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Normal human skin is superior to monkey esophagus substrate for detection of circulating BP180-NC16A-specific immunoglobulin G antibodies in bullous pemphigoid

S. Emtenani^{#1}, H. Yuan^{#2,3}, C. Lin², M. Pan³, J. E. Hundt¹, E. Schmidt^{1,4}, L. Komorowski⁵, J. R. Stanley^{#2}, and DR C. M. Hammers^{#1,2,4,§}

¹Luebeck Institute of Experimental Dermatology (LIED), University of Luebeck, Luebeck, Germany

²Dept. of Dermatology, University of Pennsylvania, Philadelphia, PA, USA

³Dept. of Dermatology, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

⁴Dept. of Dermatology, University of Luebeck, Luebeck, Germany

⁵Institute of Experimental Immunology, EUROIMMUN, Luebeck, Germany

These authors contributed equally to this work.

Summary

BACKGROUND: Bullous pemphigoid (BP) is the most common autoimmune subepidermal blistering skin disease. Two antigens were identified as targets of circulating autoantibodies (abs), BP180 and BP230, with BP180 being a critical transmembrane adhesion protein of basal keratinocytes of the epidermis. The non-collagenous domain 16A (NC16A) of BP180 is the immunodominant epitope in BP patients, and anti-BP180-NC16A immunoglobulin G (IgG) abs correlate to disease activity. Routine serologic testing and follow-up of BP relies on indirect immunofluorescence (IIF) of serum abs, commonly performed on monkey esophagus (ME), and/or ELISA testing on recombinantly produced fragments of BP180 and BP230 (BP180-NC16A, BP230-C/N).

OBJECTIVES: Following our clinical observation that many IIF stainings showed negative results when performed on ME, especially when BP180-NC16A but not BP230-C/N antibodies were detected by concurrent ELISA testing, we hypothesized that NC16A epitopes may not be well represented on ME substrate.

METHODS: Sera from different BP cohorts were tested by IIF on ME and normal human skin (NHS). To confirm findings, affinity-purified anti-BP180-NC16A/BP230 polyclonal antibodies and recombinant anti-BP180-NC16A/BP230 monoclonal antibodies (mAbs) were used.

§corresponding author (hammers@web.de).

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RESULTS: For sensitive detection of BP180-NC16A-specific IgG abs, sections of NHS are superior to the widely used ME. Confirmation comes from polyclonal affinity-purified anti-BP180-NC16A/BP230 abs, and by monoclonal abs cloned from an active BP patient.

CONCLUSION: Use of NHS is preferable over ME in routine IIF testing for BP. These results are of clinical relevance because anti-BP180-NC16A IgG titers are correlated to disease activity and important to be detected reliably for screening, diagnosis and follow-up of BP patients.

Introduction

Bullous pemphigoid (BP), that mainly affects the elderly, is the most common autoimmune subepidermal blistering skin disease. Patients typically feature tense blisters, but urticarial plaques and pruritus may precede active blistering by weeks to months¹. Two hemidesmosomal autoantigens were identified as BP180 (BP antigen, BPAG, 2) and BP230 (BPAG1, BPAG1e), with BP180 being a critical transmembrane protein mediating adhesion of the basal keratinocytes to dermal adhesion proteins such as laminin 332². The non-collagenous domain 16A (NC16A) of the BP180 ectodomain is the immunodominant epitope in BP patients, and antibody titers against BP180-NC16A correlate to disease activity, as opposed to anti-BP230 antibody titers³⁻¹⁰. This finding of a correlation of BP180 but not BP230 antibody titers to disease activity is consistent with an exclusively intracellular expression of BP230 in keratinocytes, making it inaccessible for immunoglobulins (and the immune system) unless keratinocytes are damaged¹.

Routine diagnostic confirmation and follow-up of BP relies on direct immunofluorescence (DIF) testing of perilesional biopsies, indirect immunofluorescence (IIF) of circulating serum antibodies (abs) on monkey esophagus (ME), and/or ELISA testing on recombinantly produced fragments of BP180 (i. e., BP180-NC16A) and BP230 (C-/N-terminal fragments in the MBL system¹¹, C-terminal fragment only in the EUROIMMUN system¹²). There are no commercially available test systems for non-NC16A parts of the BP180 ectodomain and the rod-like middle domain of BP230 (BP230-M).

Following our clinical observation that many IIF stainings showed negative results when done on ME, especially when BP180-NC16A- but not BP230-specific antibodies were detected by concurrent ELISA testing, we hypothesized that NC16A epitopes may not be well represented on ME substrate and that ME is not the best choice for serological work-up by immunofluorescence.

By testing sera from BP patients that had, by ELISA, i) both BP180-NC16A and BP230-C/N IgG, ii) BP230-C/N IgG only, and iii) BP180-NC16A IgG only, we here demonstrate that for sensitive detection of BP180-NC16A-specific IgG autoantibodies, sections of normal human skin (NHS) are superior to the widely used ME substrate. Additional confirmation comes from affinity-purified polyclonal anti-BP180-NC16A and anti-BP230-C IgG antibodies, and by monoclonal antibodies against both BPAGs cloned from an active BP patient. These results are of clinical importance and relevance, because BP180-NC16A IgG antibody titers are correlated to disease activity, and, despite development of sensitive and specific ELISA test systems, immunofluorescence testing is still widely used in initial diagnosis and follow up of patients.

Material and Methods

Patients and serum samples

Following the principles of the Declaration of Helsinki, sera for immunofluorescence testing were obtained from active BP patients with typical clinical presentation and positive test results in BP180-NC16A and/or BP230-C/N IgG ELISA testing (MBL) after obtaining written informed consent and Institutional Review Board approval (Shanghai Jiao Tong University School of Medicine Research Ethics Committee, Clinical Ethics Approval 61/2014; University of Pennsylvania, protocol 802188; University of Luebeck, protocol 12–178). Sera were tested on MBL kits because here both the BP230-C- and the BP230-N-terminus are available as substrates, and negative BP230 titers on this ELISA system indicates absence of both BP230-C and BP230-N reactivity; EUROIMMUN only uses a BP230-C fragment as substrate^{11,12}. For initial full serum immunofluorescence studies, 10 patients with concurrent BP180-NC16A and BP230-C/N IgG reactivity, 7 patients with BP230-C/N (but no BP180-NC16A) IgG reactivity, and 12 patients with BP180-NC16A (but no BP230-C/N) IgG reactivity (all by MBL ELISA testing according to the manufacturers' instructions) were included. For subsequent affinity purification of antigen-specific antibodies and related indirect immunofluorescence (IIF) tests, an additional 2 sera with high reactivities against both BPAGs were included.

Indirect immunofluorescence of patient sera

Normal human skin (NHS) was acquired from dermatologic surgery procedures using Institutional Review Board-reviewed protocols (Shanghai Jiao Tong University School of Medicine Research Ethics Committee, Clinical Ethics Approval 61/2014; University of Luebeck, protocol 06–109) and cryosectioned onto glass slides (5 µm thickness). Monkey esophagus (ME) sections were obtained from SCIMEDX. Slides were blocked with TBS-Ca²⁺/1% BSA at room temperature (RT) for 30 min. Slides were washed with TBS-Ca²⁺ three times and incubated with serum samples diluted 1:100 (and, additionally, 1:10 in cases of negative immunofluorescence results at 1:100) in TBS-Ca²⁺/1% BSA at RT for 60 min. Bound antibodies were detected with anti-human IgG F(ab')₂ FITC conjugate (dilution 1:100; Bio-Rad). Finally, slides were washed as above and mounted with DAPI Fluoromount-G mounting medium (SouthernBiotech).

Affinity purification and IIF of BP-antigen-specific antibodies from human sera

His-tagged BP180-NC16A-4X and BP230-C fragments were expressed as described previously^{5,12}. Antigens were coupled to magnetic beads (Dynabeads™ His-Tag Isolation & Pulldown; ThermoFisher Scientific) following the instructions of the manufacturer. Serum was diluted 1:10 in DPBS/0.05% Tween 20, pH 7.1, and incubated with antigen-loaded beads for 90 min. After extensive washing with DPBS/0.05% Tween 20, pH 7.1, antigen-specific antibodies were eluted with 76 mM citric acid, pH 2.2, and immediately neutralized with 2M TRIS buffer, pH 11.1. Original BP sera, eluates of antigen-specific affinity purifications, and the sample flow-throughs were analyzed for complete affinity purification by ELISA testing (EUROIMMUN), according to manufacturers' directions and adjusted to the respective original serum concentrations used. IIF for bound IgG was performed on ME

substrates (SCIMEDX; EUROIMMUN), adjusting samples to the respective original input serum concentrations and using above IIF protocol.

Isolation of monoclonal antibodies from a BP patient

Using previously described methods^{13,14}, antibody phage display (APD) libraries were constructed from peripheral mononuclear cell-RNA, obtained from a patient with active BP (confirmed by clinical features, DIF, IIF, ELISA; University of Pennsylvania, IRB# 704768). In brief, the PCR-amplified variable heavy and light chains were assembled via overlap-PCR, digested with *Sfi-I* (Roche), and ligated into the pComb3X vector (Scripps Institute, La Jolla). After electroporation into XL1-Blue E.coli (Agilent), phage libraries were panned on BP180/230-ELISA wells (MBL). Bound polyclonal phages were eluted with 76 mM citric acid, amplified, and re-panned for up to four rounds. All isolated monoclonal antibodies (mAbs; in form of single chain variable fragments, scFv) were sequenced, individually re-tested on BP180/230 ELISA substrates (MBL, EUROIMMUN) for binding, using an anti-M13 ELISA (for monoclonal phage) and an anti-HA-ELISA (for HA-tagged scFvs, after expression in TOP10F' *E. coli*, Invitrogen). Because both BP230 clones used in this study reacted on both the MBL BP230-C/N and the EUROIMMUN BP230-CF-ELISA system, we concluded that both clones react against the BP230-C domain (clones termed BP230-C-mAb1 and BP230-C-mAb2). Monoclonals against BP180-NC16A were termed BP180-NC16A-mAb1 and BP180-NC16A-mAb2.

Monoclonal IIF

NHS cryosections of 5 µm thickness were blocked with TBS-Ca²⁺ (Bio-Rad) plus 1% BSA (Sigma-Aldrich) at RT for 30 min. Slides were washed with TBS-Ca²⁺ three times and incubated with samples diluted in TBS-Ca²⁺ plus 1% BSA at RT for 60 minutes. Binding of scFvs was detected through staining with rat anti-HA mAb (3F10; dilution, 1:100; Roche), followed by an Alexa Fluor 594-conjugated anti-rat IgG (dilution, 1:200; Invitrogen). Analogously, IIF was performed on ME substrates (SCIMEDX; EUROIMMUN), following above protocol.

Double IIF

For double IIF, the above detailed protocols for IIF staining of affinity-purified polyclonal IgG (dilution 1:40 each) and monoclonal scFv (dilution 1:1,000) were serially executed on the same tissue substrates.

Sequence analyses and alignments

All sequence analyses are based on publicly accessible sequence data at NIH NCBI BLAST®, available online at <https://blast.ncbi.nlm.nih.gov> (Protein BLAST®; last accessed on 04/19/18, 11:58 PM). Alignments are based on NIH NCBI BLAST®, EMBL-EBI Clustal Omega (at <https://www.ebi.ac.uk/Tools/msa/clustalo/>; last accessed on 04/19/18, 11:59 PM) and SnapGene® Version 4.1.4 for Mac (GSL Biotech LLC).

Results

BP230-positive sera show linear basement membrane zone immunofluorescence staining on both monkey esophagus and normal human skin sections whereas most BP sera only positive for BP180-NC16A antibodies stain only normal human skin

Sera from patients with BP230 antibodies only or both BP230 and BP180-NC16A antibodies showed linear basement membrane zone (BMZ) staining at the dermal-epidermal junction (DEJ) on both ME and NHS (Table 1). In contrast, most sera (10/12) from patients with only BP180-NC16A antibodies showed no IF signal on ME, but always on NHS. These data suggest that the BP180-NC16A is not expressed well or not expressed at all in ME. A minority of BP sera with only BP180-NC16A antibodies (2/12) did however show linear staining in ME, likely indicating presence of additional antibodies against stretches of BP180 or BP230 not covered by the recombinant fragments used for coating of commercial ELISA plates. To study this seemingly incongruent issue in more detail we affinity-purified BP180-NC16A- and BP230-C-specific antibodies and performed additional experiments with sera depleted of both BP180-NC16A- and BP230-C-specific antibodies.

Affinity-purified anti-BP180-NC16A polyclonal antibodies show BMZ staining on sections of normal human skin, but not on monkey esophagus

Antigen-specific antibodies against BP180-NC16A and BP230-C were affinity purified from two BP patient sera. To evaluate the effectivity of affinity purification (AP), ELISA was conducted with the original BP sera (before AP), the eluates from AP, and the sample flow-throughs. As illustrated in Figure 1, all APs depleted sera specifically for the antigen reactivity chosen, resulting in mono-specific eluates for downstream testing.

IIF was performed on NHS and ME substrates using BP180-NC16A-/BP230-C-specific polyclonal antibodies from above APs. As already suggested by our findings from Table 1, eluates from BP230-C-APs stained both NHS and ME substrates (**Fig. 2A, 2B**), whereas eluates from BP180-NC16A-APs stained the DEJ on NHS only, but not on ME (**Fig. 2C, 2D**). We conclude, that for detection of pathophysiologically relevant BP180-NC16A-specific antibodies, NHS is a more sensitive substrate than ME. Interestingly, after double-depletion of BP180-NC16A and BP230-C specific antibodies from the same sera, linear staining at the DEJ can still be seen on both NHS and ME substrates (Supplemental Fig. 1), suggesting additional reactivities against epitopes outside of the BP180-NC16A and BP230-C domains that result in positive linear IIF staining. Of note, in the two sera studied here, these non-BP180-NC16A and non-BP230-C abs however did not contribute to the majority of the IIF titer (Supplemental Table 1).

Monoclonal antibodies cloned from an active BP patient confirm that monkey esophagus is not bound by anti-NC16A reactive antibodies

ELISA testing results for four monoclonal antibodies (mAbs) obtained from antibody phage display cloning and recombinant expression are shown in Figure 3A. Immunofluorescence analysis of anti-BP230 mAbs (clones BP230-C-mAb1 and BP230-C-mAb2) revealed positive staining along the BMZ on both NHS and ME (Fig. 3B). IIF testing on ME was negative for anti-NC16A mAbs BP180-NC16A-mAb1 and BP180-NC16A-mAb2, whereas

on NHS linear BMZ staining was found (Fig. 3C). Taken together with our results from full serum immunofluorescence testing and affinity-purified polyclonal serum antibody testing, we interpret these findings as that ME is likely to be devoid of human NC16A epitopes.

We confirmed our observations from **Figures 2** and **3** by performing double IIF stainings of both NHS and ME substrates with polyclonal anti-NC16A-specific antibodies affinity purified from two patients (BP-1, BP-2) and the recombinant monoclonal anti-NC16A antibody clone 1 (BP180-NC16A-mAb1 in Fig. 3A) (Supplemental Fig. 2).

BP180-NC16A is genetically different in monkeys and humans

Although humans and monkeys are both primates, genetic differences exist which result in different coding amino acids (aa) in the NC16A domains of BP180. Highest homology to human NC16A is found in *Pongo abelii* and *Gorilla gorilla gorilla*, with 97% identity (Table 2); commercially used ME usually comes from Rhesus macaques (e.g., *Macaca mulatta*; personal communication), which has only 71% homology. Interestingly, BP230 and the non-NC16A stretches of the BP180 protein have much higher homologies between monkeys and humans, which is in line with our experimental IF findings (**Fig. 2B**; **Fig. 3B**; Supplemental Fig. 1B and 1D, Supplemental Table 1) and previous observations.

Discussion

Diagnosis of autoimmune blistering skin diseases is typically based on clinical examination, histology of a lesional skin biopsy, serum testing by indirect immunofluorescence (IIF) on monkey esophagus (ME), and direct immunofluorescence (DIF) testing of a perilesional biopsy¹⁵. For bullous pemphigoid, the most common autoimmune blistering skin condition, specific and sensitive ELISA kits for detection of BP180-NC16A and BP230-C/N IgG autoantibodies facilitate diagnosis, and, in the case of BP180-NC16A IgG testing, allow for estimation of disease activity when followed over time¹.

As we observed from routine serum testing that many sera with present BP180-NC16A but absent BP230-C/N reactivity had no basement membrane zone-staining in IIF tests on ME, we hypothesized that ME is devoid of (human) BP180-NC16A epitopes relevant to human disease. This hypothesis would also be in line with results from experimental systems for BP that employ mice^{16,17}.

We confirm our hypothesis by indirect immunofluorescence studies of polyclonal patient sera (Table 1), with affinity-purified polyclonal anti-BP180-NC16A and anti-BP230-C antibodies (**Fig. 1**, **Fig. 2**), and, with recombinantly produced anti-BP180-NC16A and anti-BP230-C monoclonal antibodies (Fig. 3). Additional evidence comes from double IIF studies (Supplemental Fig. 2) and genetic comparison of the BP antigens in different monkey species and other mammals (Table 2).

Our findings confirm previous findings demonstrating that, on ME substrate, BP230-specific antibodies contribute most to the overall indirect immunofluorescence titer (as opposed to BP180 antibodies)¹⁸, and that use of ME for the serological diagnosis of BP has a rather low sensitivity of 60–80% in general¹⁹. The continued use of frozen sections of ME substrate in

routine autoimmune blistering skin disease testing seems to be based on traditional use of protocols, although it has been suggested early that frozen sections of normal human skin (NHS) are as least as sensitive as monkey- or guinea pig-derived tissues in serological BP diagnosis when taken from appropriate anatomical locations of human individuals (salt-split skin is sometimes used as well but our work did not evaluate it since it is not routinely used)²⁰. We here extend these findings by presenting data that suggest an even higher sensitivity of NHS, when compared to ME, in detecting disease-relevant BP180-NC16A antibodies.

A limitation of our study is that patients may feature additional antibody reactivities against epitopes in domains of the BP antigens that are not covered by standard commercially available ELISA systems^{3,21}. This is likely to be the case in the patients that have, by standard ELISA testing, BP230-C/N antibodies only (and no BP180-NC16A abs), but clinically active disease: Because the BP230 protein is exclusively of intracellular localization, targeting of non-NC16A-domains of the BP180 ectodomain by circulating autoantibodies may pathophysiologically be of critical relevance²². Of note, those non-NC16A-parts of the BP180 ectodomain feature higher homology between monkey and human tissue, potentially enabling detection of those antibodies also by using ME substrate (Table 2). Similarly, these additional non-NC16A serum reactivities may explain the observed positive ME staining in a few BP180-NC16A-positive but BP230-C/N negative sera (patients BP28 and BP29 in Table 1), a finding supported by our double-depletion experiments (Supplemental Fig. 1). Alternatively, those sera may have additional reactivities against the middle portion of BP230, BP230-M, that is not covered by commercial ELISA systems.

Despite these limitations that should be addressed more rigorously in future studies, we think that in BP patients the use of NHS as a sensitive substrate for serum antibody screening, detection, and titration is advantageous over the use of ME, also with regard to a desirable replacement and reduction of the use of animals in research and routine diagnostics²³. As expression of the BP180 protein differs in the different anatomical locations of human skin and mucous membranes^{20,24}, use of NHS from specific sites for diagnostic purposes may be advisable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What's already known about this topic?

- BP180-NC16A is the immunodominant epitope in patients with bullous pemphigoid (BP), and anti-BP180-NC16A autoantibody titers correlate with disease activity.
- Monkey esophagus (ME) substrate is the most widely used substrate for screening, diagnosis and follow-up of BP patients in dermatological routine labs.
- Published data indicates rather low sensitivity of ME substrate in immunofluorescence testing for BP.

What does this study add?

- Use of normal human skin (NHS) is required to reliably detect anti-BP180-NC16A abs in immunofluorescence testing of human sera.
- The human BP180-NC16A domain is poorly, if at all expressed in ME.

Figure 1:

Affinity purification (AP) of BP180-NC16A and BP230-C specific antibodies from two representative bullous pemphigoid sera. Eluates from BP180-NC16A-APs (**A**, **C**) and BP230-C-APs (**B**, **D**) demonstrated monospecific reactivity against the BP230-C or BP180-NC16A antigens, showing good effectivity of our AP procedure.

Figure 2:

Indirect immunofluorescence (IIF) testing of affinity-purified serum antibodies on normal human skin and monkey esophagus substrates. By IIF microscopy, affinity-purified BP230-C-specific polyclonal antibodies (abs) showed binding along the basement membrane zone (BMZ) of sections of both normal human skin (NHS) (**A**) and monkey esophagus (ME) (**B**) substrates; arrowheads point to binding of abs at BMZ. In contrast, BP180-NC16A affinity-purified antibodies stained only on NHS (**C**), but not on ME (**D**) substrate; arrowheads point to binding of abs at BMZ. The observed binding patterns on NHS and ME were confirmed by double IIF with affinity-purified polyclonal IgG and a monoclonal scFv (Supplemental Fig. 2).

Figure 3:

Monoclonal antibodies confirm findings from affinity-purified polyclonal antibody testing by indirect immunofluorescence. Antibody phage display-derived scFv monoclonal antibodies (mAbs) have a hemagglutinin (HA)-tag and were confirmed for binding specificity by anti-HA ELISA on BP230-C and BP180-NC16A substrates (A). Deposition of two anti-BP230-C mAbs (BP230-C-mAb1 and BP230-C-mAb2) was observed on NHS and ME substrates (B). On the contrary, IIF on ME substrate was completely negative for both anti-NC16A mAbs tested (BP180-NC16A-mAb1 and BP180-NC16A-mAb2) (C), but positive for NHS. All mAbs were adjusted to the same concentration of 1 $\mu\text{g}/\mu\text{L}$ before preparing shown dilution series.

Table 1

Twenty nine BP patients from the Shanghai Department of Dermatology at the Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, were assayed by routine ELISA testing and by indirect immunofluorescence (IIF) on monkey esophagus (ME) and normal human skin (NHS). Titers tested by IIF testing on ME and NHS were 1:100 for all sera and, additionally, 1:10 for cases with negative IIF readings at 1:100.

ID	Sex	Age	BP180-NC16A ELISA	BP230-C/N ELISA	ME IIF	NHS IIF
BP01	M	91	106.5	107.5	+	+
BP02	M	79	199.6	119.0	+	+
BP03	F	81	164.2	154.0	+	+
BP04	M	79	115.6	93.6	+	+
BP05	M	75	116.6	97.6	+	+
BP06	M	89	164.1	75.1	+	+
BP07	F	91	171.5	114.1	+	+
BP08	F	84	194.7	109.2	+	+
BP09	M	86	124.7	123.9	+	+
BP10	F	68	182.0	95.2	+	+
BP11	M	82	4.8	114.5	+	+
BP12	F	91	5.5	93.2	+	+
BP13	F	67	2.9	41.6	+	+
BP14	F	74	5.0	72.9	+	+
BP15	M	78	0.2	36.9	+	+
BP16	F	68	2.6	88.4	+	+
BP17	F	61	8.3	127.8	+	+
BP18	M	71	239.9	5.1	–	+
BP19	M	29	172.8	4.7	–	+
BP20	F	79	84.7	1.4	–	+
BP21	M	84	107.1	6.8	–	+
BP22	M	74	106.9	4.2	–	+
BP23	F	86	153.6	7.2	–	+
BP24	M	60	151.3	8.6	–	+
BP25	F	37	116.6	1.8	–	+
BP26	M	62	128.4	4.4	–	+
BP27	M	77	37.0	3.7	–	+
BP28	M	79	109.2	5.6	+	+
BP29	M	51	153.1	4.8	+	+

Table 2

Genetic comparison of human BP180, its NC16A domain, and BP230, over different species.

	Human BP180-NC16A fragment (73 aa)	Human BP180 (Collagen XVII, alpha 1; 1497 aa)	Human BP230 (BPAG1e; 2649 aa) *
<i>Homo sapiens</i>	100%	100%	100%
<i>Pongo abelii</i>	97%	97%	99%
<i>G. g. gorilla</i>	97%	98%	99%
<i>Rhinopithecus bieti</i>	71%	95%	98%
<i>Macaca mulatta</i>	71%	91%	99%
<i>Canis lupus familiaris</i>	56%	82%	95%
<i>Mus musculus</i>	55%	77%	99%
<i>Cavia porcellus</i>	48%	82%	92%

* NCBI Reference Sequence NP_001714.1