino acids 562-590). To express a recombinant product containing these cleavage sites, we expressed the entire light chain of HK, including bradykinin from Lewis, Buffalo and Fischer rats (aa 358 -621) in an *E. coli* expression system which cannot glycosylate proteins, GST-HK4-6. The purified recombinant fusion protein expressed in *E.coli* were incubated with plasma kallikrein and the cleavage was monitored by reduced LDS electrophoresis by the loss of the 62 kDa band. We found no differences in the laser densitometric scan analysis between the three strains of rat HK (Figure 6).
Figure 6. The rate of kallikrein cleavage of E.Coli expressed HK recombinants as observed using SDS PAGE. Quantitative densitometric analysis of the stained protein bands on standard reduced SDS PAGE was performed on the recombinant GST-HK4-6 from three rat strains when cleaved by kallikrein as described in Methods. Lewis (circles), Fischer (squares), Buffalo (triangles). Mean ± SEM, n=3. The disappearance of a 62 kDa band was followed to quantify the cleavage rate. No difference in the cleavage rates were observed.

This result is consistent with the hypothesis that in the absence of glycosylation, the change from Ser to Asn in Lewis rat HK is not per se responsible for the more rapid hydrolysis of Lewis rat HK.

Kallikrein proteolysis of purified CHO cell expressed recombinant (His)₆-HK4-6. We constructed a polyhistidine tagged HK cDNAs HK from all three strains species of rats and expressed them in mammalian cells (CHO cells), which are capable of glycosylation. The purified recombinant proteins were incubated with plasma kallikrein and the cleavage of the (His)₆-tag HK fusion protein was monitored by reduced SDS electrophoresis and Western blot technique (Figure 7 top). Laser densitometric analysis revealed an increased rate of cleavage in the Lewis rat HK compared to Buffalo and Fischer HK as observed by the disappearance of the (His)₆-tagged protein as it is cleave off at the kallikrein cleavage sites (Figure 7 bottom).
**Figure 7. The rate of kallikrein cleavage of CHO cell expressed HK light chain.**  (top) Western blot detects a specific 40 kDa band of the recombinant (His)$_6$-HK4-6 in all three strains and shows the disappearance of the (His)$_6$ tag band as it is cleaved by kallikrein.  (bottom) Quantitative densitometric analysis of the (His)$_6$-HK4-6. The rate of cleavage as a function of time was analyzed. Lewis (circles), Fischer (squares), Buffalo (triangles). Mean ± SEM, n=3. The Lewis recombinant (His)$_6$ tagged HK cleavage was significantly faster than observed in the Fischer or Buffalo rat recombinants.

The difference between Lewis and Buffalo recombinant proteins and Lewis and Fischer were significant at 15, 30, and 60 minutes. No statistical difference was found at any point between Fischer and Buffalo rats.

**Confirmation of N-linked carbohydrate site in HK domains 4-6 in the Lewis rat and its absence in Fischer or Buffalo.** We observed that the nucleotide sequence of the Lewis rat contains a NXT consensus sequence for N-glycosylation at the amino acid positions 511-513 (NTT), which was absent in the Fischer and Buffalo rat sequence (STT). No other consensus sequences for N-linked carbohydrates were found in HK domains 4-6 in all three rat HK proteins, although there are 7 putative O-linked carbohydrates present in all three strains based on identity sequencing with human HK$^{21}$. The (His)$_6$-HK4-6 proteins of the 3 rat lines were expressed in CHO cells and were subjected to specific N-carbohydrate cleavage using N-glycosidase F (Figure 8).

**Figure 8.** Left. N-Glycosidase F cleavage of CHO expressed HK4-6. Purified CHO expressed (His)$_6$-tagged Lewis (Lew), Fischer (Fisch) and
Buffalo (Buff) were subjected to cleavage by N-glycosidase F as described in the Methods. The samples were subjected to LDS PAGE electrophoresis and stained for protein (top, “Gel”) or subjected to Western blot technique and tested for the presence of (His)$_6$-tagged protein (bottom “Blot”). Incubated in the absence of N-glycosidase F (-), in the presence of N-glycosidase F (+). 40 kDa and 37 kDa are the size of the protein bands determined by computer analysis comparing to migration of the known standards. Right. Tunicamycin inhibition of N-glycosylation of CHO cell expressed (His)$_6$-tagged Lewis rat HK. Purified CHO expressed (His)$_6$-tagged Lewis HK4-6 cultured in the absence (-) or presence (+) of tunicamycin were subjected to LDS-PAGE (Gel) and Western blotted (Blot), and detected for the presence of (His)$_6$-tag as described above.

In the Lewis protein, the glycosidase subjected 40 kDa protein shifted to 37 kDa on the LDS gel indicating a 3 kDa shift, appropriate for cleavage of 1 carbohydrate chain. Prior to N-glycosidase treatment, the recombinant proteins were of equal size (40 kDa) in all three rat strains. No shift was observed using the Fischer or Buffalo recombinant proteins. Similar treatment of transferrin, a control protein containing 2 N-linked carbohydrates, resulted in a 7 kDa shift (73 kDa to 66 kDa) which agrees with what is reported for this protein (not shown).

Further confirmation of the presence of N-glycosylation in CHO-expressed Lewis (His)$_6$-HK4-6 was sought by culturing the cells in tunicamycin, a known specific inhibitor of N-glycosylation in eukaryotic cells. Both by gel electrophoresis and by Western blot (Figure 8) we observed that the treated cell line showed a loss of 3 kDa in comparison to controls without tunicamycin, and this loss can be attributed to the loss of one N-glycosylation chain.

Confirmation of the loss of susceptibility of kallikrein cleavage of CHO-expressed Lewis (His)$_6$-HK4-6 cultured in the absence and presence of tunicamycin. We tested the hypothesis
that N-deglycosylation would restore the expressed protein to normal rates of kallikrein cleavage. We tested kallikrein cleavage of the Lewis rat light chain recombinant which was cultured in the presence of tunicamycin and thereby lacked the N-linked carbohydrate, against the same Lewis light chain recombinant product produced in the absence of tunicamycin, thereby retaining the N-linked carbohydrate. As demonstrated using LDS-PAGE (Figure 9, top), the disappearance of the 40 kDa product containing the N-linked carbohydrate was faster than that observed in the disappearance of the 37 kDa product, lacking the N-linked carbohydrate.

**Figure 9 The rate of kallikrein cleavage of CHO cell expressed Lewis HK light chain cultured in the absence and presence of tunicamycin.** Purified CHO expressed (His)₆-tagged Lewis HK4-6 cultured in the absence (-) or presence (+) of tunicamycin were subjected to time dependent cleavage by kallikrein. (top) Protein stained gels indicate a more rapid disappearance of a 40 kDa band of the recombinant (His)₆-HK4-6 in the absence of tunicamycin and a less rapid disappearance of a 37 kDa (His)₆-HK4-6 band as it is cleaved by kallikrein. The “std” indicates the position of the 39 kDa gel standard. (bottom) Quantitative densitometric analysis of the Lewis (His)₆-HK4-6. The rate of cleavage as a function of time was analyzed. Cultured in the absence of tunicamycin (open circles), and in the presence of tunicamycin (closed circles). Mean ± SEM, n=3. The Lewis recombinant (His)₆ tagged HK cleavage with the additional N-linked carbohydrate was
significantly faster than that observed in its absence. Quantification of the data of 3 separate experiments of each recombinant (Figure 9, bottom) indicated significant slowing to a normal rate of cleavage (as observed for the Fischer and Buffalo rat products, Figure 7). This experiment indicates that the presence of the N-linked carbohydrate at amino acid 511 alters the susceptibility of the recombinant protein to kallikrein cleavage. It is not consistent with the hypothesis that the amino acid change alone or the missing O-linked carbohydrate in the light chain is the cause of the observed phenomena.

DISCUSSION

Bradykinin is known to enhance vascular permeability and edema. To be relevant to human Crohn’s disease, an animal model must show not only similar pathologic changes, but should display genetic susceptibility. PG-APS-induced enterocolitis appears to meet this requirement since Lewis but not Buffalo or Fischer rats develop chronic granulomatous inflammation. In this experimental enterocolitis model we have shown selective in-vivo consumption of contact system proteins in susceptible Lewis rats and functional relevance of this pathway through modulation of these pathologic and laboratory changes by a plasma kallikrein inhibitor.

In previous studies we demonstrated an increased rate of plasma kallikrein cleavage of Lewis rat plasma HK compared with Buffalo. We now confirm differential cleavage of HK by plasma kallikrein by measuring faster bradykinin formation in Lewis rat plasma than in Buffalo. Surprisingly, the sequences containing the cleavage sites for plasma kallikrein were identical. However complete nucleotide analysis of domains 4-6 revealed a transition in the single
nucleotide 1586 (AGT to AAT) resulting in a change from Ser$^{511}$ Buffalo or Fischer to Asn$^{511}$ Lewis. Although this amino acid change is not in a region cleaved by kallikrein, nor in the prekallikrein binding area, this change could result directly or indirectly result in conformational changes, which might alter susceptibility to proteolysis. The asparagine is part of a NTT sequence, which is a consensus sequence for N-glycosylation (NXT) and would form the only such site in the rat HK light chain. In contrast, if we had expressed full length HK, we would have to consider the three other N-glycosylation consensus sequences in the heavy chain.

We therefore expressed HK domains 4-6, aa 358-621, which includes the bradykinin sequence and the site of the mutation amino acid 511 in the Lewis, Buffalo and Fischer rats, using competent E. coli in which N-glycosylation does not occur. These results indicate that the amino acid change from Ser to Asn does not directly account for the change of cleavage rate.

Based on these results, we expressed the protein in mammalian cell system (CHO cells), which is capable of glycosylation. After laser densitometry of Western blots analysis of all three experiments, we found an increased rate of HK cleavage by plasma kallikrein (Figure 7) in Lewis rats compared to Buffalo or Fischer HK. These results indicate that this amino acid substitution is responsible for more rapid cleavage, presumably due to N-glycosylation. We show that the predicted new carbohydrate chain exists in Lewis rat HK by cleaving the CHO cell expressed protein containing N$^{511}$ with N-glycosidase F, which is specific for N-linked carbohydrate chains. Loss of the carbohydrate was evident by a more rapid migration on SDS gel electrophoresis. This change did not occur in protein containing Fischer or Buffalo rat HK4-6 expressed in CHO cells which contain S$^{511}$.

N-glycosylation of asparagine occurs as a co-translational process within the luminal
compartment of the rough endoplasmic reticulum (RER) in which preformed dolichol oligosaccharide precursor complexes are transferred to consensus amino acid sequences of the formula NX(T,S or C) where X = any amino acid except proline. The efficacy of glycosylation is T>S>C\textsuperscript{34,35}. 

In contrast, O-glycosylation occurs individually and primarily occurs in the luminal compartment of the Golgi. This synthesis occurs subsequent to completing all N-glycosylation modifications of asparagine\textsuperscript{36}. Reportedly\textsuperscript{37} by examination of peptide sequences around O-glycosylation sites, P, A, S or T were often present at 3 amino acids towards the N-terminal (-3 position) and 1,3 and 6 amino acids towards the C-terminal end (+1, +3, +6 positions). Statistically, S and T are equally O-glycosylated in glycoproteins. Human HK is known to be O-glycosylated at the T\textsuperscript{515} (of human HK sequence) homologous to the rat T\textsuperscript{513} in Fischer, Buffalo or Brown Norway rat HK (Table 1). All have the sequence STT in the series around the glycosylation site. Serine is in the -2 position within the triad, perhaps favoring O-glycosylation at T\textsuperscript{513} in the rat Fischer and Buffalo rat sequence. Additionally, Wang et al\textsuperscript{38}, on examining peptide substrates for O-glycosylation of threonine, found that residues containing XXTTX containing peptides were the best substrate. This agrees with T\textsuperscript{513} in rat HK containing an O-linked carbohydrate in the STT sequence. 

Thus in Lewis HK, sequentially during synthesis, N-glycosylation of N\textsuperscript{511} in the sequence NTT must precede the O-glycosylation at T\textsuperscript{513}. Tunicamycin prevents N-glycosylation of the only asparagine in (His)\textsubscript{6}-HK4-6. The loss of 3 kDa in the protein expressed in the culture of Lewis (His)\textsubscript{6}-HK4-6 containing tunicamycin is consistent with the failure of N-glycosylation at N\textsuperscript{511}. 

Two problems remain in our analysis of the effect of the S\textsuperscript{511}N mutation in Lewis rat HK.
The apparent molecular size of HK determined by Western blot is 110 kDa. The prediction based on one extra N-linked oligosaccharide chain is that Lewis HK would have a molecular size of 113 kDa. We postulate that the N-linked carbohydrate chain at N511 sterically interferes with the Gal-N-acetyl transferase responsible for O-glycosylation on T513 due to the sequential processing. This hypotheses fits with the hypothesis of “site-specific tolopolological modulation”39 where glycolysis at one protein site can direct the maturation of synthesis of the glycoprotein at subsequent sites. The distance between these two acceptor amino acids must be small since they are separated by a single amino acid. Elhammer et al.40 reported that the α-N-acetylgalactosaminytransferase (α-N-GalNAc-T) common for synthesis of all O-glycan chains binds to 8 amino acids in the substrate, 3 residues preceding and 4 following the Ser or Thr to be glycosylated. We suggest that the change of S to N and/or the presence of glycosylation at N511 conformationally or sterically hinders the ability of α-N-GalNAc-T to glycosylate T513 in the Golgi system. Thus we propose that Lewis rat HK contains a N511 glycosylation but not a T513 glycosylation, but Fischer and Buffalo rats have a T513 glycosylation and lack one glycosylation having an S rather than N at amino acid 511. This would result in the expression of glycosylated HK products in all species of similar molecular weight observed by Western blot of plasma HK.

The second issue is since Lewis HK is N-glycosylated at N511 and presumably lacks an O-linked carbohydrate at T513, the predicted expression product after culture in the presence of tunicamycin would contain an O-linked carbohydrate chain on T513 as a result of N511 not being glycosylated. We might have expected the proteins to have the same mobility as the CHO cell-expressed (His)6-HK4-6 of Fischer or Buffalo rat (40 kDa). Instead we find a product with the mobility of N-glycosidase-F digested Lewis (His)6-HK4-6 (37 kDa). This finding suggests that
the sequence of NTT is less favorable for O-glycosylation than STT. Indeed, in the case of Buffalo and Fischer rats, the presences of S\textsuperscript{511}TTS\textsuperscript{514} is known to be favorable for O-glycosylation since threonine and serine adjacent to T\textsuperscript{513} which form a beta turn where most O-glycosylations occur\textsuperscript{41}. Thus the N\textsuperscript{511} to T mutation would be expected to decrease the probability of O-glycosylation at T\textsuperscript{513}.

This analysis indicates that Lewis rat plasma HK probably exists as an N-glycosylated N\textsuperscript{511} which is associated with a T\textsuperscript{513} which bears no carbohydrate. Buffalo or Fischer plasma HK contain S\textsuperscript{511} with no carbohydrate and T\textsuperscript{513} bearing an O-linked oligosaccharide. Thus although both sequences have approximately the same molecular weight, they differ at two attachment sites for carbohydrate. The presence of N-linked carbohydrate appears to be responsible for the conformational change in Lewis plasma HK that facilitates a more rapid cleavage by kallikrein. Evidence by recombinants expressed in bacteria, N-linked carbohydrate deglycosylation and expression in the presence of tunicamysin indicates that it is not the amino acid change itself but rather the extra carbohydrate which results in a change in susceptibility to cleavage.

The addition or loss of protein function as a result of abnormal N-glycosylation by a change in the amino acid sequence has been reported for other plasma glycoproteins. The loss of an N-glycosylation at an NXT to an NXS results in an isoform of antithrombin III (β antithrombin III) resulting in enhanced heparin affinity\textsuperscript{34}. In contrast, the substitution of an N for I in antithrombin Rouen-III\textsuperscript{42} establishes an additional N-glycosylation in the molecule, reducing its affinity for heparin. Two mutants of Factor VIII establishing new N-glycosylation sites M to T, or I to T, results in an expressed mutant glycosylated protein with loss of functional activity\textsuperscript{43}. We have delineated a rat HK where the mutation of S\textsuperscript{511} to T results in a new N-glycosylation which
in turn modifies the susceptibility of HK cleavage by plasma kallikrein. This finding is a unique example of an effect of N-glycosylation on a substrate cleavage susceptibility resulting in enhanced functional activities of a protein.

Examination of the protein database revealed that there are four species (human, bovine, murine, and rat) with complete sequences of HK available (Table 1). Two different sequences have been reported for rat HK which differ only in amino acid 511, S or N (see fig. 1 in ref \textsuperscript{21}). Both sequences are listed as originating from Rattus norvegicus without strain designation. The other species, human, bovine and murine all have a single amino acid Ser at position 511 or its equivalent, indicating further that the N substitution is the mutant form. This mutation could render the Lewis rat more susceptible to inflammation by contributing a more rapid release of bradykinin. Therefore Lewis rats would be at an evolutionary disadvantage with respect to developing autoimmune and environmentally induced disorders. Lewis rats have enhanced susceptibility to a number of experimental inflammatory conditions, including PG-APS-induced arthritis, adjuvant-induced arthritis, collagen induced arthritis, autoimmune encephalitis and autoimmune thyroiditis\textsuperscript{44-48}. However, this mutation may be associated with enhanced clearance of proteins.

Over the past two years, bradykinin has been proposed as a proangiogenic agent. Parenti et al.\textsuperscript{49} have shown that bradykinin binding to the B1 receptor promotes angiogenesis in the rabbit cornea by upregulation of endogenous FGF-2. The role of bradykinin in inflammation has been well documented. Pain, increased blood flow and edema are features of the acute inflammatory process that are attributable to bradykinin\textsuperscript{22}. Several studies have documented the vasodilator effects\textsuperscript{23,24} as well as the increase in vascular permeability and plasma extravasations evoked by
kinins\textsuperscript{25}. Furthermore, we have shown that a bradykinin receptor 2 blocker modulates the experimental inflammatory arthritis in Lewis rats and decreases interleukin 1 production in joints\textsuperscript{50}.

Since increased cleavage of HK yields a higher bradykinin concentration, it is likely that this difference accounts in part for the susceptibility of Lewis rats to chronic inflammation. We have shown that kininogen deficient rats on a Lewis genetic background developed an attenuated form of enterocolitis after injection of PG-APS\textsuperscript{51}, which is consistent with the results of this study. Further genetic experiments are underway to confirm that the mutation in Lewis rats is closely correlated with the increased reaction to inflammation stimulated by bacterial products.

**REFERENCES**


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Table 1

HK sequence comparison between species

<table>
<thead>
<tr>
<th>Species</th>
<th>ID</th>
<th>Protein Sequence</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
<td>KGHUH1 (PIR)</td>
<td>...SSSEDSTTPSAQ...</td>
</tr>
<tr>
<td>Bovine</td>
<td>KGBOH2 (PIR)</td>
<td>...SSYEDSTTSSAQ...</td>
</tr>
<tr>
<td>Mouse</td>
<td>O08677 (Swiss-Prot)</td>
<td>...SSSEYSTTTST-Q...</td>
</tr>
<tr>
<td>Rat</td>
<td>A27115 (PIR)</td>
<td>...SSSSEDTTTST-Q...</td>
</tr>
<tr>
<td>Rat</td>
<td>A25486 (PIR)</td>
<td>...SSSEDNTTST-Q...</td>
</tr>
</tbody>
</table>

*PIR or Swiss-Prot database*
Table 2

DNA Primers

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Strand</th>
<th>Corresponding Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPK89A 5'-GCCCATTACAATAACAGAATGGAATC-3'</td>
<td>Sense</td>
<td>99 nt in intron before exon 10 (aa 358).</td>
</tr>
<tr>
<td>SPK89D 5'-AGGCTTTTTCTGATCAGTGATGATG-3'</td>
<td>Antisense</td>
<td>Coding sequence for aa 496-502.</td>
</tr>
<tr>
<td>SPK89E 5'-CGGGATCCATGGTCATGGTCACGGTGC-3'</td>
<td>Sense</td>
<td>Coding aa 477-483.</td>
</tr>
<tr>
<td>SPK89F 5'-CGGAATTCGATTTTTGTAAGATTTCTACGC-3'</td>
<td>Antisense</td>
<td>Corresponding to 31 nt into 3’nTR.</td>
</tr>
<tr>
<td>SPK89G 5'-GCGAGATCTACATCGATGATAAGA-3'</td>
<td>Sense</td>
<td>Coding sequencing aa 358-363</td>
</tr>
</tbody>
</table>

The 531 bp PCR product coding for the human HK protein sequence, aa 358-502 was generated with SPK89A and SPK89D. The 492 bp PCR product coding for the HK protein sequence, aa 478-621, was generated with SPK89E and SPK89F. The 819 bp PCR product was generated using SPK89F and SPK89G\(^5^2\).
The mutation S511N leads to N-glycosylation and increases the cleavage of high molecular weight kininogen in rats genetically susceptible to inflammation

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