Male-Specific Submaxillary Gland Protein, a Lipocalin Allergen of the Golden Hamster, Differs from the Lipocalin Allergens of Siberian and Roborovski Dwarf Hamsters

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Salivary gland · Male-specific submaxillary gland protein

Abstract

Background: An increasing number of asthma cases upon exposure to hamsters and anaphylactic reactions following hamster bites are being reported, but the allergens responsible are still poorly characterized. In the Golden hamster, male-specific submaxillary gland protein (MSP), a lipocalin expressed in a sex- and tissue-specific manner in the submaxillary and lacrimal glands, is secreted in the saliva, tears and urine. The purpose of this study was to determine if MSP is an allergen, to identify IgE-reactive proteins of different hamster species and to analyse potential cross-reactivities. **Methods:** Fur extracts were prepared from four hamster species. Hamster-allergic patients were selected based on a history of positive IgE-test to hamster epithelium. The IgE-reactivity of patients' sera was investigated by means of immunoblot and ELISA. IgE-reactive proteins in fur extracts and

the submaxillary gland were identified using anti-MSP antibodies, Edman sequencing or mass spectrometry. MSP was purified from Golden hamster and recombinant MSP was expressed in *E. coli*. **Results:** Four patients had IgE-antibodies against 20.5-kDa and 24-kDa proteins of Golden hamster fur extract, which were identified as MSP. IgE-reactive MSP-like proteins were detected in European hamster fur extract. Three patient sera showed IgE-reactive bands at 17–21 kDa in Siberian and Roborovski hamster fur extracts. These proteins were identified as two closely related lipocalins. Immunoblot inhibition experiments showed that they are cross-reactive and are different from MSP. **Conclusion:** MSP lipocalin of the Golden hamster was identified as an allergen, and it is different from the cross-reactive lipocalin allergens of Siberian and Roborovski hamsters. Our findings highlight the need for specific tools for the in vitro and in vivo diagnosis of allergy to different hamster species.

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Introduction

Apart from cats and dogs, which represent the most frequent animals to be kept as pets in industrialized countries, a number of other mammalian furry pets have become increasingly popular. They are called 'exotic pets' or 'new companion animals' and include rodents (hamsters, guinea-pigs, chinchillas, gerbils, mice and rats), mustelids (ferrets), leporids (rabbits) or even miniature pigs. In parallel to the emergence of these new pets, clinical cases were published in the literature reporting on allergic symptoms caused by these animals [1]. It is difficult to evaluate the incidence of these allergies. Indeed, the prevalence of exotic pets in households is not well known, the molecules implicated in allergic sensitization are mostly not characterized and purified allergen components are not commercially available for diagnosis. The use of animal hair or epithelial extracts does not allow for distinguishing reliably between different animals due to cross-reactive molecules which generate false-positive results. On the other hand, poor standardization of extracts can give false-negative results.

The three most popular pet hamster species are the Golden or Syrian hamster (*Mesocricetus auratus*) and the two dwarf hamsters, the Siberian or Djungarian hamster (*Phodopus sungorus*) and the Roborovski hamster (*Phodopus roborovskii*). The European, common or black-bellied hamster (*Cricetus cricetus*) is less popular as a pet. The Golden hamster is the main species used in animal research experiments. A number of studies have reported bronchial asthma upon exposure to hamsters as well as the occurrence of anaphylactic reactions after hamster bites [2–10]. Most cases are related to Siberian hamsters; only 1 study reported the occurrence of symptoms with other hamster species, namely the Roborovski, Golden and European hamster [4]. It appears, however, that the allergens originating from these four species are not identical. Indeed, patients allergic to the Siberian hamster did not react to commercial skin-prick test (SPT) preparations made from the European or the Golden hamster [2, 3, 5, 7, 8, 10].

A recent report of anaphylaxis induced by the bite of a Siberian hamster [7] described an allergen in the fur, salivary glands and urine, which seems to exist as 3 isoforms of 18, 21 and 23 kDa. So far, no allergens have been described for the Golden or Roborovski hamsters. In the latter, a lipocalin called roborovskin has been identified in the urine [11], but its distribution in the body fur, its tissue of origin and whether it might be an allergen have not been investigated. In the Golden hamster, a male-spe-

cific submaxillary gland protein (MSP) has been identified, which is secreted in the saliva and excreted in the urine of males. MSP, which exists in glycosylated as well as non-glycosylated forms, is a well-characterized lipocalin, having the closest identity with the odorant-binding lipocalins [12–15]. Low levels of MSP are also expressed female-specifically in the lacrimal glands and secreted in the tears of females [14]. The presence of MSP in the body fur of Golden hamsters and whether it is an allergen have not been investigated.

Most allergenic proteins of animal origin are members of the lipocalin family, sharing low-to-modest sequence identities but with a highly conserved tridimensional structure [16]. The aim of this work was to study whether the lipocalin MSP is implicated in allergy to the Golden hamster and to identify IgE-reactive allergens in Siberian and Roborovski dwarf hamsters and analyse potential cross-reactivities.

Material and Methods

Patients and Sera

Patients were selected from the outpatient population of the National Unit of Immunology and Allergology and the Unit of Pneumology of the Centre Hospitalier in Luxembourg, based on a history of specific IgE to hamster epithelium e84 (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden). The source material for the ImmunoCAP is a mixture of epithelia from 3 hamsters: European, Golden and Siberian. Six sera were found to be positive and 4 patients could be re-contacted for further clinical assessment and SPT. The study was approved by the National Committee for Medical Research Ethics.

Patient 1 (P1) had kept a male hamster of unknown species for 2 years, and she had asthma and rhinoconjunctivitis upon contact with it (table 1). Patient 2 (P2) had two female dwarf hamsters of unknown species for 1 and 2 years, respectively. She also had asthma and rhinoconjunctivitis upon contact with her hamsters. Patient 3 (P3) was lost from follow-up. Patient 4 (P4) experienced wheezing, conjunctival pruritus, labial and palpebral oedema a few minutes after kissing her Golden hamster, and subsequently presented with pruritus, rhinitis and allergic conjunctivitis upon contact with her pet. Patient 5 (P5) had asthma and rhinitis upon contact with his hamster; the symptoms started about 10 months after the acquisition of the pet. Patient 6 (P6) had perennial asthma and rhinoconjunctivitis. He had a cat at home and had had a hamster of unknown species in childhood (at 10–14 years of age), but without presenting any clearly hamster-related allergic symptoms. At the time of follow-up for SPT and additional investigations, none of the 4 patients had a hamster at home.

Sera from 16 cat- and/or dog-allergic and 10 guinea-pig-allergic patients were collected for control purposes. The selection was based on the presence of specific IgE to cat dander e1 or dog dander e5 (ImmunoCAP) in the case of cat/dog-allergic patients (range 1.0 to >100 kUA/l to cat dander, mean 21.1; range 0.4 to 29.5 kUA/l to dog dander, mean 6.1). The sera of guinea-pig-allergic patients

Table 1. Patient data

	P1	P2	P3	P4	P5	P6
Sex, male/female; age, years	female; 42	female; 44	male; 52	female; 51	male; 23	male; 36
Symptoms	asthma, rhinoconjunctivitis	asthma, rhinoconjunctivitis	n.d.	asthma, rhinitis, conjunctivitis, palpebral oedema	asthma, rhinitis	no symptoms at the time of exposure
Pet hamster	male, unknown species	2 female dwarf species	n.d.	Golden hamster of unknown sex	unknown	unknown
IgE hamster epithelium, kUA/l	5.6	11.2	16	0.45	4.6	17
SPT, mm						
Golden hamster (Stallergenes)	6 × 5 (1,800 µg/ml)	5 × 4	n.t.	5.5 × 6	negative	25 × 15
European hamster (ALK)	8 × 6 (300 µg/ml)	4 × 3.5	n.t.	7 × 4.5	n.t.	8 × 12
Siberian hamster	5 × 4 (10 µg/ml)	4 × 2.5 (1 µg/ml)	n.t.	negative (10 µg/ml)	n.t.	negative (10 µg/ml)
Roborovski hamster	9 × 5 (10 µg/ml)	5 × 5.5 (10 µg/ml)	n.t.	negative (10 µg/ml)	n.t.	5 (10 µg/ml)
nMSP	n.t.	negative (10 µg/ml)	n.t.	4.5 × 3 (0.1 µg/ml)	n.t.	7 × 6 (1 µg/ml)
Allergies to other animals	rabbit, guinea-pig, cat, dog, horse	cat, dog	n.d.	cat	n.d.	cat, dog

n.d. = No data; n.t. = not tested.

were selected on the basis of positive specific IgE to guinea-pig epithelium e6 (ImmunoCAP; range 5.9–41 kUA/l, mean 18.1). Twenty-two additional sera were collected from mite and/or pollen-allergic patients with no history of allergy to furred animals.

Protein Extractions

The body fur of male Golden hamsters (bred and maintained at the Centre for Cellular and Molecular Biology animal house) was shaved using an electric razor, stirred with autoclaved Milli-Q water for 1 h and the extract was collected after centrifugation. The submaxillary gland (SMG) extracts of male Golden hamsters were prepared by homogenization (2.5% w/v) in chilled 20 mM Tris-HCl (pH 7.4) and centrifugation at 28,000 g for 30 min at 4°C as described [12, 15]. Golden hamsters were handled according to the Centre's Institutional Animal Experimentation Ethics guidelines.

Adult male (Siberian and Roborovski) dwarf hamsters were obtained from a local breeder. Their fur was cut with scissors and the proteins were extracted under rotation overnight with phosphate-buffered saline (PBS) containing protease inhibitors (protease inhibitor cocktail tablets, Roche, Mannheim; Germany). The SMGs were harvested immediately after sacrifice and frozen at –20°C. They were disrupted with beads (TissueLyser II, Qiagen, Hilden, Germany) and extracted in buffer containing 50 mM TrisHCl (pH 8.0), 150 mM NaCl and protease inhibitors. The animals were handled according to the European guidelines for experimental animals. European hamster hair and skin scrape were obtained from Allergon (Ängelholm, Sweden).

Production of Native and Recombinant MSP

Natural MSP (nMSP) lipocalins present in adult male Golden hamster SMG extracts (as 24-kDa, 20.5-kDa and 30-kDa forms, which differ in the presence or absence of N-glycosylation [13–

15]) were purified employing a rapid procedure. The proteins precipitated from the extract within an 85–100% (NH₄)₂SO₄ saturation were collected by centrifugation, dissolved in 20 mM Tris-HCl containing 150 mM NaCl (pH 7.4) and resolved in a gel-filtration column (1.6 × 95 cm; Sephadex-G75 superfine; GE Healthcare, Buckinghamshire, UK) equilibrated with the same buffer. Eluted fractions were checked in SDS-PAGE and if required, by Western blot using anti-MSP antibodies. Selected fractions of the most prominent and broad protein peak of gel-filtration, in which the 3 MSP forms elute (with considerable overlap but free of other proteins) were pooled. The final pool contained 24-kDa MSP (N-glycosylated) and 20.5-kDa MSP (non-glycosylated) as the major components, along with low levels of a 30-kDa, higher N-glycosylated form of MSP.

The cDNA encoding the 157 aa long, mature polypeptide of MSP was amplified by RT-PCR using total RNA isolated from the SMG of the male Golden hamster and specific primers based on the known cDNA sequence of MSP (AF183407.2) [13, 14]. The amplified cDNA of the mature MSP was cloned in pET21a vector at NdeI/XhoI sites, such that the sequence of the encoded recombinant MSP (rMSP) included a C-terminal 6X His-tag. BL21 (DE3) *Escherichia coli* cells transformed with the above plasmid construct were cultured, and the expression of rMSP (induced by the addition of isopropyl β-D-1-thiogalactopyranoside) and its purification from the cell lysate were performed exactly as described earlier [17]. Pure rMSP eluted as a monomer in a single peak (at approx. 20 kDa) in the final gel-filtration step.

SDS-PAGE, IgE-Immunoblotting and IgE-Inhibition

Proteins were separated by using 15% SDS-PAGE under reducing conditions and transferred to PVDF membranes (Millipore, Bedford, Mass., USA). Briefly, patient sera were diluted 1:3 or 1:6

in blocking buffer (Tris-buffered saline/0.05% Tween 20 and 3% bovine serum albumin; Sigma-Aldrich, Diegem, Belgium). Proteins blotted on the membrane were incubated overnight at room temperature with the diluted patient sera. Bound IgE-antibodies were detected with anti-human IgE-antibodies labelled with alkaline phosphatase (Southern Biotech, Birmingham, Ala., USA). Specific bands were visualized by colour development using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Promega, Leiden, The Netherlands). MSP was detected using polyclonal rabbit antibodies (IgG), which were purified by protein-A sepharose affinity from the crude anti-serum raised earlier against pure N-glycosylated 24 kDa natural MSP [13]. An anti-rabbit IgG antibody labelled with alkaline phosphatase (Sigma-Aldrich) was used as a secondary antibody for detection. Two-dimensional (2D)-gel electrophoresis was performed with immobilized pH gradient strips according to the manufacturer's instructions (Bio-Rad, Nazareth, Belgium). The proteins were checked for the presence of glycosylation using a commercial kit (glycoprotein staining kit, Pierce, Erembodegem, Belgium).

For IgE inhibition assays, patients' sera were diluted and incubated with 20 µg of different extracts or 10 µg of purified nMSP for 2 h at room temperature before being added to the membrane strips for the detection of IgE-binding proteins.

Detection of Specific IgE Antibodies by ELISA

Specific IgE to native MSP, recombinant MSP and hamster albumin (Sigma) were quantified by ELISA. Briefly, the proteins were coated overnight at a concentration of 5 µg/ml in PBS to microtitre plates (NUNC MaxiSorp™, Roskilde, Denmark). Unspecific binding sites were blocked by the addition of blocking buffer (3% BSA in PBS) for 30 min. Patient sera were diluted 1:3 or 1:6 in blocking buffer and added to the wells for 2 h. Between each step, the plates were washed with washing buffer (Tris-buffered saline/0.05% Tween-20). The binding of specific IgE was measured by addition of biotin-labeled anti-human IgE (Southern Biotech) for 1 h, followed by alkaline-phosphatase-labeled streptavidin (BD Biosciences, Erembodegem, Belgium) for 30 min. Plates were developed by addition of para-nitrophenyl phosphate (p-NPP) (Sigma) and optical density was read at 405 nm. A series of 22 sera from patients allergic to mites and/or pollen were used as negative controls. The quantification of specific IgE to hamster fur extracts was performed as described above, except that 8 µg of protein/ml were coated to the microtitre plate.

SPT with Extracts and Native Allergens

Commercial SPT solutions were used from the following suppliers: ALK, Horsholm, Denmark containing *C. cricetus* (European hamster) and Stallergenes, Antony, France, containing *M. auratus* (Golden hamster) hair extracts. The protein concentrations of the commercial SPT solutions were quantified by Bradford assay (Bio-Rad). Hair (fur) extracts from the two dwarf hamsters *P. sungorus* (Siberian hamster) and *P. roborovskii* (Roborovski hamster) prepared in the laboratory were sterilized by filtration and diluted in human serum albumin diluent (ALK) with the following concentrations of 0.1, 1, 10 and 50 µg/ml. nMSP was diluted in the diluent to 0.1, 1 and 10 µg/ml. The diluent was used as negative control, histamine chloride (10 mg/ml) was used as a positive control and skin reactions were read after 20 min. Prick tests yielding a papule ≥3 mm above the negative control were rated positive.

Protein Sequencing and Mass Spectrometry

For N-terminal sequencing, proteins were separated by SDS-PAGE and transferred onto PVDF membranes by semi-dry transfer using CAPS-buffer [3-(cyclohexylamino)-1-propanesulfonic acid, CAPS 10 mM, methanol 20%]. Purified proteins were subjected to N-terminal sequencing by automated Edman degradation on a PROCISE 49X HT protein sequencer (Applied Biosystems, Foster City, Calif., USA). For analysis by mass spectrometry, IgE-reactive spots were cut out from 2D-gels stained with Gelcode Blue (Pierce, Erembodegem, Belgium).

For mass spectrometry analyses, the isolated spots were digested in gel with trypsin (sequencing grade, Roche). Digested peptides were analysed as described [18] on a MALDI TOF/TOF instrument (Ultraflex I, Bruker Daltonics, Bremen, Germany). Proteins were identified by comparison of the mass lists of their peptide mass fingerprints with the NCBI database and the *in silico* digestion of the corresponding sequences (peptide mass, ExPASy, Swiss Institute of Bioinformatics, Lausanne, Switzerland). Some unknown digested peptides were *de novo* sequenced on a second MALDI TOF/TOF instrument (AB Sciex 5800, Framingham, Mass., USA) to extend the sequence coverage and improve the characterization of the isoforms.

Results

IgE-Reactive Proteins in Fur Extracts from Four Hamster Species

The aqueous body-fur/hair extracts of four hamster species (Golden, European, Siberian and Roborovski) were separated by SDS-PAGE and immunoblotted with sera from 6 patients (fig. 1) who had been found positive to hamster epithelium (e84) with ImmunoCAP. Sera from P2, P3, P4 and P6 but not from P1 and P5 detected IgE-reactive proteins at approx. 20 kDa and 24 kDa in Golden hamster fur (fig. 1a). Similar bands were visible in the European hamster fur extract with sera from P3 and P4 (fig. 1b).

In the Siberian hamster fur extract, the sera from P1, P2 and P5 detected major IgE-reactive bands in the 17- to 21-kDa region (fig. 1c). These IgE-reactive bands are very similar to those described by Torres et al. [7] in Siberian hamster fur extract. The sera of the same 3 patients also detected an IgE-reactive band in Roborovski hamster fur at 21 kDa (fig. 1d) as well as low-molecular-weight bands of about 6 kDa in the Siberian and Roborovski hamster fur extracts. The serum of P2 was strongly reactive to Siberian and Roborovski hamster fur, but it also showed reactivity to Golden hamster fur.

The fact that P3 and P4 reacted only to the Golden and European hamsters in the IgE immunoblots, but not to the two dwarf hamster species, suggests that the allergens of the Golden and European hamsters might be different from those of the Siberian or Roborovski hamsters.

Fig. 1. IgE-reactive proteins in fur extracts from four hamster species. **a–d** Proteins were separated by SDS-PAGE under denaturing conditions and analysed on IgE immunoblot using sera from 6 patients allergic to hamsters. MSP was detected by polyclonal rabbit antibodies. Numbered lanes correspond to the sera of P1–P6. N = Negative control; R = rabbit anti-MSP antibody.

Detection of MSP by Rabbit Anti-MSP Antibodies

MSP lipocalin is known to be present in the SMG, saliva and urine of male Golden hamsters as the N-glycosylated 24-kDa and non-glycosylated 20.5-kDa major forms, along with a minor, higher N-glycosylated form of approximately 30 kDa [13–15]. As the IgE-reactive bands detected in Golden hamster fur corresponded to the molecular weight expected for MSP, polyclonal rabbit antibodies raised against MSP were used to detect MSP in the different fur extracts (fig. 1a–d, lane R). Major protein bands were detected in the Golden hamster fur extracts at 20 kDa and 24 kDa as well as a minor band at 30 kDa. Similar potentially cross-reactive proteins were recognized at 20 kDa and 24 kDa in the European hamster fur extracts. No protein bands were detected in the fur extracts of the two dwarf hamsters.

The immunoblots using MSP antibodies suggest that the 20-kDa and 24-kDa IgE-reactive allergens in Golden hamster fur are likely to be MSP lipocalins, and the similar molecular-weight IgE-reactive proteins detected in

European hamster fur are closely related to MSP lipocalins. Overall, the results showed that the IgE-reactive MSP/MSP-like allergens are present in the fur of Golden and European hamsters, and that their epitopes are different from the IgE-reactive 21- to 17-kDa allergens present in the fur of Siberian and Roborovski hamsters.

Immunoblot Inhibition Studies

In order to confirm that MSP indeed corresponds to the IgE-reactive protein in the fur of the Golden hamster, a series of immunoblot inhibition experiments were carried out on blotted Golden hamster fur proteins (fig. 2a). The binding of IgE antibodies in P4, who had a Golden hamster, was completely inhibited by pre-incubation with purified native MSP proteins, confirming that the 24/20-kDa IgE-reactive proteins share common IgE-reactive epitopes with MSP. However, pre-incubation with Siberian or Roborovski hamster fur extracts did not significantly affect the binding.

Fig. 2. IgE immunoblot inhibition experiments. Golden, Siberian and Roborovski hamster fur extracts were separated by SDS-PAGE under denaturing conditions and analysed by IgE immunoblot using 2 patients sera. **a** Serum of P4, who had a Golden hamster, was preincubated without inhibitor (lane –) or with 20 µg of Golden hamster fur extract (lane G), with 10 µg of nMSP (lane M), with 20 µg of Siberian (lane S) or Roborovski (lane R) hamster fur extract. **b, c** Serum of P2, who had a dwarf hamster, was pre-incubated without inhibitor (lane –) or with 20 µg of Golden hamster fur extract (lane G), with 20 µg of Siberian (lane S) or Roborovski (lane R) hamster fur extract.

Fig. 3. SDS-PAGE profile of purified MSP proteins and IgE binding to purified hamster proteins by ELISA. **a** Coomassie-stained SDS-PAGE profile of male Golden hamster SMG extract and purified rMSP and nMSP proteins. Purified nMSP shown is a mixture

of the 24-kDa (glycosylated), 20.5-kDa (non-glycosylated) and 30-kDa (glycosylated) forms [13]. **b** IgE-binding to rMSP, nMSP and serum albumin was assayed by ELISA using the sera of 6 hamster-allergic patients, P1–P6.

IgE-reactivity to fur extracts of Siberian and Roborovski hamsters was further investigated by inhibition and cross-inhibition experiments (fig. 2b, c) using the serum of P2 who had a dwarf hamster. The reactivity of P2's serum to Siberian hamster fur extract was not affected by pre-incubation with the Golden hamster fur extract, but IgE-binding was completely inhibited by the addition of Siberian or Roborovski hamster fur extract (fig. 2b). In the reverse experiment (fig. 2c), pre-incubation with Siberian hamster fur extract significantly re-

duced IgE-binding to the Roborovski extract, but could not completely inhibit it. Complete inhibition was seen after pre-incubation with the Roborovski fur extract. Pre-incubation with Golden hamster fur extract had no effect.

MSP Is Specifically Recognized by IgE-Antibodies from Hamster-Allergic Patients

All 6 hamster-sensitized patients were tested for IgE-reactivity to nMSP, rMSP and hamster serum albumin by ELISA. Figure 3a shows nMSP purified from SMG con-

Fig. 4. Detection of IgE reactivity to hamster fur proteins by ELISA. IgE-binding to native proteins extracted from fur of Golden, Siberian and Roborovski hamster was assayed by ELISA using the sera of P1–P6.

taining glycosylated (24 and 30 kDa) and non-glycosylated (20.5-kDa) forms [13–15] and rMSP purified after expression in *E. coli*. The sera of all patients except P1 reacted with recombinant and native MSP (fig. 3b). P2 and P6 had also IgE to hamster serum albumin. Twenty-two patients without allergy to animal dander, but with allergies to mite and/or pollen did not show any reactivity to the nMSP or rMSP allergens. In addition, 16 cat- and dog-allergic patient sera as well as 10 guinea-pig-allergic patient sera were tested for potential cross-reactivity with MSP, but did not show any IgE-reactivity (data not shown). The MSP lipocalin sequence (GI:19881387) was aligned to known mammalian lipocalin allergens. The most closely related lipocalin allergen was found to be Cav p 3 (42% identity), followed by Cav p 2 (37%), while Can f 6, Can f 4, Bos d 2, Equ c 1 and Fel d 4 had 34–30% identity.

SPT Reactivity to MSP and Fur Extracts from Four Different Hamster Species

SPTs were performed with the fur extracts of four hamster species: commercial extracts from Golden and European hamster and extracts prepared in-house from the fur of Siberian and Roborovski hamsters (table 1). P1 and P2 reacted strongly with all 4 extracts. P4 who had a Golden hamster reacted only to the Golden and European hamster extracts. She also reacted to nMSP at 0.1 µg/ml. P6 reacted strongly to the Golden and European hamster extracts as well as to native MSP at 1 µg/ml. The reactivity of P1 to the extracts of the Golden and European hamsters is

at variance with the IgE immunoblot experiment. The reactivity of P2 to European hamster fur might be explained by her sensitization to serum albumin, which is present in this extract. Of note, when we determined the protein concentrations of the commercial SPT solutions, they were found to be 30–180 times higher than the concentrations of the in-house extracts which produced a positive reaction.

Detection of IgE Reactivity to Hamster Fur Proteins by ELISA

The immunoblot experiments analysed patients' reactivity to denatured proteins whereas in the SPTs, patients reacted to native proteins. In order to analyse specific IgE to native hamster fur proteins, all patient sera were tested by ELISA using protein extracts obtained from the fur of Golden, Siberian and Roborovski hamsters (fig. 4). Sera from P1 and P5, which were negative to Golden hamster fur on immunoblot, showed a weak reaction to Golden hamster fur on ELISA. The serum from P4 reacted exclusively to Golden hamster fur. This patient did not react in the SPT to the fur extracts of the 2 types of dwarf hamster. The different results obtained by ELISA and immunoblot for P1 and P5 may be explained either by the recognition of structural epitopes, which are denatured in immunoblot and/or by a higher sensitivity in ELISA.

Characterization of IgE-Reactive Proteins from the Siberian and Roborovski Dwarf Hamsters

IgE-reactive bands in fur extracts from Siberian and Roborovski hamsters were analysed by N-terminal sequencing. The 21-kDa bands gave the following result: NDYAELEG for the Siberian hamster and NDYAELEGXDTIA for the Roborovski hamster (X being an unidentified amino acid).

The quality of the obtained sequences was poor due to the low abundance of the proteins in the fur extracts. The MSP allergen, which we found in the fur extracts of the Golden hamster, is known to be abundantly expressed in SMG and is secreted in the saliva [12–15]. We therefore decided to screen the SMGs of Siberian and Roborovski hamsters for the presence of IgE-reactive proteins similar to those detected in their fur extracts. Allergens of molecular weight similar to the ones detected in the fur could be found in the SMGs of both Siberian and Roborovski hamsters (fig. 5a, c). A 2D-gel electrophoresis analysis followed by IgE immunoblot showed that the IgE-reactive bands of both species dissociated into several spots at different acidic isoelectric points (fig. 5b, d).

These IgE-reactive spots were further analysed by N-terminal sequencing and in-gel trypsin digestion and

Fig. 5. Detection and analysis of IgE-reactive proteins in the SMG extract of Siberian and Roborovski hamsters. SMG proteins (20 µg) were separated by SDS-PAGE under denaturing conditions and immunoblotted using the serum of P2. **a, b** Siberian hamster. **c, d** Roborovski hamster. N = Negative control; P = patient serum. SMG proteins (250 µg) were separated by 2D-gel electrophoresis and stained with Coomassie Blue. Arrows and numbers designate the spots analysed by MS/MS.

mass spectrometry. Figure 6 shows the amino acid sequences obtained for these spots. No sequence information was obtained for spot 1 (Siberian hamster) as its N-terminal was found to be blocked and it was resistant to enzymatic digestion. The sequence of the N-terminal region obtained for spot 2 (21 kDa), spot 3 (19 kDa) and spot 4 (17 kDa) of the Siberian hamster were identical, except for one mismatching residue at the 26th position (G) in spot 4. Earlier, Torres et al. [7] had sequenced only the 8 amino acids of the N-terminal stretch of 23- and 21-kDa IgE-reactive proteins in the fur, urine and saliva of the Siberian hamster. The sequence (NDYAELEG) obtained by them is completely included in our sequenced N-terminal stretch of spots 2 and 3 of Siberian hamster SMG. The peptide mass fingerprints of the trypsin-digested IgE-reactive spots of the Siberian hamster showed that one of the sequences (spot 2) is identical to the protein sequence deduced from a cDNA of a putative odorant-binding lipocalin (Pho s 21 kDa; GI:530376029),

which was cloned recently from the Siberian hamster SMG by Torres et al. [19]. Two of the internal peptide sequences determined by Torres et al. [7] are found within the sequence of spot 4. The few mismatches between the sequences of spots 3 and 4 and their mismatches with that of spot 2 (GI:530376029) indicate the presence of at least 3 isoforms of IgE-reactive allergens in the Siberian hamster.

For the Roborovski hamster, the 36 N-terminal amino acids of the 21-kDa IgE-reactive SMG protein (in 1D SDS-PAGE) were determined as NDYAELEGN/KWDTIAIAADNND/AKIKEEGPM/LRLYVREL/H. This N-terminal sequence was highly similar, but not identical to the sequence of roborovskin, a lipocalin protein earlier identified in the urine of Roborovski hamsters, the sequence of which was also determined by Edman degradation and mass spectrometry [11].

Analysis of the IgE-reactive spots obtained by 2D-gel electrophoresis with the Roborovski SMG extract iden-

Siberian hamster		
Ph sung GI:530376029	NDYAELEGKWDITIAAADNDAKIKEEGPLRLYVRELYCNCSEMEVTFYVNANNQCSKTTVIGYKQADGTYRTQFEGDN	80
Ph sung spot 2	NDYAELEGKWDITIAAADNDAKIKEEGPLR...ELYCNCSEMEVTFYVNANNQCSKTTVIGYKQADGTYRTQFEGDN	
Ph sung spot 3	NDYAELEGKWDITIAAADNDAKIKEEGPLR.....QADGTYRTQFEGDN	
Ph sung spot 4	WDITIAAADNDAKIKEEGPLR.....QADGTYRTQFEGDN	
Ph sung GI:530376029	RFQPVYATPENIVFTSKNVDRAGQETNLIFVVGKSQPLTPEQHEKLVFAHENNIPEENIHNVLATDTCPK	151
Ph sung spot 2	RFQPVYATPENIVFTSKNVDRAGQETNLIFVVGKSQPLTPEQHEKLVFAHENNIPEENIHNVLATDTCPK	
Ph sung spot 3	RFQPVYATPENIVFTSK...AGQETNLIFVVGKSQHLTPEQHEKLVFAHENNIPEENIHNVLATDTCPK	
Ph sung spot 4	RFQPVYATPENIVFTNK...AGQETNEMVVGKSQHLTPEQHEK.....NIPEENIHNVLATDTCPK	
Roborovski hamster		
Roborovskin	DNYAELQGEWDTLALAADNDKJKEEGPJRVYREJYCNCSEMEVTFYVNANNQCSKTEVTGYKQADGTYRKTTFEGDN	80
Ph robo spot 3	NDYAELEGKWDITIAAADNDAKIKEEGPLR.....QTFEGDN	
Ph robo spot 4	NDYAELEGKWDITIAAADNDAKIKEEGPLR.....ADNDKIKEEGPMR.....QTFEGDN	
	ADNDKIKEEGPMR.....QTFEGDN	
Ph robo spot 5	NDYAELEGKWDITIAAADNDAKIKEEGPMR.....QADGTYRTQFEGDN	
	NDYAELEGKWDITIAAADNDAKIKEEGPMR.....QADGTYRTQFEGDN	
Roborovskin	TFKPVYATPEQVFTSKNVDRAGQETNJJXXXXKSEHJTPEQHEKJVKAJHNNJPEENJHNVJATDTCPK	151
Ph robo spot 3	.FEPVYJTPENJFVTSK...AGQETNJJFVVGK..HJTPEQHEK.....QJPEENJHNVJATDTCPK	
	JFQPVYATPENJVFTR...AGQETNJJFVVGKSHJSTPEQHEK.....QJPEENJHNVJATDTCPK	
Ph robo spot 4	JFQPVYATPENJVFTR.....HJTPEQHEK.....QJPEENJHNVJATDTCPK	
	JFQPVYATPENJVFTR.....SHJTPEQHEK.....QJPEENJHNVJATDTCPK	
Ph robo spot 5	JFQPVYATPENJVFTRNVDRAGQETNJJFVVGK..HJTPEQHEK.....QJPEENJHNVJATDTCPK	
	JFQPVYATPENJVFTRNVDRAGQETNJJFVVGKSHJTPEQHEK.....QJPEENJHNVJATDTCPK	

Fig. 6. Analysis of protein sequences of IgE-reactive spots isolated from 2D-gel electrophoresis of Siberian and Roborovski hamster SMGs. The protein spots were subjected to Edman sequencing and mass spectrometry analysis. Translated cDNA (GI:530376029) of a Siberian hamster SMG lipocalin and the sequence of roborovskin, a lipocalin isolated from urine of Roborovski hamster [11] were used as references for the mass spectrometry analysis. Iso-baric leucine (L) and isoleucine (I) amino acids cannot be distinguished by mass spectrometry and are indicated by J in the roborovskin and peptide sequences of IgE-reactive spots 3–5, except for

in the N-terminal region where they were resolved by Edman degradation. L/I ambiguities within the peptide sequences obtained by mass spectrometry for IgE-reactive spots 2–4 of the Siberian hamster were tentatively resolved by deducting them from the translated cDNA sequence (GI:530376029) of the Siberian hamster. Peptides determined by Torres et al. [7] are underlined. Shaded amino acids indicate the differences between the sequences determined in this study and the reference sequences. X = Unresolved residue; dot = no sequence information.

tified peptides related to roborovskin and also confirmed the data obtained for the N-terminal sequence of the SMG 21-kDa IgE-reactive band resolved in 1D SDS-PAGE. Spots 1 and 2 were resistant to peptide mass fingerprints, but their N-terminal sequences determined by Edman degradation (data not shown) were identical to the N-terminal sequence determined above. The sequence information obtained for spots 3–5 of the IgE-reactive 21-kDa SMG protein of the Roborovski hamster indicated the presence of heterogeneity (mismatches among the spots). Thus, several sequence isoforms also exist in the Roborovski hamster, explaining the ambiguous sequence obtained from Edman degradation of the 21-kDa IgE-reactive band of SMG extract and also

its heterogenous migration pattern in 2D SDS-PAGE. The isoforms of Roborovski and Siberian hamster allergens do not seem to be glycosylated; they were negative to periodic acid-Schiff stain for glycoprotein (data not shown).

The results of sequencing and mass spectrometry analysis of the IgE-reactive proteins of Siberian and Roborovski hamsters indicated that they are closely related lipocalins with a sequence identity between sequenced stretches of approximately 90%, and they exist as multiple isoforms. In amino acid sequence alignments, Pho s 21 kDa of the Siberian hamster shows some identity with MSP and Cav p 3 (41%), followed by Cav p 2 (39%), Bos d 2 (34%) and Can f 4, Fel d 4 and Equ c 1 (29–25%).

Discussion

Several hamster species are commonly kept as domestic pets. They all belong to the family Cricetinae, but are classified into different genera which form individual clades. The Golden hamster, of the *Mesocricetus* genus, and the dwarf hamsters of the *Phodopus* genus separated about 13 million years ago [20]. The 2N chromosome numbers of different hamsters vary substantially: the Golden has 44, the European 22, the Siberian 28 and the Roborovski 34 chromosomes [21]. There are other marked differences between these four species, the most prominent being their size, with the European hamster being the largest. It has been described that patients allergic to the Siberian hamster did not react to commercial SPT solutions [2]; such solutions are only available from the Golden and European hamsters.

In this study, we analysed the IgE-reactivity to the fur extracts of four hamster species in order to determine the allergens implicated in allergic reactions to the Golden hamster and define potential cross-reactivity between the allergenic molecules of different hamster species. MSP lipocalin of the Golden hamster, known to be present in the SMG and lacrimal glands, saliva, tears and urine, has now also been detected in body fur. At present, it is unclear whether the MSP in fur is locally synthesized in the glands or cells of the skin or if its presence in the fur is due to contamination by body fluids like saliva and urine which contain MSP [12–15]. Purified native and recombinant MSP were recognized in ELISA by IgE-antibodies in sera from 5 hamster-allergic patients. P4, who was exposed to a Golden hamster, reacted strongly to native MSP in the SPT. MSP seems to be a specific allergen indicative of hamster allergy because it is not recognized by sera from cat-, dog- or guinea-pig-allergic patients. Moreover, MSP-like allergens were also detected in the fur of European hamsters. Sequence identities of MSP to lipocalins of other furry pets are relatively low (max. 42%), and a general cross-reactivity seems very unlikely, although the existence of patient-dependent cross-reactive epitopes cannot be excluded.

IgE immunoblot inhibition studies were performed using sera of 2 patients, 1 sensitized to the Golden hamster and the other to dwarf hamsters. The results confirmed that MSP is an allergen and that it lacks cross-reactivity with the allergens in the fur of Siberian and Roborovski hamsters. This also confirmed the previous reports of lack of cross-reactivity between allergens of Golden and Siberian hamsters [2, 3, 5, 7]. However, we noted a strong IgE cross-reactivity between the fur allergens of the Siberian and Roborovski hamster.

For the Siberian hamster, an allergen had been partially described by Torres et al. [7], who determined the N-terminal sequence for 8 residues as well as 2 short internal peptide sequences for 23- and 21-kDa IgE-reactive proteins, which they detected in the fur, saliva and urine of the Siberian hamster. The N-terminal sequence could not be obtained for an 18-kDa IgE-reactive protein from the same sources, but its internal peptides were identical to those of the 23-kDa and 21-kDa IgE-binding proteins. The authors predicted the existence of 3 isoforms of this allergen, which was classified as a lipocalin based on the sequence information of only 3 peptides. Recently, the same authors cloned a cDNA from the SMG of the Siberian hamster. The encoded lipocalin contains the N-terminal peptide, but shows 4 amino acid deviations from the internal peptides determined previously [7]. The protein, Pho s 21 kDa, has been expressed in *E. coli*, and the binding of IgE-antibodies from 13 patients allergic to Siberian hamster could be demonstrated [19]. Our data confirm the expression of the postulated cDNA at protein level and, based on our extensive sequence data, provide additional evidence for the existence of at least 2 more isoforms, 1 containing the peptides initially described by Torres et al. [7].

The allergens from the Roborovski hamster, another popular pet hamster, were previously unknown. Our analysis of a 21-kDa IgE-reactive protein found in the fur and also abundantly in the SMG of the Roborovski hamster resulted in the identification of this allergen protein as roborovskin, which is a lipocalin, previously reported by Turton et al. [11] in the urine of Roborovski hamsters of both sexes. Our 2D-gel electrophoresis analysis of this IgE-reactive 21-kDa lipocalin allergen showed that it is comprised of several isoforms, resolving as different spots at different acidic isoelectric points. Moreover, we found that the protein sequence of different 2D-gel electrophoresis spots had slight mismatches with each other and also with the sequence of roborovskin obtained in Turton et al. [11]. These differences might be explained by several factors: in their study, Turton et al. [11] purified roborovskin from urine using anion exchange chromatography for MS and MS/MS analysis. Although their source material was different, it is possible that the ion-exchange chromatography resulted in purification of a single isoform and elimination of other variants of roborovskin. Overall, our data show evidence for the existence of at least 3 isoforms of roborovskin, and confirm them to be allergens. Presently, it is unclear whether the observed differences in migration in 2D-gel electrophoresis of the lipocalin allergen isoforms of Siberian and Roborovski

hamsters are only due to protein sequence heterogeneity or also to other post-translational modifications. The different isoforms do not seem to be glycosylated since they were negative to glycoprotein staining.

In conclusion, MSP, a tissue- and sex-specifically expressed lipocalin now found to be also present in body fur, is a major allergen of the Golden hamster. It shares only about 40% amino acid sequence identity with the major lipocalin allergens of the Siberian and Roborovski hamsters. On the other hand, the amino acid sequences of the lipocalin allergens from Siberian and Roborovski dwarf hamsters are highly identical (approx. 90%). This finding is in line with clinical observations on a lack of concordance of SPT reactivity of hamster-allergic individuals using commercial extracts. The number of patients analysed in this study being limited, these results have to be confirmed within a larger group of patients with a well-documented clinical history of exposure to different pet hamster species. However, the results pre-

sented here as well as in earlier studies clearly establish the need for a SPT formulation using Siberian and Roborovski hamster hair extracts in addition to the existing formulations containing the hair extracts of Golden or European hamster. The specificity of the in vitro IgE diagnosis will also be substantially improved by the isolation and availability of species-specific allergen components in their native or recombinant form.

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Disclosure Statement

All authors state that they do not have any conflict of interest.

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