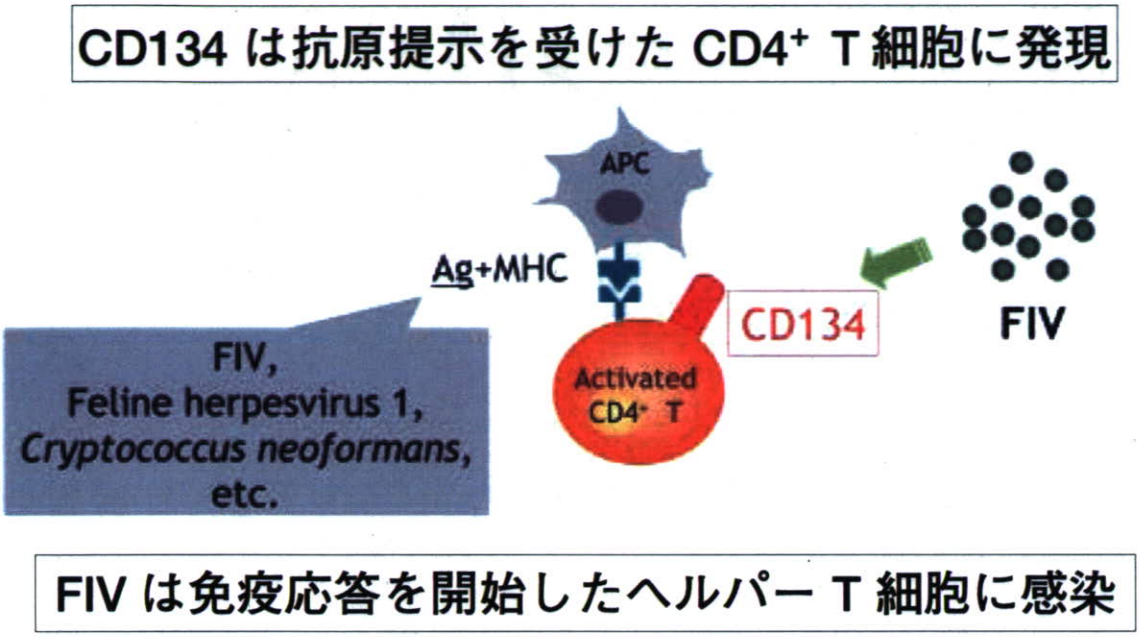

図 4 CD134 分子の分布と免疫不全
CD134 分子は抗原提示を受けた CD4 陽性細胞に発現するので，FIV は免疫応答を開始したヘルパー T 細胞に感染することができる．この時の抗原は FIV 自身であっても良いし，他の微生物・病原体であっても良い．

とになり，このことがスクリーニングをわずか 1 週間で，しかも繰り返さずに完了することに繋がっている．

3. FIV の受容体同定

2.で紹介した改良版発現クローニング法は，本来は抗体が認識する細胞表面蛋白質を同定するものであるが，原理的には細胞表面蛋白質を認識するものは抗体である必要はない．そこで，ウイルスで蛋白質を認識させる状況を考え，ディッシュにコートしたウイルスで細胞のトラップができないかと考えた．抗 FIV Env 抗体を用いて FIV をディッシュにコートし，このディッシュに FIV 感染に高感受性である浮遊系細胞（MYA-1 細胞株）[6] を入れたところ，細胞は浮遊系のものであるにもかかわらずディッシュに良く付着した．このことは，**2.**の手法がウイルスに対しても応用しうることを示している．そこで FIV 高感受性細胞（MYA-1 細胞株）由来 cDNA library を浮遊系の細胞に発現させ，FIV でコートしたディッシュでスクリーニングを行った結果，CD134 という分子を FIV への細胞付着をもたらす細胞表面蛋白質として同定した．

CD134 分子を細胞に発現させると，**図 3** で示すように FIV の細胞への吸着が促進された．また CD134 発現細胞ではウイルスが非常に良く増殖した．CD134 分子と FIV Env 蛋白質を共発現させると，細胞融合が起こることが分かった．つまり CD134 分子は単に FIV と結合する分子であるというだけでなく，FIV の細胞内への侵入を担う受容体であることが分かった[8]．

これに先立ち，FIV の感染がケモカイン受容体 CXCR4 に対する抗体や生理的リガンド(SDF-1)，特異的拮抗剤(AMD3100 など)により阻害されることが知られていた[12]．そこで，CD134 分子の発現によって見られる FIV の感染と CXCR4 との関係について調べた．その結果，CD134 分子を介した FIV の感染は CXCR4 特異的拮抗剤によって阻害されることが分かった．また FIV と CD134 分子との結合は CXCR4 の有無に関係なく起こるが，CD134 分子なしで FIV と CXCR4 の結合を見ることはできないことも分かった．すなわち，FIV の感染に細胞側の CD134 と CXCR4 の 2 つの分子が関わっており，CD134 分子が第一受容体，CXCR4 分子が第二受容体であることが分かった[8]．

ちなみに FIV の受容体同定に用いた cDNA library は **2.** で紹介した発現クローニング法で用いたものと同じである．つまり cDNA library には CD4 分子の cDNA が含まれていたが，スクリーニングで CD4 分子はクローニングされてこなかった．念のため CD4 分子を浮遊系細胞に発現させたが，この細胞と FIV との結合は認められなかった．FIV の感染には CD4 分子は特に関わっていないと考えられる．

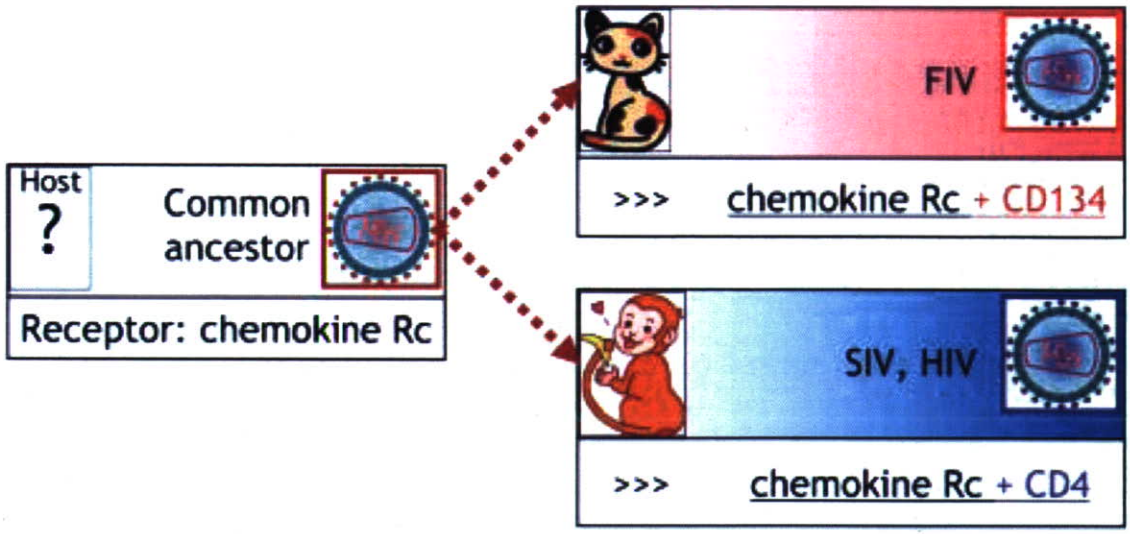

図5　ウイルスの進化と受容体（仮説）

（左）かつてある宿主にいたレンチウイルスの祖先．このウイルスはケモカイン受容体のみで細胞に感染することができた．

（右上）ケモカイン受容体を介してネコに感染．その後，より増殖しやすいようにケモカイン受容体とは別のCD134分子を第一受容体として選んだ．

（右下）ケモカイン受容体を介して霊長類に感染．その後，より増殖しやすいようにケモカイン受容体とは別のCD4分子を第一受容体として選んだ．

4. CD134分子の発現分布と免疫不全

HIVやSIVがCD4分子を第一受容体とし，ヘルパーT細胞に強い感染指向性を示すことは理解しやすい．ではCD134分子ではどうだろうか．

CD134分子は活性化していないnaïveのヘルパーT細胞には発現していないが，抗原提示を受け活性化したヘルパーT細胞に発現するようになる分子である[13]．CD134分子が発現されると，抗原提示細胞上のCD134L（リガンド）によりT細胞は更に活性化を受けることとなる[14]．第一受容体がCD4ではないFIVがin vitroでCD4陽性細胞に強い感染指向性を示し，またin vivoで感染ネコのCD4陽性細胞から検出されるのは，CD134分子の発現が主にCD4陽性細胞内であるからであると考えられる．

CD134分子を第一受容体としているということはFIVの巧みな免疫回避法を思わせる（**図4**）．CD134発現細胞を標的に感染していくということは，その時活性化しているヘルパーT細胞すなわちFIV抗原特異的に免疫応答を開始したヘルパーT細胞にFIVは感染していくことを意味する．もう少し言い換えるならば，FIVは自分を排除するための細胞そのものに高効率で感染するのである．また，FIV感染ネコに他の微生物，例えばネコヘルペスウイルスやクリプトコッカスが感染した場合には，それらの微生物に対して免疫応答を開始したヘルパーT細胞をもFIVは感染の標的としていくことになる．恐らくはFIVはCD134分子を第一受容体とすることでFIV自身が増殖しやすい環境を作り，また他の微生物存在下ではその微生物の増殖を促進し，二次感染や日和見感染といったエイズ様症状を引き起こすのであろう．

"FIVは活性化したFIV抗原特異的ヘルパーT細胞に感染していく"という表現に少し疑問を感じる人もいるかもしれない．（当然ではあるが）ある個体の初感染時にはFIV抗原への免疫応答は起こっていないからである．しかし周囲や体内に外来抗原が全くなく，免疫系が全く動いていないという個体の状況は考えにくい．初感染時に限っては，その時たまたま活性化していた細胞にFIVは感染していくと思われる．ここで少しワクチン研究について触れるが，不活化FIV等の投与がチャレンジ時のウイルス感染を増強したという報告が多い[15]．恐らく抗原投与自体がFIVの感染標的を増やし（免疫系の活性化→ヘルパーT細胞の活性化＝CD134発現細胞の増加となる），十分な免疫が与えられていない場合はウイルスがよく増える結果となる，ということであろう．

このように免疫応答を行っている細胞への感染指向性は，FIVだけでなく実はHIVやSIVも有する特徴であると考えられる．ただし分子メカニズムは若干異なる．HIVやSIVは恒常的にヘルパーT細胞に発現しているCD4分子を第一受容体とするので，この段階で感染指向性に差は出ないであろうが，細胞の活性化に伴い第二受容体であるケモカイン受容体の発現量は増加するので，活性化したヘルパー

T細胞にHIVやSIVは感染しやすいはずである．実際，この現象は報告されているものである[16)17)]．

5. ウイルスの進化と受容体

ここで，FIVの第一受容体がCD134分子であることと，3つの免疫不全レンチウイルスFIV・HIV・SIVの祖先が共通と考えられていること，ケモカイン受容体は動物間で高度に保存されていること，そして免疫不全レンチウイルスの中にはごく稀に（第一受容体がない細胞にも）ケモカイン受容体のみを介して感染できる株があることを踏まえ，レンチウイルスの進化と受容体についての私や現京大宮沢孝幸先生の仮説を紹介したい（**図5**）．

仮説とは次のようなものである．まず，かつてある宿主に，現在のレンチウイルスの祖先となるようなウイルスがいた．このウイルスはケモカイン受容体のみで細胞に感染できるウイルスであった．そしてある時このウイルスが，動物間で高度に保存されているケモカイン受容体を介して様々な動物種に感染した．そして各動物種内でウイルスはより増殖しやすいようにケモカイン受容体とは別の分子を（今で言うところの）第一受容体として選ぶようになった．ネコの世界ではCD134分子が，霊長類の世界ではCD4分子が第一受容体として選ばれた．レンチウイルスにとってある宿主でよりよく増殖するためにはヘルパーT細胞を感染標的とする必要があり，ネコと霊長類の世界で若干異なる分子機構がとられた，ということではないだろうか．

6. 他のウイルスの感染指向性

2.で紹介した改良版発現クローニング法は，条件が揃えば他の様々なウイルスの受容体同定に応用可能であると考えられる．実際にネコカリシウイルスでも可能であることを既に確認している[18)]．また，FIVの場合もカリシウイルスの場合もウイルス粒子（ウイルス液）を用いてスクリーニングを行っているが，ニパウイルスの細胞への結合を担うG蛋白質の組換え発現蛋白質によっても本法でその受容体（Ephrin B2）[19)20)]の同定が可能であることを確認済みである．さらにウイルスや蛋白質でコートを行う場合，特異的抗体であらかじめディッシュをコートしておけば（タグ付組換え蛋白質の場合は抗タグ抗体でも可），超遠心や精製等をしておく必要はない．ディッシュ上で濃縮と精製が起こるからである．条件が満たされれば，ウイルスでなくてもバクテリアや原虫，細胞リガンドの結合標的の同定も可能なはずである．

この方法を更に少し違った形でエボラウイルスの受容体同定に応用してみた．詳細は次のようである．エボラウイルスの細胞への吸着・侵入を担う表面糖蛋白質（GP）は，マウス白血病ウイルスなどのレトロウイルスのエンベロープの代わりとして機能することが知られている（このようなウイルスはシュードタイプウイルスと呼ばれている）．またリンパ球細胞株はエボラウイルスやこのシュードタイプウイルスに殆ど感染しない．そこで，このリンパ球細胞株（浮遊系）を標的細胞としてエボラウイルス高感受性細胞株（Vero E6）から得られたcDNAライブラリーを発現させ，シュードタイプウイルスの感染を指標にして標的細胞に感染感受性をもたらす細胞因子をスクリーニングした．そして改良版発現クローニング法を，シュードタイプウイルスが感染した細胞の選択に用いた．つまり，この時シュードタイプウイルスはその感染レポーターを膜蛋白質（ここではネコCD2）としたものを用い，ディッシュは抗ネコCD2抗体でコートしたものを用いた．形成されたコロニーに蛍光蛋白質（GFP）を感染レポーターとしたシュードタイプウイルスを感染させ，GFP陽性となったコロニーを選び出し，インテグレートしているcDNAを調べた．その結果，受容体型チロシンキナーゼAxlが同定された[21)]．エボラウイルスの感染におけるAxl分子の役割については現在解析中である．

このような実験を行う場合，通常は薬剤耐性遺伝子等を感染レポーターとしたウイルスを用いて細胞の選択を行うが，cDNA libraryを発現する標的細胞に感染性がわずかでもあると，目的のcDNAの発現に関係なく細胞が生き残ることになるので，スクリーニングの効率は激減してしまう．しかし膜蛋白質をレポーターとしてディッシュ上にコロニー形成を起こさせると，第二の選択（ここではGFP発現ウイルスによる選択）が可能となるので，標的細胞に多少の感染感受性があってもスクリーニングの効率は低下しない．第二の選択でGFPの発現量（強弱）に着目すればウイルスの感染を促進する因子の同定も可能なはずである．

7. おわりに

非常に単純（で意義も疑問視されそう）な思いつきが本研究の始めにあった．周囲の暖かい目で見守られ，人前で発表できる形になったことは嬉しい．紹介した知見などが少しでも皆様のお役に立てたのならばなお嬉しいことである．

謝　辞

本稿で紹介した研究は，東京大学農学生命科学研究科獣医微生物学教室および同医科学研究所ウイルス感染分野で行われたものである．明石博臣教授（東大），宮沢孝幸准教授（現京大），河岡義裕教授（東大）および高田礼人教授（現北大）をはじめ多くの方々のご支援をいただいたことにお礼申し上げます．また杉浦奨励賞にご推挙いただきました河岡義裕教授，明石博臣教授，見上彪先生（現内閣府）に深く感謝いたします．さらに，本研究を評価して頂いた日本ウイルス学会に感謝いたします．

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〔ウイルス 第57巻 第1号, pp.75-82, 2007〕

Feline immunodeficiency virus tropism

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Feline immunodeficiency virus (FIV) induces a disease similar to acquired immunodeficiency syndrome (AIDS) in cats, yet in contrast to human immunodeficiency virus (HIV), CD4 is not the viral receptor. We identified a primary receptor for FIV as CD134 (OX40), a T cell activation antigen and costimulatory molecule. CD134 expression promotes viral binding and renders cells permissive for viral entry, productive infection, and syncytium formation. Infection is CXCR4-dependent, analogous to infection with X4 strains of HIV. Thus, despite the evolutionary divergence of the feline and human lentiviruses, both viruses use receptors that target the virus to a subset of cells that are pivotal to the acquired immune response. Further, we applied the new method for FIV receptor to Ebola virus entry factors with some modifications, and identified receptor-type tyrosine kinases, Axl and Dtk (members of Tyro3 family). Distribution of the molecules matches well with the Ebola virus tropism.

Available online at www.sciencedirect.com
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Vaccine 25 (2007) 1334–1341
Vaccine
www.elsevier.com/locate/vaccine

Prime-boost immunization with DNA followed by a recombinant vaccinia virus expressing P50 induced protective immunity against *Babesia gibsoni* infection in dogs

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Received 24 January 2006; received in revised form 26 July 2006; accepted 27 September 2006
Available online 10 October 2006

Abstract

A heterologous prime-boost immunization regime with priming DNA followed by recombinant vaccinia virus expressing relevant antigens has been shown to induce effective immune responses against several infectious pathogens. In this study, we constructed a recombinant plasmid and vaccinia virus, both of which expressed P50 of *Babesia gibsoni*, to investigate the immunogenicity and protective efficacy of a heterologous prime-boost immunization against canine babesiosis. The dogs immunized with the prime-boost regime developed a significantly high level of specific antibody against P50 when compared with the control groups, and the antibody level was strongly increased after a booster immunization with a recombinant vaccinia virus. The prime-boost immunization regime induced a specific IgG2 antibody response and IFN-γ production in dogs. Two weeks after the booster immunization with a recombinant vaccinia virus expressing P50, the dogs were challenged with *B. gibsoni* patasites. The dogs immunized with the prime-boost regime showed partial protection, manifested as a significantly low level of parasitemia and a 2-day delay of the peak parasitemia. These results indicated that such a heterologous prime-boost immunization approach might be useful against *B. gibsoni* infection in dogs.

Keywords: *Babesia gibsoni*; DNA vaccine; Vaccinia virus

1. Introduction

Babesia gibsoni is a tick-borne hemoprotozoan parasite that causes piroplasmosis in dogs. This parasite causes severe disease and sometimes death [1–3]. *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe, and the Americas [4,5]. The disease is frequently present in dogs and has recently become a serious clinical problem [6–9]. For the control and alleviation of *B. gibsoni* infection in dogs, vaccination is generally considered to be the most effective means. Until recently, most vaccine development efforts against protozoan diseases aimed at stimulating the humoral immune response, for example, the production of protective antibodies. However, for intracellular protozoan infections such as malaria or babesiosis, it is known that the induction of immune responses at both the humoral and cellular levels is required for protection against these pathogens [10,11]. Therefore, a vaccine may be needed to induce both types of immune responses and provide optimal protection.

Previous studies have shown the use of an intramuscular injection of plasmid DNA inducing both antibodies and cell-mediated immune responses in a number of animal models. The use of DNA vaccines has been implemented to elicit durable responses against intracellular pathogens, such

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doi:10.1016/j.vaccine.2006.09.083

as *Plasmodium* or *Toxoplasma* [12,13]. However, a DNA vaccine used alone could induce only modest cell-mediated immune responses, including antigen-specific killer T cell responses in animals. To improve the DNA vaccines, various strategies have been considered, such as cytokine augmentation and ballistic epidermal delivery; however, the cell-mediated immune response induced with a DNA vaccine alone remained modest [14,15]. In other cases, recombinant vaccinia viruses expressing antigens derived from pathogens have often been used to induce both antibodies and cell-mediated immune responses [16–18]. However, in a mouse malaria model, immunization with a recombinant vaccinia virus alone failed to induce a protective immune response [19,20].

Recently, it has been demonstrated that a prime-boost immunization regime with a DNA plasmid and a recombinant vaccinia virus, both of which expressed the same antigen of pathogens, could induce a strong immune response, including cell-mediated immunity [21–23]. Using a mouse malaria model, it was shown that immunization with priming DNA followed by a recombinant vaccinia virus, both of which expressed pre-erythrocytic antigens, induced complete protection against sporozoite challenge [24]. Therefore, it was considered that such a heterologus prime-boost immunization regime might provide the basis for preventative and therapeutic vaccination against protozoan diseases of dogs. However, in dogs, the number of reports of the use of a prime-boost regime is quite limited [25].

In our previous studies, we identified an immunodominant surface protein P50 expressed on the *B. gibsoni* merozoites [26,27]. P50 is a type-I transmembrane protein, which showed no homology to the other proteins including apicomplexan or other protozoan parasite. Although, the function was unknown, we demonstrated that the P50 was recognized as an immunodominant antigen by the host immune system in dogs infected with *B. gibsoni* [26]. Furthermore, we showed that the antiserum against recombinant P50 produced in a rabbit significantly inhibited the parasite growth on *B. gibsoni*-infected canine red blood cell-substituted severe combined immunodeficiency (Ca-RBC-SCD) mice. These results indicated that P50 is a useful vaccine candidate for the control of canine *B. gibsoni* infection. In this study, we constructed the recombinant plasmid and vaccinia virus expressing P50 to investigate the immunogenicity and protective efficacy of a heterologus prime-boost immunization regime against *B. gibsoni* infection in dogs.

2. Materials and methods

2.1. Parasites

The NRCPD strain of *B. gibsoni* parasites was used [28,29]. The *B. gibsoni*-infected red blood cells (RBCs) used for challenges were collected from a dog experimentally infected with *B. gibsoni*.

2.2. Construction of vaccine plasmid

The entire P50 gene in pBluescript SK(+) vectors [26] was recovered after digestion with *Eco*RI and *Xho*I, blunt-ended with a Klenow fragment of DNA polymerase, and then inserted into the blunt-ended *Eco*RI restriction enzyme site under the control of the CAG promoter of the mammalian expression vector pCAGGS [30,31], designated as pCAGGS-P50. pCAGGS-P50 was amplified in DH5 α *Escherichia coli*, and the purification was performed using the QIAGEN Plasmid Mega Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The expression of P50 was analyzed using rabbit kidney (RK13) cells *in vitro* prior to an immunization trial of the dogs. The pCAGGS-P50 plasmid or control empty plasmid pCAGGS was transfected to RK13 cells using a lipofectine reagent (Gibco BRL, Rockville, MD, USA) by the standard method [32]. The expression of P50 was analyzed by the indirect immunofluorescence antibody test (IFAT) or Western blotting using anti-P50 monoclonal antibody (MAb) 3ID [27] as a primary antibody 2 days after transfection. IFAT and Western blotting were performed as described in our previous paper [26].

2.3. Construction of the recombinant vaccinia virus expressing P50

The entire P50 gene in pBluescript SK(+) vectors [26] was recovered after digestion with *Eco*RI and *Xho*I, blunt-ended with a Klenow fragment of DNA polymerase, and then ligated into the *Sal*I site of the vaccinia virus transfer vector pAK8 [33]. The structure of the recombinant plasmid pAK8-P50 was checked by restriction enzyme analysis. Rabbit kidney (RK13) cells infected with the vaccinia virus LC16mO (mO) strain [33] were transfected with the pAK8-P50 using a lipofectine reagent (Gibco BRL). Thymidine kinase-negative (TK-) viruses were isolated by a plaque assay on 143TK-cells in the presence of 5-bromo-2′-deoxyuridine at a concentration of 100 μg/ml [33]. The recombinant vaccinia virus expressing P50 (vvP50) was propagated in RK13 cells in Eagle's minimum essential medium (Sigma, Tokyo, Japan) supplemented with 7.5% fetal bovine serum (FBS). To analyze the expression of P50 *in vitro*, RK13 cells were inoculated with five plaque-forming units (PFU) of vvP50 or mO per cell. Two days after inoculation, the cells were harvested and then subjected to IFAT or Western blotting as described above.

2.4. Dogs and immunization

Purebred female-specific pathogen-free (SPF) Beagle dogs (14–15 months old) purchased from the Chugai Medical Animal Institute (Nagano, Japan) were used. All dogs were physically examined by the veterinarian of the Obihiro University of agriculture and veterinary medicine and received the routine vaccination including canine parvovirus, canine adenovirus (types 1 and 2) and distemper. Nine dogs

were randomly divided into three groups ($n = 3$). One group was immunized with pCAGGS-P50 and vvP50. The negative control group was immunized with the empty plasmid pCAGGS and the parent vaccinia virus mO strain. The remaining group was used as the no-immunization control. The dogs were immunized with DNA thrice followed by a recombinant vaccinia virus booster immunization because this immunization schedule showed most effective result for the induction of a strong immune response against malaria infection than other vaccination regimes in humans [34]. Dogs were injected intramuscularly (i.m.) in the quadriceps muscle with the plasmid DNA. A 1-ml insulin syringe with a 21G-in. needle was used for all injections, and each single dose consisted of 200 μg dissolved in 1 ml of PBS containing 25% (w/v) sucrose. The dogs were immunized with plasmid DNA three times at 2-week intervals. Two weeks after the last plasmid DNA immunization, the dogs were boosted intravenously (i.v.) with an injection of either 5×10^8 PFU of the recombinant vaccinia virus vvP50 or mO. After the DNA or recombinant vaccinia virus immunization, dogs were examined every day for 14 days by a veterinarian and no side effect were observed.

2.5. Reactivity analysis against B. gibsoni merozoites

Two weeks after the booster immunization with the recombinant vaccinia virus, serum samples were collected from dogs. The reactivity of the sera against *B. gibsoni* parasites was analyzed by Western blotting using merozoites purified from infected dog RBCs. Purification of the merozoites, sample preparation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were performed as previously described [27].

2.6. Determination of the total IgE response by enzyme-linked immunosorbent assay (ELISA)

Total IgE of the immunized dogs were measured using a capture ELISA (Dog IgE ELISA Quantitation kit, Bethyl Laboratories, Montgomery TX, USA) to analyze the allergic reaction caused by the immunization of DNA and recombinant vaccinia virus. The ELISA was performed following the manufacture's instructions. The sera collected at pre-immunization and 2 weeks after the each immunization (day-56, -42, -28 and -0 of Fig. 4) were used for analysis. All serum samples were used in 1:200 dilutions. The concentration of total IgE was calculated from the standards (ranges: 10,000–7.8 ng/ml).

2.7. Challenge infection

A purebred female SPF Beagle dog was used for the donor of the *B. gibosni* parasites for the challenge infection. The fresh infected RBCs were collected at acute phase of the experimentally infected donor dog (approximately 13% parasitemia, 14 days post-infection), and washed with PBS three times by centrifugation, and then adjusted 2×10^8 ml^{-1} in PBS. The infected RBCs were used for the challenge within in 1 h from the blood collection. Two weeks after the booster immunization, dogs were i.v. injected with 2×10^8 of *B. gibsoni*-infected RBCs (NRCPD strain) in the intermediate ccephalic vein. Parasitemia in peripheral blood and the packed cell volume (PCV) were monitored at 1-day intervals.

2.8. Determination of the IgG subclass by the ELISA

The increase in the antibody responses against the P50 protein in dogs was measured using the ELISA with glutathion *S*-transferase (GST)-P50 as an antigen. The ELISA was performed as described in our previous paper [35]. All serum samples were used in 1:200 dilutions for ELISA. The antigen-specific isotype profile was determined using horseradish peroxidase (HRP) conjugated anti-dog IgG, IgG1, and IgG2 antibodies (Bethyl Laboratories) as the secondary antibodies. All HRP-conjugated secondary antibodies were used in 1:4000 dilution. The IgG subclass against P50 of the sequential sera collected from a dog infected with *B. gibsoni* was also analyzed.

2.9. Determination of the peripheral IFN-γ response

The serum samples collected at 0 and 8 days post-infection were used for the assay. The samples were kept at −80 °C until use. IFN-γ was measured using a capture ELISA (R&D Systems, Minneapolis, MN, USA) for dogs following the manufacturer's instructions.

2.10. Statistical analyses

The parasitemia and antibody response in the dog group immunized with P50 and the immunized and non-immunized controls were statistically analyzed by the Student's *t*-test with Stat view.

All animal experiments described in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

3. Results

3.1. Expression of P50 in vitro by a vaccine plasmid

To investigate whether the pCAGGS-P50 plasmid expresses P50, we transfected the plasmid to RK13 cells and analyzed it by IFAT and Western blotting. In the IFAT analysis, the anti P50 MAb 31D [27] specifically reacted to the RK13 cells transfected with the pCAGGS-P50 plasmid but not to the cells transfected with the control pCAGGS plasmid (Fig. 1). In Western blotting with the MAb 31D a specific band with a molecular weight of 50 kDa was detected in

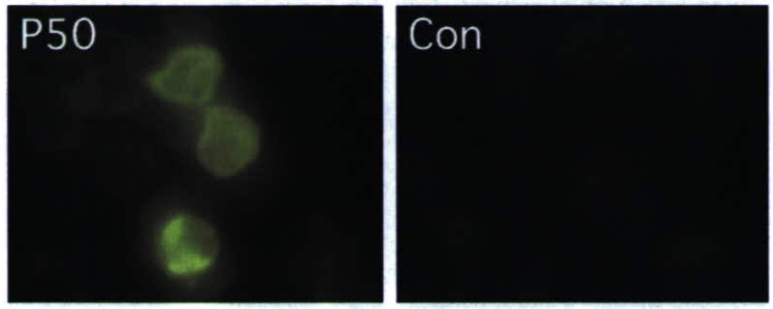

Fig. 1. Expression of P50 in RK13 cells transfected with pCAGGS-P50. The expression of P50 was analyzed by IFAT using MAb 31D recognizing P50. P50, cells transfected with pCAGGS-P50; con, cells transfected with control plasmid pCAGGS.

the RK13 cells transfected with the pCAGGS-P50 plasmid (data not shown). The molecular weight of P50 expressed by pCAGGS-P50 plasmid was similar to that of the native P50 from *B. gibsoni*.

3.2. Expression of P50 in vitro by the recombinant vaccinia virus

RK13 cells were infected at 5 PFU/cell with a vvP50 or with a control vaccina virus mO. After incubating for 2 days, cells infected with vvP50 were analyzed by Western blotting or IFAT using MAb 31D. In Western blotting, a specific band with a molecular weight of 50 kDa was detected (Fig. 2A). The molecular weight of the recombinant P50 expressed by vvP50 was similar to that of the native P50 from *B. gibsoni*. In the IFAT analysis, specific fluorescence was detected in the cells infected with vvP50 but not in the cells infected with the control mO (Fig. 2B).

3.3. IgG subclass against P50 of a dog experimentally infected with B. gibsoni

The IgG subclass against P50 of the sequential serum samples collected from experimentally *B. gibsoni*-infected dog was measured. As shown in Fig. 3, the IgG2 antibody response against P50 was detected as a major subclass in comparison with the IgG1 antibody response. A higher IgG2 subclass response was detected until the acute and chronic phases of infection.

3.4. Peripheral IgG and IFN-γ response in immunized dogs

To confirm the immunogenicity of the vaccine plasmid and virus, sera were collected 2 weeks after each immunization from dogs and examined by ELISA, IFAT, or Western blotting. Fig. 4 shows the antibody responses determined by ELISA with GST-P50 protein as the antigen. As shown in Fig. 4a, the IgG response against the P50 protein did not show significant increase after each immunization with the vaccine plasmid pCAGGS-P50 ($P > 0.2$ versus day of first immunization). Two weeks after the booster immunization with the recombinant vaccinia virus vvP50, the mean optical density (OD) significantly increased ($P < 0.0001$, versus day of first immunization). Neither the vaccine-immunized control group nor the non-immunized control group developed a specific antibody response against P50. The isotype of the antibody was also analyzed. As shown in Fig. 4b and c, the IgG2 responses were determined as a major subset when compared with those of IgG1. To determine the antigenicity of the vaccine, the sera obtained 2 weeks after the booster immunization with vvP50 were examined for IFAT or

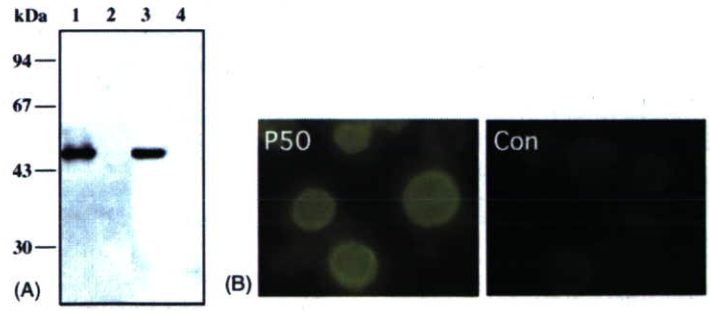

Fig. 2. Expression of P50 in RK13 cells infected with vvP50. (A) Western blot analysis of P50 expressed in RK13 cells. The expression of P50 was detected using MAb 3 1D. Lane 1, lysates of recombinant vaccinia virus vvP50-infected cells; lane 2, control lysates of vaccinia virus mO-infected cells; lane 3, lysates of *B. gibsoni*-infected RBCs; lane 4, control lysates of healthy-dog RBCs were used as the antigen. (B) IFAT analysis of P50 expressed in RK13 cells. The cells were stained with MAb31D. P50, cells infected with vvP50; con, cells infected with the control parent virus mO.

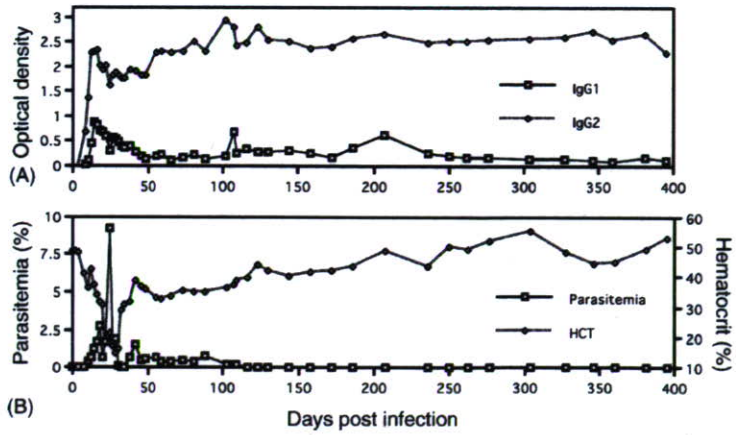

Fig. 3. Determination of the IgG subclass against P50 of the sequential sera from a dog infected with *B. gibsoni*. (A) IgG subclass response against P50 of a dog infected with *B. gibsoni*. (B) Parasitemia and hematocrit (HCT) value of the same dog.

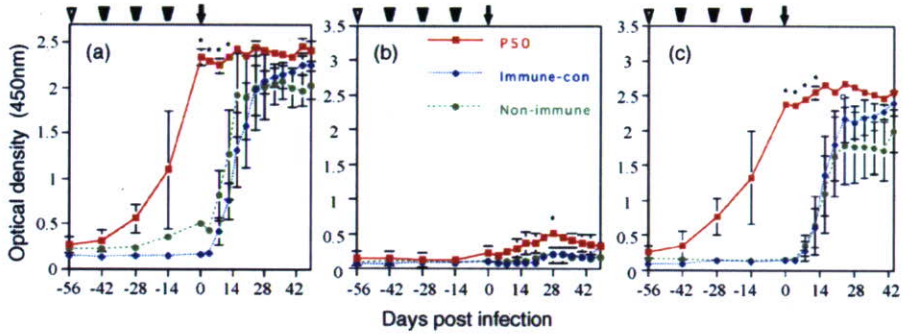

Fig. 4. Determination of the antibody response against P50 of dogs immunized with P50. (a) Total IgG, (b) IgG1, and (c) IgG2. P50, sera collected from dogs immunized with pCAGGS-P50 and vvP50; immune-con, sera collected from dogs immunized with control antigen pCAGGS and mO; non-immune, sera collected from non-immunized control dogs. The dogs were immunized with DNA at day-56, -42 and -28 (white arrowhead), and immunized with recombinant vaccinia virus at day-14 (solid arrowhead). Then, the dogs were challenged with 2×10^8 of *B. gibsoni*-infected RBCs at day-0 (white arrow). The asterisks show the significant difference ($P < 0.05$) between the dog groups immunized with P50 and the control groups. The results are shown as the mean values, and the error bars represent the standard deviations.

Western blotting using *B. gibsoni* merozoites as the antigen. In IFAT analysis, all sera derived from dogs immunized with the vaccine plasmid and virus strongly reacted to the *B. gibsoni* merozoites, but sera derived from the control dogs did not (data not shown). As shown in Fig. 5, the sera from dogs immunized with pCAGGS-P50 and vvP50 specifically recognized the 50-kDa band of the native P50 protein of *B. gibsoni* merozoites. The peripheral IFN-γ response of dogs after the challenge infection was analyzed by ELISA. As shown in Table 1, at 0 days post-infection, all dog groups showed an undetectable level of IFN-γ. At 8 days post-infection, the dog group immunized with pCAGGS-P50 and vvP50 showed a significantly higher level of the IFN-γ response but not detected in control dogs immunized with pCAGGS and mO or non-immunized.

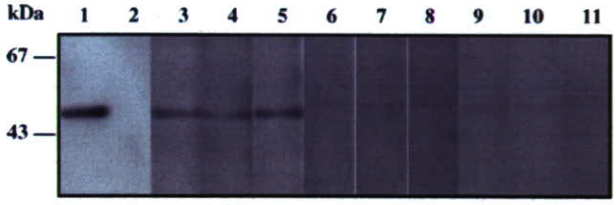

Fig. 5. Western blot analysis of the reactivity of the sera collected from dogs immunized with P50. Lanes 1 and 2, MAb 31D; lanes 3–5, each serum collected from dogs immunized with pCAGGS-P50 and vvP50; lanes 6–8, each serum collected from dogs immunized with control pCAGGS and mO; lanes 9–11, each serum from non-immunized control dogs. Lanes 1 and 3–11, lysates of *B. gibsoni*-infected RBCs; lane 2, control lysates of healthy-dog RBCs were used as the antigen.

Table 1
Peripheral IFN-gamma response of the immunized dogs infected with *B. gibsoni*

Dog group ($n = 3$)	IFN-γ response (pg/ml)	
	Day 0	Day 8
P50	U.D.[a]	57.3 ± 11.08[b]
Immune-con	U.D.	U.D.
No-immune	U.D.	U.D.

[a] U.D.: under detectable level.
[b] Result was shown in mean ± S.D.

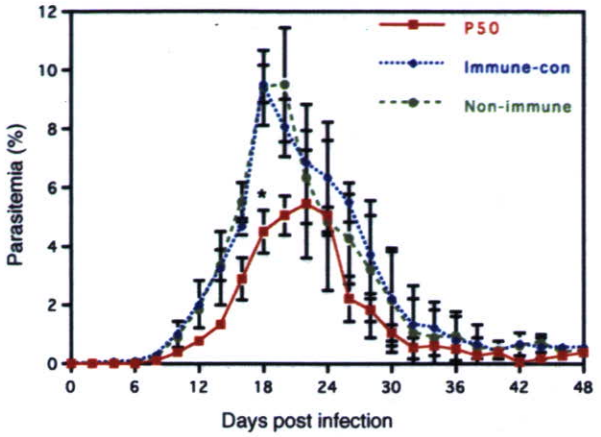

Fig. 6. Parasitemia of dogs after challenge infection with *B. gibsoni*-infected RBCs. P50, dogs immunized with pCAGGS-P50 and vvP50; immune-con, dogs immunized with control plasmid pCAGGS and mO; non-immune, non-immunized control dogs. The results are shown as the mean values, and the error bars represent the standard deviations. The asterisks show the significant difference ($P < 0.05$) between the dog groups immunized with P50 and the control groups.

3.5. Allergic reaction of the immunized dogs

To determine the allergic reaction of the immunized dogs, serum total IgE concentration of immunized period (day-56–0 of Fig. 4) was analyzed. All serum samples showed the concentration of less than 7.8 ng/ml (under detectable level) of serum total IgE responses (figure not shown).

3.6. Protective efficacy against challenge infection

Two weeks after the booster immunization with the recombinant vaccinia virus vvP50, the dogs were challenged by an intravenous injection of the NRCPD strain of *B. gibsoni*-infected RBCs collected from an experimentally infected dog on day 0. Parasitemia in peripheral blood was monitored every 1–2 days. Parasite growth was significantly inhibited ($P < 0.05$, on day 20) in dogs immunized with P50 in comparison with that in control immunized and mO or not immunized (Fig. 6). There was no significant difference between the two control groups ($P > 0.2$). At the peak of parasitemia, the ratio of the inhibitory effect was 48.5% compared to that of the immunization control group and 49.2% compared to that of the non-immunized group. The PCV of venous blood was measured to determine the anemia level. There was no significant difference between the group immunized with P50 and the immunized and non-immunized control groups.

4. Discussion

For protection against animal babesiosis, the IFN-γ production by $CD4^+$ T cell considered to play important roles [10,36]. In the animal has resolved acute infection, activated macrophage produced cytokines such as interleukin 12 (IL-12) and IL-18 that promote IFN-γ production by $CD4^+$ T cells. In turn, IFN-γ is required for activation of the macrophages to produce babesiacidal molecules such as nitric oxide, and enhancing an opsonizing IgG2 antibody response. Thus, $CD4^+$ T cell-mediated immune response and IFN-γ was considered to be important for maintaining protective immunity against babesiosis. Traditionally, vaccine formulations were designated to mainly induce antibodies; however, recent efforts have aimed at inducing both cellular and humoral immune responses. In recent studies, it has been reported that a heterologous prime-boost immunization regime priming DNA followed by a recombinant vaccinia virus, both of which expressed the same antigen, appears to be particularly promising at inducing cellular and humoral immune responses [21–23]. Moreover, this immunization regime is capable of inducing greatly enhanced and persistent level of $CD8^+$ and $CD4^+$ T cells. These observations have been extended to protozoan parasite infection in animal models [25,37–39].

Previously, we identified the immunodominant protein P50 of *B. gibsoni* and demonstrated that the antiserum against P50 significantly inhibited parasite growth in Ca-RBC SCID mice infected with *B. gibsoni* [40]. These results indicated that P50 might be a useful vaccine candidate for the control of canine *B. gibsoni* infection. In this study, we determined the immunogenicity and protective efficacy of P50 using a heterologous immunization regime with priming DNA followed by a recombinant vaccinia virus in dogs. The dogs immunized with P50 developed a strong immune response and showed partial protection against experimental intravenous inoculation of *B. gibsoni*-infected RBCs.

For the vaccination trial against *B. gibsoni* infection, we generated the mammalian expression vector pCAGGS-P50 and the recombinant vaccinia virus vvP50. In Western blotting, it was confirmed that both vaccine components correctly expressed the 50-kDa band in RK13 cells. To examine the immunogenicity of these vectors, a group of dogs ($n = 3$) was immunized three times with priming DNA and once with a boost of the recombinant vaccinia virus. A previous study showed that this immunization schedule was most effective for the induction of a strong immune response against malaria infection than other vaccination regimes in humans [41]. After a booster immunization with vvP50, the dogs developed a significantly higher level of IgG antibody against P50 than that before the booster immunization. These antibody responses were higher than those induced in control dog groups after a challenge infection with *B. gibsoni* parasites. These results indicated that the immunization regime used in this study was an effective method for dogs as well. The sera collected from dogs after the booster immunization showed strong reactivity against *B. gibsoni* merozoites in IFAT and specifically reacted to the native P50 in Western blotting. These results demonstrated that P50 expressed by those vectors in dogs is similar to the native P50 in terms of its molecular structure and antigenicity. We also determined the type of immune response elicited in dogs by

measuring the ratio of IgG1 and IgG2 anti-P50 antibodies. In our previous study, we analyzed IgG subclass of the dogs immunized with recombinant P50 expressed in insect cells by baculovirus with saponin as adjuvant. In that case, the IgG1 and IgG2 antibody responses showed similar level [42]. In this study, in contrast, the dog group immunized with P50 showed an increase in the IgG2 response over the IgG1 subclass. The response of the IgG subclass was similar to that of a dog recovered from the acute infection of *B. gibsoni*. The higher IgG2 response was maintained at all infection periods after challenge. Moreover, the P50-immunized dog group showed a significantly higher level of IFN-γ after challenge infection with the parasite. These results might indicate an induction of IFN-γ producing $CD4^+$ T cell immune response in dogs immunized with P50, although we could not segregate whether the immunological response induced by *B. gibsoni* endogenous P50, pCAGGS-P50 or vvP50.

To confirm the protective efficacy of the immunization with priming pCAGGS-P50 followed by vvP50, the dogs were inoculated with *B. gibsoni*-infected RBCs derived from i.v. infected dogs. The dogs immunized with P50 showed partial protection, manifested as a significantly low level of parasitemia; complete protection, characterized as the absence of apparent parasitemia, was not observed. Furthermore, the dogs immunized with P50 showed a 2-day delay of the peak parasitemia and 49.5% inhibition on parasite growth when compared to the control groups. These results indicated that P50 might be a useful candidate for the development of a vaccine for the control of canine *B. gibsoni* infection.

Despite the fact that the dogs immunized with P50 showed significantly low level of parasitemia, PCV level did not showed significant difference and severe anemia was not protected. This reduction of parasitemia was rather short-term and limited to a very narrow window. Thereafter, the immunized dogs showed little overall protection against effects such as anemia compared to the control groups. Therefore, further study of the pathogenesis of canine *B. gibsoni* infection will be required to develop an effective vaccine.

Acknowledgements

We are grateful to J. Miyazaki of Osaka University for the pCAGGS plasmid. This work was supported by a grant from The 21st Century COE Program (A-1) and a Grant-in-Aid for Scientific Research (no. 13556049) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. S. Fukumoto was supported by a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

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Veterinary Parasitology 151 (2008) 268–278
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Gene silencing of ribosomal protein P0 is lethal to the tick *Haemaphysalis longicornis*

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Received 12 September 2007; received in revised form 6 November 2007; accepted 7 November 2007

Abstract

Ribosomal protein P0 has been demonstrated to be a multifunctional protein in the large subunit of eukaryotic ribosome. In this study, a gene encoding ribosomal protein P0 termed HlP0 was isolated from a full-length salivary gland cDNA library previously constructed from the tick *Haemaphysalis longicornis*. The full-length cDNA of the HlP0 gene is 1141 bp, with an open reading frame (ORF) of 963 bp. The ORF of the HlP0 gene encodes a putative protein of 320 amino acid residues, with a predicted molecular mass of 35 kDa. Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that the HlP0 gene transcript was expressed in all developmental stages and all tissues dissected from 4-day-fed adult ticks. Antibodies raised against recombinant HlP0 recognized a native protein with an expected molecular size of 35 kDa in all tested tissues. RNA interference of HlP0 gene was carried out by injecting ticks with PBS, green fluorescent protein (GFP) dsRNA, and HlP0 dsRNA. The results showed that ticks treated with HlP0 dsRNA obtained a strikingly lower body weight (2.63 ± 1.21 mg vs. 226.75 ± 74.80 mg in the PBS-injected group and 231.15 ± 51.32 mg in the GFP dsRNA-injected group, Student's *t*-test, $P < 0.01$), a lower engorgement rate (4% vs. 100% and 94.11%, respectively), and higher mortality (96% vs. 2.5% and 10.4%, respectively) after blood-sucking than the control groups. This suggests that ribosomal protein P0 is required for the blood ingestion and subsequent viability of *H. longicornis*. This is the first report of ribosomal protein P0 from ticks.

Keywords: *Haemaphysalis longicornis*; Ribosomal protein P0; RNA interference

1. Introduction

Ticks are hematophagous arthropods that infest many host species. Once they attach to the host, their blood-sucking can cause irritation and infection of the skin, anemia, and paralysis (Vedanarayanan et al., 2004). More importantly, they transmit a variety of pathogens, such as viruses, bacteria, spirochetes, rickettsia, and protozoans to their hosts, which has been a major constraint to the improvement of the livestock industries. Moreover, 14 tick-borne diseases have been reported in international travelers, and the incidence of travel-associated tick-borne diseases is increasing as more people travel (Jensenius et al., 2006). To control ticks and tick-borne diseases, the frequent use of chemicals is not sustainable for environmental, medical, and

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0304-4017/$ – see front matter
doi:10.1016/j.vetpar.2007.11.015

economic reasons. Vaccination is, thus, considered to be a promising alternative measure to control ticks. With respect to tick vaccine development, the mechanisms of tick activities, i.e., feeding, oviposition, and molting, need to be characterized. To date, a large array of studies has been carried out on the function of bioactive molecules from different tissues of ticks (Willadsen, 2004). Simultaneously, a series of proteins responsible for the secretion of proteins has been characterized (Karim et al., 2004, 2005). Nevertheless, proteins associated with protein synthesis in ticks remain unknown. It has been reported that the overall mass and protein in the salivary glands increased about 25-fold during tick feeding in female *Amblyomma americanum* (Shipley et al., 1993). New gene expression was observed in the salivary glands of female ticks after attachment, mating, and feeding (Oaks et al., 1991). This change in the protein expression level is directly related to the function of ribosomes.

As an important component of the translation of protein, the ribosome is universal to all organisms. Ribosomes in mammals consist of four RNA species and a vast number of ribosomal proteins (RPs). Among the RPs in eukaryotic cells, acidic phosphoproteins P0, P1, and P2 form a complex, presumably as the pentameric form $(P1)_2/(P2)_2/P0$ (Shimizu et al., 2002). P0 protein herein is a multifunctional protein. It contributes RNA-binding domain to the binding of $(P1)_2/(P2)_2/P0$ complex to 28S rRNA (Uchiumi and Kominami, 1992), and is associated with the interaction of the complex with the elongation factor, eEF2 (Justice et al., 1999). Moreover, the dephosphorylated P0 detaches from the ribosome and is transported to the nucleus, where it showed apurinic/apyrimidinic endonuclease (APE) activities that are involved in DNA repair (Sanchez-Madrid et al., 1981; Yacoub et al., 1996). In order to describe the function and significance of ribosomal protein P0, the disruption of protein P0 was previously performed using P-element insertion (Frolov and Birchler, 1998) and C-terminal truncation (Griaznova and Traut, 2000; Hagiya et al., 2005). In addition to the above methods, interference with the gene by double-stranded RNA (dsRNA) transfection has been employed in the study of P0 in mosquito cells (Jayachandran and Fallon, 2003).

RNA interference (RNAi), the sequence-specific degradation of mRNA mediated by homologous dsRNA, has become a valuable tool for determining the biological role of genes. To date, RNAi of genes has been performed in several kinds of arthropods, including mosquito (Attardo et al., 2003; Boisson et al., 2006), fruit flies (Boutros et al., 2004), silkworm (Ohnishi et al., 2006), and ticks (Hatta et al., 2007; Zhou et al., 2006).

In this study, we isolated a ribosomal protein P0 from the adult tick *Haemaphysalis longicornis* and investigated its transcription profiles in different developmental stages and tissues. Moreover, through RNAi of ribosomal protein P0 gene, we determined that normal expression of P0 is essential for the feeding and survival of ticks. The down-regulation of P0 led to the failure of blood-sucking and subsequent death of ticks.

2. Materials and methods

2.1. Ticks

The Okayama strain of hard tick *H. longicornis* (Fujisaki, 1978) was infested on the ears of Japanese white rabbits (SPF, Japan Laboratory Animals, Tokyo, Japan). To obtain desired tissues from partially fed ticks, unfed adult ticks were allowed to feed for 4 days on rabbits and then manually detached. The recovered ticks were immediately subjected to dissection under a microscope, and tissues were collected in a cold PBS buffer. The collected tissues were stored at −80 °C until use.

2.2. Sequencing and analysis of the gene

A full-length salivary gland cDNA library was constructed using the vector-capping method as described previously (Kato et al., 2005), and a total of 10,000 recombinant transformants from the library were randomly selected and partially sequenced to form the database of expressed sequence tags (Harnnoi et al., 2007). From the database, two sequences containing a ribosomal P0 encoding insert were selected and fully sequenced using an automated sequencer (ABI prism 310 Genetic Analyzer, Applied Biosystems) (Boldbaatar et al., 2006) by three primers, T7 forward, P0 gene-specific (HlP0-con, listed in Table 1), and T3 reverse primers. The obtained full-length cDNA sequence of ribosomal P0 was then analyzed using a basic local alignment search tool (BLAST) (NCBI: http://www.ncbi.nlm.nih.gov).

2.3. In vitro expression of recombinant HlP0 and preparation of the anti-rHlP0/TrX serum

The HlP0 gene was PCR amplified using primers HlP0-E-F and HlP0-E-R (Table 1), and non-directionally cloned into the *Bam*H I site of the expression vectors pET32a and pGEX-4T-3 (Amersham Pharmacia

Table 1
Gene-specific primers used in sequencing, expression, and RT-PCR amplification

Name	Sequence
HlActin-F	5′-CCAACAGGGAGAAGATGAACG-3′
HlActin-R	5′-ACAGGTCCTTACGGATGTCC-3′
HlP0-con	5′-CAAGATCTCCAAGGGCACG-3′
HlP0-E-F	5′-CCGGATCCATGGTCAGGGAGGACAAGACC-3′
HlP0-E-R	5′-CAGGATCCGAGCTCAGTCGAAGAGTCCG-3′
HlP0i-U1	5′-GAGACATCCTTGGCTCACTCTC-3′
HlP0i-U2	5′-GGATCCTAATACGACTCACTATAGGGAGACATCCTTGGCTCACTCTC-3′
HlP0i-D1	5′-GGATCCTAATACGACTCACTATAGGAGAGGCGGAGGGAGCTCAGTC-3′
HlP0i-D2	5′-AGAGGCGGAGGGAGCTCAGTC-3′
GFPi-U1	5′-ATGGTGAGCAAGGGCGAGGAGC-3′
GFPi-U2	5′-GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGAGC-3′
GFPi-D1	5′-GGATCCTAATACGACTCACTATAGGACTTGTACAGCTCGTCCATGCCG-3′
GFPi-D2	5′-ACTTGTACAGCTCGTCCATGCCG-3′

Biotech, Piscataway, NJ) and used to transform DH5α cells. The resultant clones, designated HlP0/GST and HlP0/TrX, respectively, were sequenced and used to transform *E. coli* (BL21) cells for protein expression. Fusion proteins were expressed according to the description of McBride et al. (2000). After identification of the positive clones, recombinant HlP0/GST (rHlP0/GST) protein was solubilized in 2% (w/v) *N*-lauroylsarcosine sodium (Sigma) and purified with glutathione sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech, USA). Recombinant HlP0/TrX (rHlP0/TrX) protein was expressed in the form of an inclusion body, which was primarily purified by sonication for immunization, as described elsewhere (Kukkonen et al., 2004). Briefly, inclusion bodies of rHlP0/TrX were completely mixed with an equal volume of Freund's complete adjuvant (Sigma) and intraperitoneally injected into mice (ddY, 8 weeks old). The mice were immunized twice again with the same dose of recombinant protein in the incomplete adjuvant at days 14 and 28. Eight days after the third injection, the titer of the antibodies was evaluated using ELISA, employing rHlP0/GST as an antigen (5 μg/ml, 50 μl/well). Subsequently, all the sera were collected from the blood of the mice.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Salivary glands, midgut, cuticle, ovary, synganglion, and fat body isolated from 4-day-fed adult ticks were sonicated and Western blotting was performed as described previously (Gong et al., 2007). The successful transfer of the proteins to the membrane was confirmed using SeeBlue Pre-Stained Protein marker plus 2 (Invitrogen, USA). The membrane was then incubated with the serum (1:300) collected as described in Section 2.3. The peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG, 1:2000) was used as the second antibody for the detection of the native protein.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of HlP0 transcriptional profiles

To investigate the HlP0 expression pattern at different developmental stages (eggs, larvae, nymphs, and adult females), partially fed ticks (except for eggs) were detached by hand for RNA extraction. Simultaneously, 4-day-fed adult ticks were manually detached and immediately dissected under the microscope for the various tick tissues (midgut, salivary glands, cuticle, ovary, synganglion, and fat body) (You et al., 2001). Total RNA from these samples was extracted using the TRIZOL reagent (Invitrogen, USA). The mRNA abundance of HlP0 in the extracted RNA samples was evaluated by RT-PCR analysis using a one-step RNA PCR kit (Takara, Japan) by HlP0 (HlP0-E-F and HlP0-E-R, Table 1) and actin gene-specific primers (HlActin-F and HlActin-R, Table 1). The RT-PCR amplification was carried out in a 10 μl reaction liquid including 200 ng RNA from each sample, and the RT-PCR program for RNA of developmental stages was 40 min at 50 °C, followed by the PCR program of 2 min of denaturation at 94 °C and 30 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min, and finally 72 °C for 7 min. The RT-PCR amplification for RNA from different tissues was performed for 60 min at 50 °C, followed by the similar PCR reaction for 40 cycles.

2.6. Generation of dsRNA and injection of ticks with dsRNA

Gene silencing of HlP0 in adult ticks was carried out as reported previously (Hatta et al., 2007). In brief, one of the extracted plasmids (described in Section 2.2) was subjected to PCR amplification using two pairs of primers, HlP0i-U1 and HlP0i-D1 and HlP0i-U2 and HlP0i-D2 (listed in Table 1). The amplification was performed using a program described in Section 2.5. The resulting PCR products were purified from agarose gel and employed as templates for dsRNA synthesis using the T7 RiboMax™ Express Large-scale RNAi Kit (Promega, Madison, WI, USA). The quality of dsRNA was checked using 1.5% agarose gel, and the concentration was quantified by spectrometry. To obtain a negative dsRNA control, plasmid pCX-EGFP (Zhang et al., 2007a) was treated with *Eco*R I, and the recovered product was subjected to similar PCR amplification to that described above employing GFP-specific primers (shown in Table 1) and synthesis of GFP dsRNA. dsRNA or PBS was injected into ticks using a method described by Hatta et al. (2007). Injected ticks were stored at 25 °C for 24 h in a moist chamber, and the alive ticks were allowed to feed on rabbits. Two rabbits were involved in the tick infestation, one for HlP0 dsRNA-injected and PBS-injected ticks (on the left and right ear, respectively), and the other one for HlP0 dsRNA-injected and GFP dsRNA-injected ticks.

2.7. Analysis to confirm HlP0 gene silencing

Four days post-infestation, four ticks each were randomly collected from the PBS- and dsRNA-injected groups. The ticks were homogenized using a mortar and pestle, one half for RNA extraction and the other half for protein preparation. The extracted RNA samples were subjected to the same RT-PCR amplification for 30 cycles mentioned in Section 2.5. The products of RT-PCR amplification were fractionated by 1.5% agarose gel and the densities of the bands were analyzed by computerizing densitometry using Image Master Program (Luminous Imager Version 2.0G, Aisin Cosmos RD Co. Ltd., Tokyo, Japan) as described elsewhere (Tanaka et al., 2004). Moreover, to confirm that the translation product of the HlP0 gene decreased due to disruption of HlP0 mRNA, the endogenous protein of the HlP0 from each group was subjected to Western blotting, employing antibodies against HlP0 recombinant protein as primary antibodies. Expression of actin protein was investigated simultaneously as an internal control, using anti-actin antibody (Sigma–Aldrich, 1:500). The density of Western blot bands from each lane was measured with Shimadzu Phoretix software (Shimadzu-biotech, Tokyo, Japan) as described previously (Takenaka et al., 2006). The relative expression ratio of HlP0 was calculated as: relative density ratio of HlP0 protein = (HlP0 signal density/actin signal density). Statistical significant of difference between controls and HlP0 dsRNA-treated group was calculated by Student's *t*-test. Both RT-PCR and Western blotting experiments were performed at least three times.

2.8. Evaluation of the RNAi effect of HlP0

The success of tick feeding was investigated by measuring the attachment rate at 2 days post-infestation, feeding periods, engorgement rate, body weight, and mortality (Zhou et al., 2006). Furthermore, two ticks were detached using forceps at 4 days post-infestation and dissected for cuticle and salivary gland collection, and the size of these tissues was compared under the microscope.

3. Results

3.1. Characterization of HlP0 cDNA

The full-length HlP0 cDNA consists of 1141 nucleotides, with an open reading frame (ORF) of 963 bp from 76 bp to 1038 bp (Access number: EU048401). The cDNA sequence contains a poly adenylation signal, AATAAA, located 14 bp upstream of the poly (A) tail (Fig. 1A). The presumptive 5′-end of the HlP0 cDNA sequence, ATCGTTT (marked with *stars* in Fig. 1A), shared six residues with the insect conserved cap site ATCA(G/T)T(C/T) (Hultmark et al., 1986). The ORF of the cDNA sequence encodes a putative protein of 320 amino acid residues, with a predicted molecular mass of 35 kDa. The deduced amino acid sequence of the HlP0 gene did not show a signal sequence (SignalP3.0). In the primary structure of HlP0, three functional domains may be recognized (Krokowski et al., 2002), including the rRNA-binding domain (position 44–67, Rodriguez-Gabriel et al., 2000), the second functional domain (182–298 amino acid residues) that is responsible for the interaction with P1/P2 proteins (Shimizu et al., 2002), and the third highly conserved region that is implicated in the interactions with elongation factors during translation (Uchiumi et al., 1986; Zhang et al., 2007b). A database search using the BLAST program revealed a high sequence similarity of the HlP0 protein to P0 proteins from various species. The alignment of the deduced

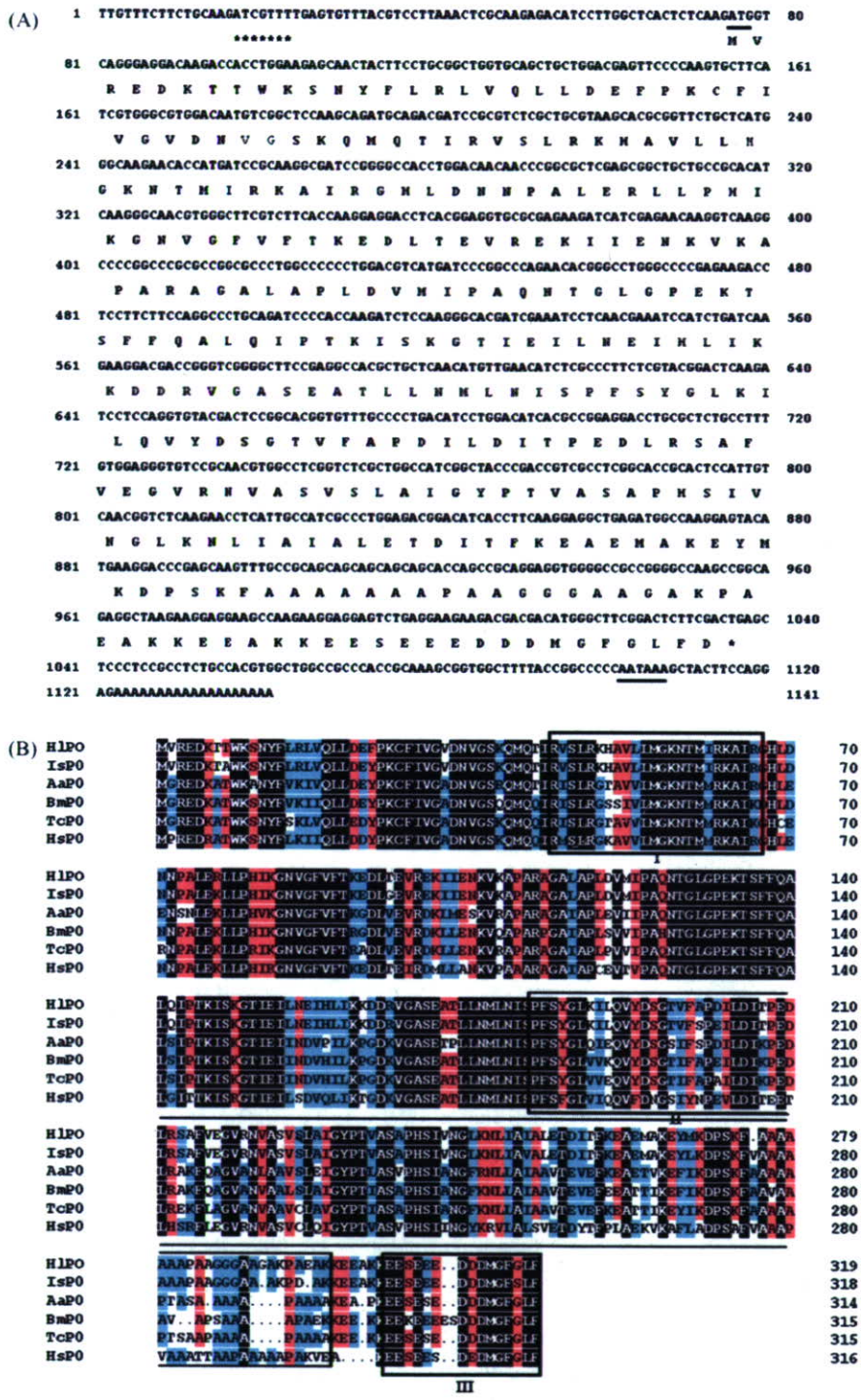

Fig. 1. Analysis of the HlP0 gene sequence. (A) Nucleotide and deduced amino acid sequence of HlP0. The 5′-end nucleotide sequence that shares similar residues with insect conserved cap site is marked with *stars*. The start codon (ATG) and AATAAA are *underlined*. (B) Alignment of the deduced protein sequence of HlP0 from *Haemaphysalis longicornis* tick with those from other species. Sequences included in the alignment and identities: IsP0 (*Ixodes scapularis*, AAY66850, 94.7%), AaP0 (*Aedes albopictus*, AAM97779, 70.64%), BmP0 (*Bombyx mori*, NP_001037123, 71.56%), TcP0 (*Tribolium castaneum*, XP_966610, 74%), and HsP0 (*Homo sapiens*, NP_000993, 68.75%). The predicted rRNA-binding domain (position 44–67), the P1/P2-binding domain (position 182–290), and the conservative C-terminal region are marked with *boxes* and indicated as domains I, II, and III, respectively.

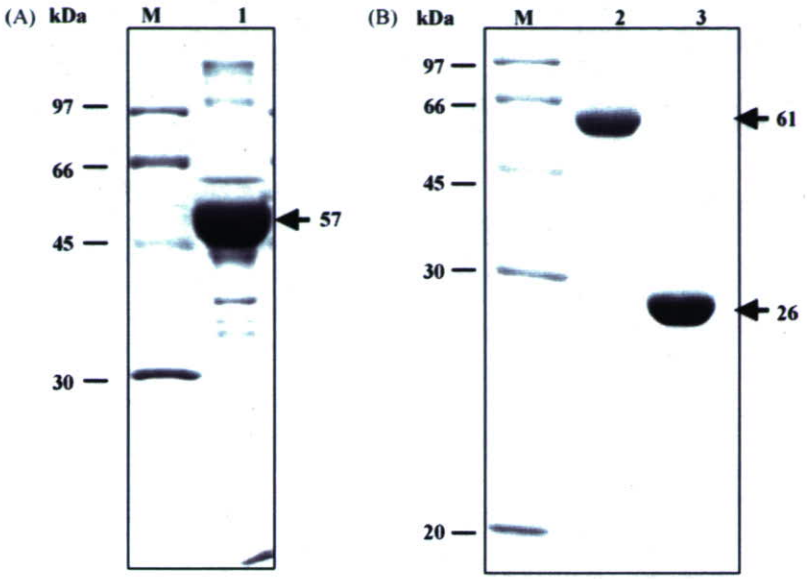

Fig. 2. Purification of recombinant HlP0 proteins. (A) Purification of rHlP0/TrX protein. (B) Purification of rHlP0/GST protein. M: protein molecular weight marker (low); lane 1: purified insoluble inclusion bodies of rHlP0/TrX protein; lane 2: purified rHlP0/GST protein; lane 3: GST protein. The expressed protein bands with expected molecular size are shown with *arrows*.

HlP0 protein sequence with that from *Ixodes scapularis* (AAY66850), *Aedes albopictus* (AAM97779), *Bombyx mori* (NP_001037123), *Tribolium castaneum* (XP_966610), and *Homo sapiens* (NP_000993) yielded identities of 94.7%, 70.64%, 71.56%, 74%, and 68.75%, respectively (Fig. 1B).

3.2. Identification of the endogenous HlP0 protein

rHlP0/Trx was expressed as inclusion bodies with a molecular mass of 57 kDa (Fig. 2A) and used to immunize mice three times. Simultaneously, rHlP0/GST was expressed as a GST fusion protein with a molecular mass of 61 kDa (Fig. 2B). Purified rHlP0/GST was used as an antigen to evaluate the titer of anti-rHlP0/Trx. Subsequently, the sera were collected and recruited to recognize native HlP0 in different tissues from 4-day-fed adult ticks. The result of Western blotting demonstrated that native HlP0 with an expected molecular mass of 35 kDa was ubiquitously detected in all dissected tissues (Fig. 3).

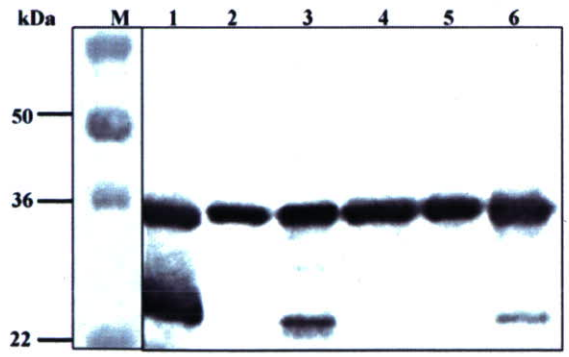

Fig. 3. Identification of endogenous HlP0 by Western blot analysis. The anti-rHlP0/TrX serum was used as the first antibody to detect the native protein in various tissues from partially fed ticks. M: pre-stained protein ladder; lane 1: midgut; lane 2: salivary glands; lane 3: cuticle; lane 4: ovary; lane 5: synganglion; lane 6: fat body.

3.3. Expression pattern of HlP0 gene

The total RNA of eggs, larvae, nymphs, and adult ticks, as well as that of different tissues dissected from 4-day-fed ticks, including salivary glands, midgut, ovary, synganglion, fat body, and cuticle, was subjected to RT-PCR analysis. Fig. 4 shows that the HlP0 gene transcript was present in the whole life cycle of ticks and ubiquitously expressed in all tested tissues.

3.4. Confirmation of HlP0 gene silencing

The disruption of HlP0 mRNA due to an injection of dsRNA was confirmed by RT-PCR of total RNA from 4-day-fed adult ticks and Western blotting. The result indicated that the transcript of HlP0 gene was obviously detected in PBS-injected and GFP-injected ticks but showed a remarkable decrease in HlP0 dsRNA-injected ticks (Fig. 5A and B). The success of RNAi was further confirmed using Western blotting for the detection of endogenous HlP0 protein. Treatment with HlP0 dsRNA

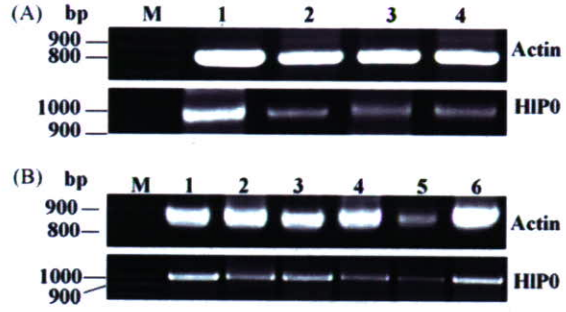

Fig. 4. Transcription profiles of the HlP0 gene in different developmental stages and tissues of partially fed ticks by RT-PCR. (A) Total RNA extracted from different developmental stages of ticks was subjected to RT-PCR. Lane 1: eggs; lane 2: larvae; lane 3: nymphs; lane 4: adult ticks. (B) Total RNA extracted from different tissues was subjected to RT-PCR. Lane 1: midgut; lane 2: salivary glands; lane 3: cuticle; lane 4: ovary; lane 5: synganglion; lane 6: fat body.

led to a significant reduction in the levels of HlP0 protein compared with ticks injected with PBS ($P < 0.0001$) and GFP dsRNA ($P < 0.005$, Fig. 5C and D).

3.5. Impact of HlP0 silencing on tick feeding and survival

Ticks injected with PBS, GFP dsRNA, and HlP0 dsRNA were placed on the ears of two rabbits; success of feeding and survival were investigated. PBS-injected ticks showed a more obvious increase in body size than HlP0 dsRNA-injected ticks 4 days post-infestation (Fig. 6A). Detached HlP0 dsRNA-injected ticks were much smaller than those in the PBS control group (Fig. 6B). The ticks from each group were weighed before and after feeding, and the results were summarized. The average weight of the unfed ticks from the HlP0 dsRNA-injected group (1.99 ± 0.13 mg) was not different from that in the PBS-injected (2.00 ± 0.17 mg) and GFP dsRNA-injected groups (1.99 ± 0.10 mg). However, there was a significant variation in tick fed body weight (2.63 ± 1.21 mg in the HlP0 dsRNA-injected group vs. 226.75 ± 74.80 mg in the PBS-injected group and 231.15 ± 51.32 mg in the GFP dsRNA-injected group, Student's t-test, $P < 0.01$, Fig. 6C). Disruption of HlP0 mRNA did not affect the attachment rate of ticks in the PBS-, GFP dsRNA-, and HlP0 dsRNA-injected groups (74.38%, 87.18%, and 70.73%, respectively) 2 days post-infestation (Fig. 6D). The ticks from the PBS- and GFP dsRNA-treated groups fed on rabbits for 5–8 days until repletion and then detached. Nevertheless, HlP0 dsRNA-treated ticks fed on rabbits for 6–12 days until they detached or died on the ear of rabbits without reaching repletion. As a result, 100% of attached ticks treated by PBS and 94.11% treated by GFP dsRNA were engorged, while only 4% of HlP0 dsRNA-injected ticks were engorged.

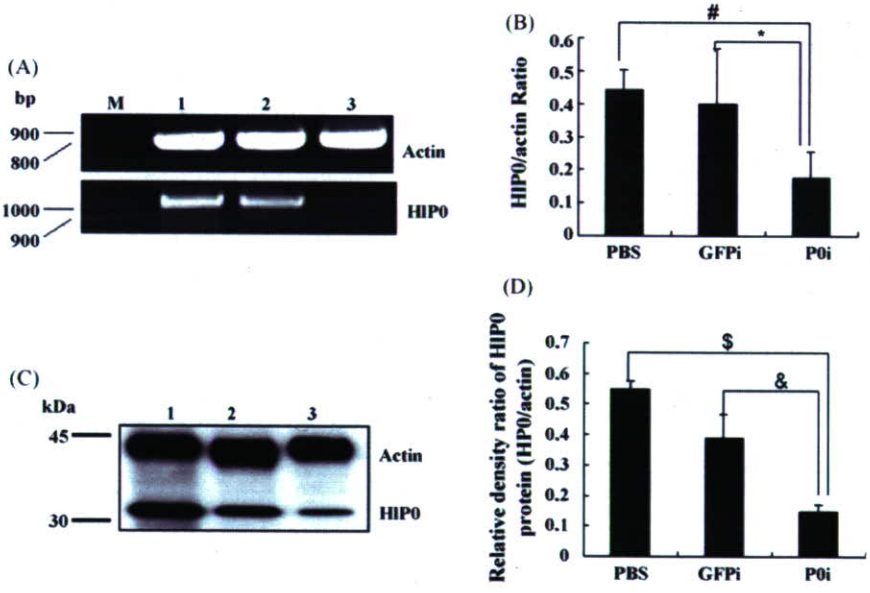

Fig. 5. Confirmation of silencing of the HlP0 gene by reverse transcription PCR (RT-PCR) and Western blotting. (A) HlP0 transcription products in RNA from 4-day-fed ticks were investigated using RT-PCR (30 cycles) with actin and HlP0 gene-specific primers. (B) Quantification of the band density obtained by RT-PCR. HlP0 band density is shown relative to actin. (C) Western blotting of native HlP0 protein from 4-day-fed ticks was carried out using anti-actin sera and anti-rHlP0/TrX sera. M: 100 bp DNA ladder; lane 1: PBS-injected ticks; lane 2: GFP dsRNA-injected ticks; lane 3: HlP0 dsRNA-injected ticks. D: density of HlP0 protein bands is shown as a ratio to actin protein. Values represent mean ± S.D. $^{*}P < 0.05$; $^{\#}P < 0.01$; $^{\&}P < 0.005$; $^{\$}P < 0.0001$ (Student's t-test).