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The screening of a basophil cDNA-library in search of novel lineage-specific proteins

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Abstract

Basophils are important mediators in many allergies. More information on their protein expression profile can give important clues as to their function. In this project, a cDNA library made from *in vitro* cultured basophil-like cells was screened in search of basophil-specific proteins. Lineage-specific proteins were often found in more than one clone, which is important since it indicated that the library has good coverage and that the lineage-specific proteins are highly expressed. To find full-length clones of interesting hypothetical proteins in the cDNA library, different primer pairs were designed. In the end, more than 150 proteins were found, and of them eleven were hypothetical proteins that might represent possible target molecules in allergy therapy.

Keywords

Basophils, cDNA, screening, primer, granula, allergy, antibody

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Jeanette Grundström

Sammanfattning

Basofiler är våra minst vanliga blodceller med en förekomst på knappt 1% av de vita blodkropparna. Detta har inneburit att det varit svårt att studera dem och därmed vet man inte så mycket om dem. Tillsammans med mastceller är basofiler de viktigaste cellerna som ligger bakom våra vanligaste allergier. Om man kan förstå mer om hur basofiler fungerar skulle det öppna för nya vägar att behandla allergier.

För att förstå basofiler bättre kan man undersöka funktionen hos basofilspecifika proteiner. De mest intressanta proteinerna är de som finns i små membranförsedda blåsor s.k. granula i basofilen. Det är bl.a. granulaproteinerna som ligger bakom de vanliga symtomen vid allergier. Proteininnehållet kan undersökas genom att skapa ett molekylärt bibliotek som representerar generna för de proteiner som basofilen innehåller. Genom att undersöka biblioteket tar man reda på vilka dessa proteiner är.

I det här projektet har ett bibliotek som är gjort från basofillika celler undersökts. Drygt 150 proteiner har hittats varav de proteiner som hittats flest gånger är granulaproteiner. Dessutom har elva hypotetiska proteiner som man inte vet så mycket om hittats. Dessa hypotetiska proteiner är intressanta mål för framtida forskning och skulle kunna vara möjliga målmolekyler i allergimediciner.

Examensarbete 20p i Molekylär bioteknikprogrammet

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1 INTRODUCTION

The basophilic granulocyte was first described by Paul Ehrlich in his work "Beiträge zur Kenntnis der granulierten Bindegewebzellen und der eosinophilien Leukocyten"[1] in the late 19th century. The basophilic granulocyte, or basophil for short, is a blood cell that matures in the bone marrow and circulates in peripheral blood. Basophils have often been described as bloodstream mast cells, since these two cell types have almost indistinguishable morphologies and they contain many similar substances, e.g. histamine[2, 3]. Despite the great similarity between mast cells and basophils they are two separate cell types. Mast cells are much more common than basophils. In contrast to basophils, mast cells mature in the tissue and seldom circulate in peripheral blood. Basophils were given their name since they stain with basic dyes like May-Grünwald-Giemsa[3] or Alcian blue. The function of basophils is unclear. However there is evidence indicating that they participate in the immunity against parasites together with eosinophils and mast cells[4]. Basophils also play an important part in allergic reactions.

Even though the basophil has been known for more than a hundred years, not much is known about its function and contents. This is mainly due to the low basophilic blood count that is less than 1% of peripheral blood leukocytes[5, 6],and that it has been hard to purify basophils[1]. Another problem for studying basophils is that they are terminally differentiated and in their mature form theycontain very little mRNA, compared to lymphocytes and monocytes, in the mature form. The basophil is also a short-lived blood cell with a lifespan of only a few days[7].

1.1 Basophils in the immune system and allergy

Without the immune system we would be extremely susceptible to infections by various microorganisms. The different cells that make up the complex network have the ability to recognize and kill a wide range of pathogens. Unfortunately the regulation of the immune system sometimes goes wrong. The cells start to recognize harmless substances or even take the body's own cells for pathogens. This is what happens in both allergy, in which basophils are important actors, and in autoimmunity.

Allergies, like hay fever and allergic asthma, are mediated by immunoglobuline E (IgE) antibodies. Individuals that have a genetic predisposition to develop IgE-mediated allergies are called atopic. An atopic individual react to common and harmless particles, called allergens, with a so-called T-helper 2 (T_H2) directed immune response. The T_H2 response promotes the production of specific IgE antibodies. As an example, an allergen could be a protein from pollen or bee venom. The first step in the development of an allergy is when the high affinity receptor for IgE (FcεRI) binds to the constant region of allergen-specific IgE, which leads to the sensitisation of basophils and mast cells. The next time the allergen enters the body, it is recognised by the specific IgE antibodies and binds to them. When several IgE antibodies bind to the same allergen, cross-linking of FcεRI leads to rapid release of preformed mediators by degranulation. The cross-linking also leads to synthesis and release of other mediators from the basophils and the mast cells. The preformed mediator histamine, together with leukotrienes (LT) and prostaglandins (PG) are the primary molecules responsible for the common symptoms in allergies. LTs and PGs are produced upon receptor cross-linking. Histamine is a vasoactive amine that increases permeability in venular capillaries, lowers blood pressure and also leads to smooth muscle contraction[7]. Also LTs and PGs alter the blood pressure and lead to bronchoconstriction[7]. If the antigen enters the blood it will rapidly spread throughout the body. This triggers histamine release that will lead to several unfortunate events, one of which is systemic lowered blood pressure. Systemic

lowered blood pressure can result in anaphylactic shock, which might be lethal. The smooth muscle contraction is very apparent in asthma, in which the contraction of smooth muscle around the bronchi results in breathing difficulties.

The progression of an allergic disease can be divided into three distinct parts: acute allergic reactions, late phase reactions and chronic allergic inflammation[6]. Acute allergic reactions are mainly mediated by mast cells while basophils seem to be more important in late phase reactions[7]. In the acute phase, mast cells in tissue and circulating basophils encounter their allergen and the FcεRI molecules are cross-linked and preformed mediators are released[7]. Both mast cells and basophils also release, among others, the cytokines interleukin-4 (IL-4) and IL-13 that are very important for the further development of the allergic reaction[8]. For example epithelial cells and fibroblasts produce the chemokine eotaxin under the positive control of IL-4 and/or IL-13 (reference 9 and 10, respectively). The late phase reaction usually appears about 2-4 h after an acute allergic response[7] when eosinophils, basophils and T_H2 cells infiltrate the site of inflammation e.g. the skin, lung or nose[1]. Basophils[11] and eosinophils are recruited by eotaxin that binds to the CCR3 on their surface. It has been shown that eotaxin triggers the basophils to release more IL-4[12] and this might in turn stimulate further production of eotaxin that attracts more basophils and eosinophils and thus the inflammation might be maintained and lead to a chronic inflammation. It seems like basophils, IL-4 and eotaxin are involved in a positive feedback loop in allergic inflammation[1].

In the first years of the 21st century, basophils have attracted more and more interest as the possible key initiator responsible for the induction of a T_H2 response to invading pathogens. Strong evidence for this is that they synthesise and release large amounts of IL-4[13] and IL-13 rapidly after activation[4]. Both these cytokines promote the T_H2 skewing of the immune system and induces IgE production in B-cells[4]. It has also been shown that basophils release IL-4 and IL-13 upon activation by non-specific super antigens called lectins[14], and upon non-specific interaction of the HIV-1 gp120 with IgE[15]. Lectins can come from plants such as beans and induce an allergic response possibly by cross-linking IgE[14]. Some parasites also release lectins that may induce the activation of basophils[4]. The defence against many helminthic parasites, like *Schistosoma mansoni*, is mediated by T_H2 profile cells and induce the production of IgE[7]. The indications that basophils might participate in the immunity against parasites is important since it gives them a natural role in the immune system, not only as the bad guys responsible for much of the inconvenience connected to allergies.

1.2 Morphology of basophils

The basophil is a cell with a diameter between 10 and 14 μm that is filled with cytoplasmic granules[2]. When the basophil is stained with a basic dye they cannot easily be distinguished from mast cells[16]. Both cell types stain metachromatically, which means that the abundant granules inside the cells are clearly showing[6]. If an electron microscope is used it can be seen that basophils have larger but fewer granules than mast cells and that the nucleus contains lobes[16]. On their surface basophils express a variety of antigens including FcεRI, the IL-3 receptor, and the chemokine receptor 3 (CCR3)[2, 5]. FcεRI binds to circulating IgE leading to the constitutive presence of antibodies on the surface of basophils. The FcεRI on basophils and mast cells is a tetramer consisting of one α-, one β- and two γ-chains[5]. The receptor is also expressed on the antigen presenting cells: Langerhans cells and peripheral blood dendritic cells, as a trimer of one α- and two γ-chains[5]. In their granules, basophils store many different substances e.g. histamine and chondroitin sulfates[17]. Another name for the chondroitin sulfates is proteoglycans. The major substances that bind to the basic dyes are the

highly abundant negatively charged proteoglycans. After activation, basophils synthesise other important mediators e.g. PGs and LTs, mainly LTC_4[18]. In addition, basophils also release the cytokines IL-4 and IL-13[17] that skews the immune system into a T_H2-type of response[8].

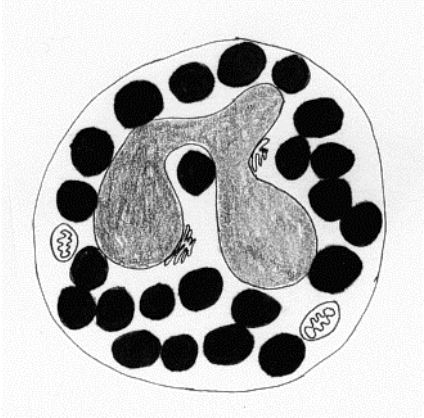

Fig 1. Schematic picture of a basophil.

1.3 Basophil differentiation

Despite many years of investigation and extensive studies of basophil differentiation, it is not yet clear exactly how the basophil develops. Basophils originate from a $CD34^+$ hematopoietic progenitor cell that differentiates and mature in the bone marrow[6]. $CD34^+$ progenitors are found in the bone marrow, peripheral blood and in cord blood[3]. In different investigations, evidence points in different directions and it has been suggested that basophils might share a common progenitor with eosinophils, mast cells or even megakaryocytes[3]. Evidence for a common basophil/eosinophil progenitor comes from the fact that different assays often display eosinophil and basophil lineages in the same colonies. Moreover basophils and eosinophils have some cytokines in common that favours the growth of both cell types both *in vitro* and *in vivo*[3]. A common progenitor for basophils and megakaryocytes is proposed by the facts that most megakaryocytic colonies synthesise histamine, express FcεRI and CCR3 on the surface and that they have the capacity to produce IL-4 and IL-13[3]. Finally, in favour of a common basophil/mast cell progenitor are the facts that they share many morphological and functional properties[3] and that basophils in patients with asthma, allergy or allergic drug reactions have a more mast cell like phenotype[19].

Cytokines direct the differentiation of blood cells in the bone marrow. Many blood cells have a specific cytokine that is needed to generate that cell type from the pluripotent stem cell. Mast cells for example need the cytokine stem cell factor[20] (SCF) and eosinophils need IL-5[21]. In contrast, basophils distinguish themselves from other blood cells since they can grow and differentiate without IL-3, which is the cytokine that promotes basophil differentiation[3]. This suggests that basophils could be a cell type for which the final maturation occurs by default[3].

1.4 Isolation of basophils

Due to the low numbers of basophils in the blood, there has been a need for an easy way to generate large amounts of basophils in another way than purifying them from peripheral blood. In 1998, Kepley and his co-workers compared different culturing conditions for interphase cells from Ficoll-Hypaque centrifuged cord blood cells, and came up with a protocol for *in vitro* culturing basophil-like cells[22]. They set up three different culturing conditions with IL-3: high IL-3 concentration, low IL-3 concentration or pulsing the cells

with IL-3 for 3-4 h. They demonstrated that pulsing with IL-3 gave the highest yield of basophil-like cells, but that both the high and low IL-3 concentrations also generated basophil-like cells. The basophil-like cells were characterised by e.g. metachromatic staining, measuring histamine content, measuring the expression of FcεRI and staining with a basophil-specific antibody.

1.5 Background to project

Due to their potential role in different allergies, basophils are currently a hot topic in immunology. If more can be understood about their participation in allergies e.g. location and mediator release, scientists can also learn how to interfere with basophils and thereby create a way to inhibit allergic diseases. Allergies are very common. Although the prevalence varies between different types of allergies, as much as 20% of the population is affected by some allergies[23], and thus new allergy drugs would help many people.

In order to learn more about basophils, effective tools to study them are needed. One of the largest problems has been to distinguish basophils from mast cells, especially when basophils has been recruited to sites of inflammation and reside in tissue, since they have almost the same morphology when stained with basic dyes. To solve this problem a specific marker for basophils has to be developed.

1.5.1 Basophil-specific antibodies

Up to date three different basophil-specific antibodies have been developed, called Bsp-1[24], 2D7[25] and BB1[26]. Bsp-1 is a monoclonal IgM antibody that recognises a 45kDa surface antigen and reacts with basophils[24]. Unfortunately Bsp-1 is not sensitive enough for immunohistochemical staining[25]. The other two antibodies are basophil-specific and are suitable for immunohistochemical staining of basophils[16]. The first antibody that was suitable for immunohistochemistry was 2D7. This antibody was produced and described by Kepley *et al*. in 1995[25]. 2D7 is a monoclonal $IgG_{1\kappa}$ antibody that recognises an antigen located in the granules. Unfortunately not much more is known about the antigen, but it is much more sensitive and specific than traditional basic dyes. In 1999, McEuen *et al*. described the monoclonal IgG_{2a} antibody BB1[26]. This monoclonal antibody recognises a granule protein that is secreted upon basophil activation, but it is also expressed on the surface. The antigen seems to be secreted in a complex with proteoglycans with a molecular mass of $5x10^6$ Da[27] and the BB1 antibody represents a means for detecting basophils that is better than basic dyes.

1.5.2 Methods to generate antigens

The search for a basophil-specific antigen can be performed in different ways. Kepley and McEuen chose to immunise mice with human basophils to get antibodies directed against basophils. By doing that, they produce many hybridomas that secrete monoclonal antibodies against many different proteins expressed in basophils. Unfortunately most of the hybridomas will not produce antibodies against basophil-specific antigens, and if a basophil-specific hybridoma is found, nothing will be known about the antigen itself, except that it is basophil specific. Another way to find basophil-specific antigens is to identify basophil proteins by analysing mRNA, and thus identifying their DNA sequence. These proteins can then be used to produce large amounts of fusion protein that can easily be purified and used to immunise rats.

To identify new basophil proteins in large scale, a cDNA library from basophil mRNA can be used. Screening this library by sequence analysis will not only lead to the identification of new proteins, but also many already known proteins will be found. This will therefore be a way to thoroughly characterise the protein content of basophils. All new proteins found can then be analysed further for different motifs indicating that they might be granule proteins. Interesting proteins can then be used for fusion protein production. It has to be taken into account that the identified proteins often come from a clone that is not a full-length clone, but that it is very probable that the whole cDNA library does contain full-length clones of the protein. Therefore sequences from the NCBI protein sequence database[28] can be used to design primers that will find full-length clones in the cDNA library.

To generate good fusion proteins many aspects have to be taken into account. Fusion proteins with glutathione-S-transferase (GST) and a His-tag can be produced rather quickly and are easily purified from the amplification vector in a nickel-chelating column. A bacterial vector is best for rapid production of fusion proteins, and there are several vectors available for GST-fusion protein production. After an appropriate vector has been chosen the primer design can start. Important to remember when the primers are designed is that the protein of interest should be in the right reading frame following the GST sequence and that there has to be two restriction sites outside the protein sequence that are recognised by different restriction enzymes. Moreover there should be a stop codon in the correct reading frame after the protein sequence, but there should not be a stop codon before the coding frame of the protein ends. In addition, the protein sequence must not contain any restriction sites for the chosen restriction enzymes, and last but not the least the primers should be specific for the desired protein.

1.6 Previous data from the Lars Hellman group

My thesis project is a part of a larger basophil project that has been going on for almost ten years in professor Lars Hellman's group. This project aims at getting a better understanding of human basophils and their role in allergic diseases and inflammation, and in the end to develop possible treatments to these diseases.

Previous to this project, human basophils had been studied in the laboratory and *in vitro* differentiated basophil-like cells had been cultured from human umbilical cord blood cells. There had been attempts to generate a cDNA library from mature human basophils that led to the discovery that basophils are terminally differentiated and contain very little mRNA in the mature state. This led to the idea that a cDNA library should be made from *in vitro* differentiated basophil-like cells. Which culturing conditions should be used was decided based on data presented in a study by Kepley et al in 1998[22]. It was decided that human cord blood cells should be cultured 9-12 days in media continuously supplied with IL-3. The basophil-like cells then have a somewhat immature phenotype that suggests that they still contain mRNA. Before the human cord blood cells were cultured they were purified by Ficoll centrifugation that excludes red blood cells, neutrophils and eosinophils. After culturing, the cells were again purified, but this time by negative selection in Magnetic Activated Cell Sorting (MACS). To confirm that the *in vitro* differentiated cells are indeed basophil-like, different tests have been made. The cells were stained with Alcian blue and showed metachromatic staining, indicating that they are basophil-like cells. Interesting is that, if cultured a little longer, the cells showed a hybrid basophil/eosinophil phenotype when stained with May-Grünwald Giemsa, a dye that stain both basophils and eosinophils but with different colours. The *in vitro* differentiated cells also expressed a surface marker, CD203c

previously known as 97A6, a marker that is only expressed by mast cells, basophils and $CD34^+$ hematopoietic progenitor cells[29].

1.6.1 Producing a cDNA library

Preceding this thesis work and making it possible for me to do my work, a cDNA library from the *in vitro* differentiated basophil-like cells had been produced in Lars Hellman's laboratory. The library was made from about 40 million basophil-like cells. This library has also been amplified to get a large volume that will give enough material for future experiments. The cDNA library was made in three steps, first by separating the mRNA from the total RNA in the cells, then by synthesising cDNA from the mRNA and last by packaging the cDNA into phage particles. For this library the phage λgt10 was used and the phages were allowed to infect an *E. coli* strain named C600 Hfl. To confirm that the library contains cDNA from basophil-like cells, it was screened for different markers. These markers are the α-chain from FcεRI and β-tryptase, and the result was that 14 clones in 100000 contained the α-chain and 11 clones in 100000 contained β-tryptase. As mentioned earlier basophils express FcεRI on the surface and recently some studies have shown that they also express β tryptase[19, 30-31], which is more commonly known as a mast cell marker. In conclusion, these experiments strongly indicate that the cells the cDNA library was made from were indeed basophil-like.

1.7 Aim

The aim of this project was to study individual clones in the unamplified phage λgt10 cDNA library and thereby characterise the proteome of the human basophil. Since the library consists of cDNA made from mRNA from *in vitro* differentiated basophils, the contents of the library should be representative for the protein expression profile in basophils.

After the primary characterisation, some interesting proteins were chosen for further investigation. The definition of an interesting protein is a protein that is most probably a granule or surface protein, and that might be basophil-specific. Then primers will be designed to perform polymerase chain reaction (PCR) amplification of full-length clones in the amplified cDNA library to produce GST fusion proteins that can be used for immunising rats.

In the future, the aim of the project could be to identify a basophil-specific protein and to produce a basophil-specific antibody against this protein. This could lead to an effective tool for studying basophils and their role in the immune system. Importantly, finding out the function of the new basophil-specific proteins will give clues about the function of basophils and the proteins might also be possible target molecules in allergy therapy.

2 MATERIALS AND METHODS

2.1 Screening of the phage λgt10 basophil cDNA library

To begin with, cultures of the *E. coli* strain C600Hfl were started. Frozen bacteria were inoculated in 10 ml LB media with 10 mM $MgSO_4$ and 0,2% maltose. The cultures were grown overnight with shaking at room temperature. On day two, the unamplified phage cDNA library was used to infect the bacterial cultures in order to isolate single clones from the library. This was performed by mixing 100 µl diluted phage cDNA library with 200 µl of C600Hfl culture. The library was diluted 100 or 200 times in LB media with 10 mM $MgSO_4$. Then the mix was incubated in a 37°C water bath for 30 min. After incubation 3 ml 48°C top agar was added to the tubes and the mixes were spread on LA-plates and grown overnight in an incubator at 37°C, 5% CO_2. The top agar contained 0,7% agarose in 50 ml LB media with 10 mM $MgSO_4$. When the culture grows, the phages will amplify inside the infected bacteria and eventually the infected bacteria will lyse. This forms a clear zone in the bacterial lawn that is called a plaque and contains many copies of the same phage. Plaques were isolated using Pasteur pipettes, one pipette for each plaque. The plaques were stored, one in each tube, with 500 µl LB-media with 10 mM $MgSO_4$ and 30 µl chloroform at 4-8°C until use.

2.2 PCR and sequencing of isolated clones

The cDNA in the phages was amplified using primers designed for the phage λgt10 in a way that has the effect that only phages with inserts will give an amplification product. The phage material was taken from the upper phase without chloroform. Only inserts will be amplified since the primers are directed towards the insert site, and if there is no insert they will only generate a fragment of about 40bp corresponding to the two primers. The primer pair was 5'-CTT TTG AGC AAG TTC AGC CTG GTT AAG –3' and 5'- GAG GTG GCT TAT GAG TAT TTC TTC CAG GGT A –3'. Primers were purchased from Invitrogen (Carlsbad, CA, USA). Amplification was performed in PCR reactions of 100 µl, where 2 µl was template from a plaque solution and 98 µl was PCR mastermix. Two different concentrations of primers were used during the project since more primers had to be ordered while the project was in process and it was decided to dilute them differently. The reason for the different dilution was that the primers were added in great excess when the concentration was 1 µg/µl and therefore some money could be saved if less primer was added to each reaction and therefore the new primers were diluted to a concentration of 300 ng/µl. The different mastermixes are presented in table 1.

Table 1. Content per reaction of the different PCR mastermixes.

Substance	Primer conc. 1 µg/µl	Primer conc. 300 ng/µl
Forward primer	1,1 µl	1 µl
Reverse primer	1,04 µl	1 µl
Taq buffer	10 µl	10 µl
dNTP (2,5mM/nucleotide)	10 µl	10 µl
$MgCl_2$ (2,5mM)	6 µl	6 µl
Double distilled water (ddW)	68,86 µl	70 µl
Taq polymerase	1 µl	1 µl

The PCR program was as follows:

94°C for 5min
94°C for 30s
62°C for 1min
72°C for 2min
Repeat steps 2-4 30 times
72°C for 10min
4°C until put in the -20°C freezer

After PCR, the reaction mixes were run on a 1% agarose gel with 0,01% ethidium-bromide. The marker λ *Bst*EII from Fermentas life sciences (Vilnius, Lithuania) was used to get a preliminary estimation of the lengths of the fragments. Both the marker and the PCR reaction solutions were mixed with Ficoll sample buffer, containing 0,25% bromophenolblue, 0,25% xylene cyanole and 25% Ficoll 400 (Pharmacia, Uppsala, Sweden) in 2 mM Ethylene diamine tetra acetic acid (EDTA). The amplified DNA was detected using a UV-board, and thus the phage clones with a cDNA insert were identified. The DNA fragments were cut out and the DNA was extracted using a QIAquick gel extraction kit from QIAgen (Venlo, the Netherlands).

Before sequencing the concentration of the DNA was determined using a Nanodrop spectrophotometer from Saveen-Werner (Malmö, Sweden). The samples were sent to Ulla Gustafson at the department of Animal Breeding and Genetics, the Swedish University of Agricultural Sciences (SLU, Uppsala, Sweden), who performed the sequencing.

After sequencing, the data was analysed using different databases. The sequences were compared against the BLASTn database of NCBI[28] to see which protein a sequence matched. The limit for homology between the found sequences and the database was set at about 95% and the database sequence had to be human. More information about the identified protein was searched for in different NCBI[28] and ExPASy[32] pages.

2.3 Isolation of cDNA from the amplified cDNA library

To simplify the PCR reaction and to obtain a better template, phage DNA from 10 ml of amplified cDNA library was purified. This is needed since the library mixture contains dead bacteria and phage capsules and other impurities that would disturb the PCR reaction if left in the solution. The cleaning was performed by spinning down the 10 ml amplified cDNA library at 10000 rpm for 5min and then transferring the supernatant to a new tube. To the supernatant, 2 µg/ml RNAse A (Fermentas life sciences, Vilnius, Lithuania) and 2 µg/ml DNAse I (Boehringer Mannheim, Mannheim, Germany) was added and the mix was incubated in a 37°C water bath for 30 min. After incubation, 1 g polyethyleneglycol (PEG) 6000 (Serva, Heidelberg, Germany) and 0,6 g NaCl was added and the tube was carefully mixed until everything was dissolved. Then the mix was incubated for 1 h on ice. After incubation, the tube was centrifuged at 10000 rpm for 10 min, and after centrifugation the supernatant was discarded and the tube was left upside down for 10 min to let as much as possible of the PEG 6000 to leave the tube. The pellet, containing aggregated phage particles, was dissolved in 500 µl sodiumchloride and magnesium (SM) buffer. SM buffer contains 100 mM NaCl, 8 mM $MgSO_4x7H_2O$ and 50 mM Tris pH7,5. The tube was then centrifuged again, this time at 13000 rpm for 2 min at 4°C. The supernatant was then transferred to a fresh tube and 5 µl 10% Sodium dodecylsulfate (SDS) and 5 µl 0,5 M EDTA was added. The mix was incubated for 15 min at 70°C. After incubation, all proteins were removed from the

supernatant. This was done by first adding 300 µl of phenol pH7 to the 500 µl of supernatant and then centrifuging for 3-5 min at 13000 rpm. Then the upper phase was transferred to a new tube and extracted with phenol: chloroform, 50:50, pH7 in the same way. The upper phase was then again transferred to a fresh tube and 50 µg/ml RNAse A was added and the mixture was incubated for 30 min in a 37°C water bath. To really be sure that all proteins and the added phenol were removed the supernatant was again extracted in the same way but this time phenol: chloroform, 50:50, pH7 was used first and the second time only chloroform was used. Then the upper phase was transferred to a new tube and 500 µl isopropanol was added and the solution was incubated for 15 min at -20°C to precipitate the cDNA. After incubation, the tube was centrifuged for 10 min at 13000 rpm and then the supernatant was carefully removed and the pellet was washed with 1 ml of 70% ethanol by carefully inverting the tubes a few times. Then the tube was centrifuged again at 13000 rpm for a few minutes. After centrifugation the ethanol was removed and the pellets were let to dry in a speedvac for about 10 min. Finally the clean cDNA pellet was resuspended in 20 µl of ddW. The whole procedure was done in two tubes simultaneously and in the end the two tubes were pooled. Before using the cDNA in PCR it was diluted 100 times to a concentration of 11,6 ng/µl.

2.4 PCR for finding full-length clones

A new PCR program was designed to match the Tm of the new primers and the DNA polymerase PFU Turbo from Stratagene (La Jolla, CA, USA) that was going to be used. PFU Turbo polymerase has better proofreading than Taq polymerase which is preferable when fusion proteins are to be produced. The error-rate for PFU Turbo polymerase is $1{,}3 \times 10^{-6}$ compared to the error rate of Taq polymerase that is 8×10^{-6} according to the manufacturer. The PCR program was as follows:
94°C for 1 min
94°C for 1 min
54°C for 1 min
72°C for 1 min 30 s
Repeated steps 2-4 35 times
72°C for 10 min
4°C until put in the -20°C freezer.
Each reaction tube contained 2 µl template, the clean cDNA from the amplified library, 1 µl forward primer (300ng/µl), 1 µl reverse primer (300ng/µl), 10 µl PFU buffer (Stratagene, La Jolla, CA, USA), 10 µl dNTP (2,5 mM/nucleotide), 75 µl double ddW and 1 µl PFU Turbo polymerase (2,5U/µl). Primers were purchased from Invitrogen (Carlsbad, CA, USA).

3 RESULTS

3.1 Screening of the unamplified cDNA library

During my project, a total of 903 plaques were collected from the LA-plates and given numbers between 1150-2053. These samples were added to the samples collected by my supervisors previous to this work. All the samples prepared in this work, including 72 samples from previous work, were analysed in PCR and gel electrophoresis. Out of these PCR reactions only 296 of the clones resulted in a PCR product, which equals 30% of the plaques. Almost all samples were sequenced and after bioinformatic research it was concluded that 173 of the cDNA sequences represented some kind of protein, which indicates that only 24% of the phages in the cDNA library actually contains protein coding information. There were 82 clones that contained cDNA inserts that were not sequenced, with numbers between 1770 and 2049. All proteins corresponding to the cDNA clones are presented in table 2.

Table 2. Proteins corresponding to the clones isolated from the basophil-like cell cDNA library[a]. The sequences were compared to the BLASTn database of NCBI[28] with a homology limit at about 95% to a human protein.

Clone number	Name of protein	Genbank accession number	Number of sequences
	Cellular structural genes		
886, 1263	Tubulin β	BC001002, AB062393	2
1483	Actin β	BC004251	1
913	Actin γ 1	BC039144	1
	Enzymes		
909	Acylphosphatase 2	BC012290	1
1294	Aldolase A	BC010660	1
1758	Aminolevulinate δ synthase 1	BC011798	1
1576	Asparagine-linked glycosylation 8 homolog	BC001133	1
1318	ATP-ase lysosomal accessory protein 1	BC000724	1
1471	Carnitine O-octanoyltransferase	BC051874	1
1142	Choline phosphotransferase	AF195623	1
1226	Cyclin dependent kinases regulatory subunit 2	BC006458	1
1242	Diacylglycerol kinase zeta	AK123378	1
1306	Disulfide isomerase	AY358646	1
902, 1396	Enolase 1	NM_080738, NM_001428	2
1348	Esterase D	AF112219	1
1310	Eukaryotic translation initiation factor 2α kinase 3	NM_004836	1
980	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3	NM_001356	1
1414	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	BC016060	1
899	Flap structure specific endonuclease 1	NM_004111	1
1437	Glia maturation factor γ	BC032819	1
1528	Glucosaminyl transferase 1	NM_001490	1
1100	Glutathione-S-transferase zeta	BC001453	1
1564	IMP cyclohydrolase	BC008879	1
1687	KRIT 1	AF388384	1
1723	Lactate dehydrogenase B	BC002362	1
1179	Methylthioadenosine phosphorylase	BC026106	1
924	NADH dehydrogenase 1α subcomplex 9	BC009311	1
1282	Ornithine decarboxylase 1	NM_002539	1
1385	Phosphatidic acid phosphatase type 2A	BC039847	1
1745	Phosphoantothenate cysteine ligase	BC062586	1
1104	Phosphoribosylaminoimidazole carboxylase	BC010273	1
921	PI-3 kinase related kinase SMG-1	BC061522	1
1267	Prolyl 4-hydroxylase α-subunit	M24487	1
1716	Protein kinase, DNA activated, catalytic polypeptide	NM_006904	1
964	SP25	D14658	1

1129	Transketolase	BC024026	1
1155	Triosephosphate isomerase 1	BC009329	1
	Genes involved in the immune system		
1167	MHC-1, HLA-Bw62	M28204	1
1601	CD74 antigen	NM_004355	1
1419	β2microglobulin	BC032589	1
920, 1260, 1346	Translationally controlled tumor protein 1	BC052333	3
1124	Proteasome activator subunit 3	NM_005789	1
1377	T1A1 cytotoxic granule associated RNA binding protein	NM_022173	1
1644	Oxidation resistance 1	BC032710	1
1730	Peroxiredoxin 2	BC000452	1
	Genes involved in lipid metabolism		
1424	Oxysterol binding protein	NM_002556	1
	Genes involved in the protein translation machinery		
1761	Ribosomal protein L3	BC012786	1
1309	Ribosomal protein L7	NM_000971	1
1278, 1596	Ribosomal protein L7a	NM_000972	2
1296, 1610	Ribosomal protein L12	NM_000976, BC050644	2
999	Ribosomal protein L13a	NM_012423	1
1351	Ribosomal protein L15	NM_002948	1
1579	Ribosomal protein L18a	NM_000980	1
948, 1430	60S ribosomal protein L23a	BC014459, BC058041	2
1109	Ribosomal protein L26	NM_000987	1
1232	Ribosomal protein S3	NM_001005	1
1252	Ribosomal protein S8	NM_001012	1
1320	Ribosomal protein S10	BC001032	1
1600	Ribosomal protein S11	BC007945	1
1459	Ribosomal protein S16	BC007977	1
1374	Ribosomal protein S18	NM_022551	1
1117	Ribosomal protein S25	NM_001028	1
1270	Ribosomal protein S27	BC002658	1
1411, 1492, 1532	Ribosomal protein large P0	NM_001002	3
905	Translation initiation factor 4A	BC012547	1
992, 1108, 1353	Translation elongation factor 1α1	BC018641	3
1741	Translation elongation factor 1β2	NM_001959	1
1674	Translation elongation factor 1γ	BC019051	1
1704	RNA polymerase III	NM_007055	1
	Growth and differentiation related proteins		
1489	Cyclin B1	NM_031966	1
1518	Structural maintenance of chromosome 1 like 1	NM_006306	1
1727	Lectin galactoside binding, soluble 1	BC020675	1
	Regulatory proteins		
1312	F-box only protein 9	NM_033481	1
1274	GM2 activator protein	X62078	1
	Ionic channels, sorting proteins and transporters		
894	Facilitated glucose transporter, member 6	BC013740	1
930	Solute carrier organic anion transporter member 3A1	AF205074	1
985	Vacuolar protein sorting 29	NM_016226	1
1670	Sorting nexin 11	NM_013323	1
1160	B-cell receptor-associated protein 31	BC014323	1
1176	Ferritin, light polypeptide	BC004245	1
1336	Chloride intracellular channel 1	NM_001288	1
1225	Epsin 4	BC004467	1
	Lineage specific proteins		
892, 911, 916, 950, 977, 1156, 1168, 1186, 1364, 1487,	Major basic protein (MBP, proteoglycan 2)	BC005929, Y00809, NM_002728	18

1566, 1581, 1608, 1743, 1754, 1765, 1766, 1768			
1269, 1560	Charcot-Leyden crystal protein	L01664	2
990, 1116	Proteoglycan 1	X17042	2
1397, 1438	MBP homolog, proteoglycan 3	NM_006093	2
927	Leukocyte associated Ig like receptor 1	NM_021706	1
1441, 1553, 1751	Eosinophil peroxidase (EPO)	X14346	3
	Mitochondrial genes		
1106	Mitochondrial ribosomal protein S15	NM_031280	1
1637	ATP synthase, H+ transporting, mitochondrial F1 complex, β polypeptide	BC016512	1
1720	Solute carrier family 25, member 5	NM_001152	1
	Proteins associated with the cytoskeleton		
1189, 1201, 1522	Thymosin β 4, X-linked	NM_021109	3
1594	Thymosin β 10	BC016731	1
1212	Diaphanous homolog 1	NM_005219	1
939	Cofilin 1	BC012265	1
1339	Transgelin 2	BC009357	1
1388	Restin	AF143235	1
	Proteins involved with RNA		
1307	RNA binding motif protein 3	BC006825	1
893	RNA binding motif protein 6	BC046643	1
984	Calcium homeostasis endoplasmic reticulum protein	BC021294	1
1135	Splicing factor prp8	AF092565	1
1389	Heterogeneous nuclear ribonucleoprotein A1	NM_002136	1
1726	Heterogeneous nuclear ribonucleoprotein C	BC003394	1
1656	Cancer susceptibility candidate 3	BC050526	1
1749	Cleavage stimulation factor	M85085	1
	Signal proteins		
1163	Adenylate cyclase-associated protein 1	BC013963	1
996	Membrane protein palmitoylated 1, 55kDa	NM_002436	1
1428	Guanine nucleotide binding protein	BC019093	1
1531	Jagged 1	NM_000214	1
	Stress induced proteins and chaperones		
1649	Heat shock 70kDa protein 5	BC020235	1
883, 1202, 1702	Heat shock 70kDa protein 8	BC008907, BC019816	3
1327	GRP78	AJ271729	1
942	T-complex protein 1, δ subunit	AF026291	1
1152	Cyclophilin B	BC032138	1
	Transcriptional factors and DNA binding proteins		
1140	Zinc finger protein 9	BC014911	1
1281	Zinc finger protein 70	NM_021916	1
912	Zinc finger protein 207	BC000962	1
1347	General transcription factor IIIC	BC060821	1
1361	Transcription factor Dp-2	BC013993	1
1544	C-myc transcription factor	L16785	1
1618	Hematopoietically expressed homeobox	NM_002729	1
1712	High mobility group nucleosomal binding domain 2	NM_005517	1
	Hypothetical and other proteins		
915	Hypothetical protein MGC13204	BC005106	1
943	Hypothetical protein MGC14156	BC007876	1
1534	Hypothetical protein MGC17943	BC020522	1
951	Hypothetical protein MGC46719	BC035727	1
1183	Hypothetical protein DKFZP434I216	BC054486	1
1238	Hypothetical protein KIAA0556	XM_044632	1
1247	Hypothetical protein FLJ10719	NM_018193	1
1321	Hypothetical protein FLJ11171	BC035005	1
1268	Hypothetical protein LOC170371	XM_378226	1

820	Hypothetical protein LOC389865	XM_374329	1
1317	Sushi domain containing	NM_022486	1
1127	GPP34 related protein	NM_018178	1
1182	Ral-GDS related protein	NM_153615	1
1264	Tetratricopeptide repeat domain 11	BC009428	1

a. The clones were sequenced and identified using the NCBI BLASTn database[28]

Most proteins identified are housekeeping proteins which have a function in the basic maintenance of the cell. Also note that the lineage specific proteins are generally more abundant than other groups. This is illustrated in fig 1.

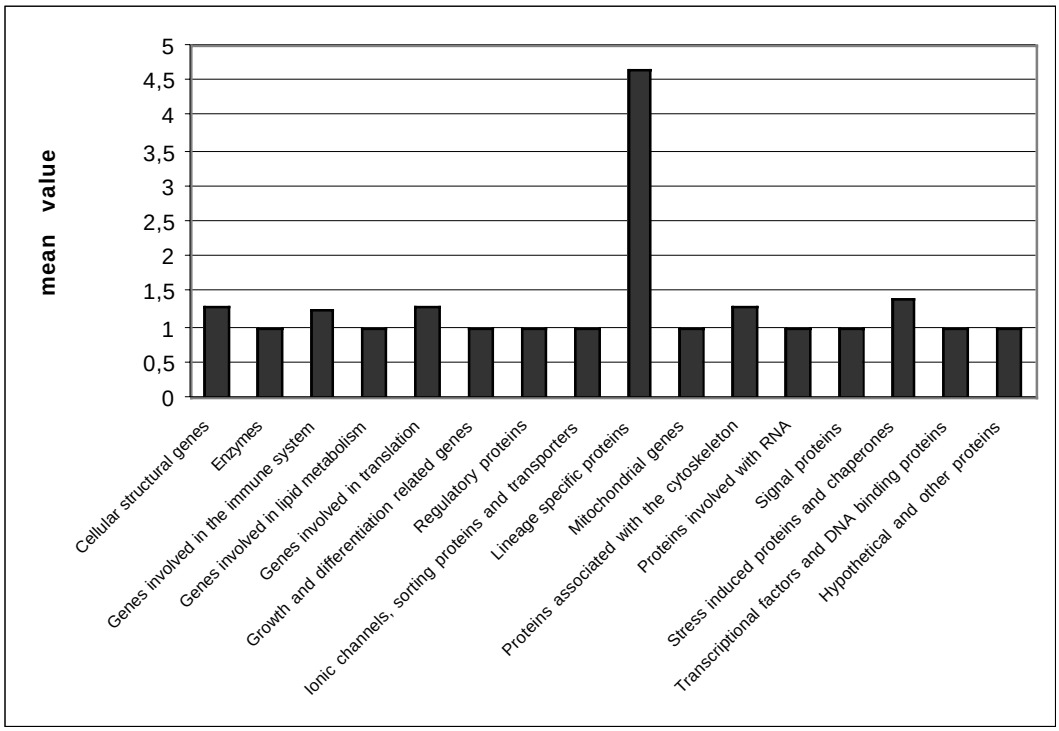

Fig 2. Average number of clones per protein in each group.

3.2 Primer design for isolation of full-length clones

Nine proteins were chosen for further investigation. These hypothetical proteins were candidates as basophil-specific proteins suitable for fusion protein production. A protein that is unsuitable for protein production could in this case be a protein that contains a very common domain that would lead to a non-specific anti-serum if used to immunise rats. To find any full-length clones of the interesting proteins in the amplified cDNA library, primer pairs were designed that would generate sequence suitable for fusion protein production. Of the nine proteins, seven were chosen from the listed proteins (Table 2). These were clones number 915, 943, 1238, 1247, 1268, 1317 and 1534. The last two proteins were identified by my supervisors, clone number 715, called Z-protein, and 750, called C21ORF4. The primer pairs were designed so that the resulting DNA would contain a *Sal*I restriction site in the 5' end and a stop codon and a *Kpn*I restriction site in the 3' end. All primers had at least 20 bases specific for the desired protein plus the restriction site in one end (Table 3).

Table 3. Primer pairs for finding full-length clones in the amplified cDNA library

Primer identity	Primer sequence 5' -> 3'	Expected length of PCR product
715 forward	ATA GGT CGA CGG GTA GCT GTT GCC ATC ATG	779 bp
715 reverse	GCG GGT ACC TCT TCA GTT GAG CTG AGA TAC	

750 forward	ATA GGT CGA CTA TGT GAA TGT ATT GAC TGG AGT	461 bp
750 reverse	ATA GGT ACC TCA GGT CCA TAG CTC TTC GGT	
915 forward	CGC GGT CGA CTG ATG CCT CCC AGA AAA AAA	729 bp
915 reverse	CGC GGT ACC TCA CAA GGA ATT GGC TTC TG	
943 forward	ATA GGT CGA CTG AGT GGA GCA CGC TGC AG	359 bp
943 reverse	ATA GGT ACC CTA GCG CTG CTC CAC TTC TT	
1238 forward	ATA GGT CGA CAT AAC ACA AGA CAG CTG GGG GAC	1019 bp
1238 reverse	TAT GGT ACC CTA ACT GCG CCA CCT TGA CCG TG	
1247 forward	AGA GGT CGA CCT GCG GGT AAA GCC AAA ACT AAA A	1046 bp
1247 reverse	TAT GGT ACC CTA TCC TCC GGA GCT CTG ACA CA	
1268 forward	ATA GGT CGA CGC ATG CAG AAG AAC CCA GGG GGC	495 bp
1268 reverse	CCT GGT ACC GCT CAC CTA CAG CTC AAT CAC	
1317 forward	ATA GGT CGA CGT AAC TAC ACC GTG AAC ATC TCC	1046 bp
1317 reverse	TAT GGT ACC CTA GTG TCT TCT CAC CTT ATT CCA	
1534 forward	GTA GGT CGA CTC ATG AAT CAG ACA GAT AAA	371 bp
1534 reverse	GGA GGT ACC TCA GTC AGA TTT GTC TTT CT	

The annealing temperatures for the different primer pairs varied between 55,6°C and 61,9°C (data not shown). In order to simplify the screening we first tested all primer pairs at a low annealing temperature, 54°C. If a low annealing temperature is chosen this will allow less specific binding of the primers, and thus a higher chance of binding. This is preferred since our primers do not match perfectly due to the restriction sites in the ends. After the PCR reaction, six of the primer pairs had generated one specific product of approximately the correct size (fig 2). Only clones nr 715, 1238 and 1268 showed signs of non-specific annealing since they gave more than one PCR product (fig 2). The primers corresponding to these clones had annealing temperatures above 59°C (data not shown).

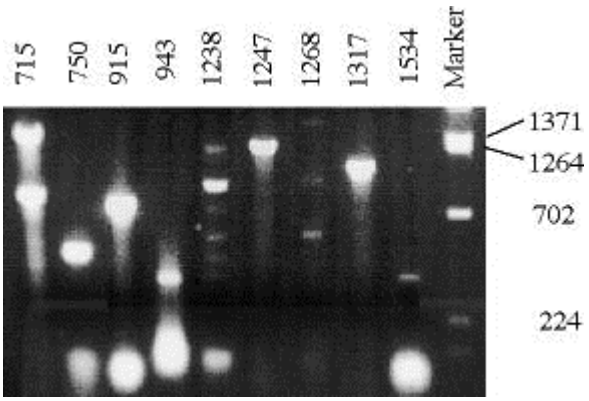

Fig 3. Agarose gel containing PCR products from amplification of the amplified cDNA library with the different primer pairs designed to find any full-length clones.

4 DISCUSSION

The aim of the project was to characterise the protein content of the basophil-like cells, the basophil proteome, and hopefully to find new basophil-specific proteins. The result was that 173 proteins were found and they are presented in table 2. Of these proteins, the majority was housekeeping genes (70%) and as much as 16% were lineage specific. However, eleven of them were hypothetical proteins that have only been predicted by bioinformatic methods or by mRNA analysis. The hypothetical proteins might represent new interesting proteins that could be basophil-specific, or they might not be proteins at all. This will be determined by the investigation that will take place after this project. The characterisation presented in this work is of course not a total representation of all proteins that the basophils contain. To get a more complete characterisation one would have to continue to screen the cDNA library until no more proteins not found before show up. As expected most proteins found are housekeeping genes, which is good. The housekeeping proteins are responsible for the basic functions of the cell, working to keep the cell alive. Many housekeeping proteins indicate that the cell is well functioning. It also indicates that the cDNA library is of good quality.

4.1 Limitations of the cDNA library

In the beginning of my project the idea was to find about 300 proteins, but since only 24% of the picked clones contained cDNA corresponding to a protein, the result was about half the expected. The poor outcome was mainly due to the fact that many of the clones that had an insert (30% of the clones), did not contain cDNA from a protein but was instead genomic DNA, mitochondrial DNA or just nonsense sequence. Since the cDNA library was made from about 40 million cells it was not possible to purify them to a large extent, which in the end leads to this type of contamination. Preferably about 100 million cells are needed to allow good purification and maybe even a test run. The cells were also slowly approaching the terminally differentiated stage with low or almost no functional mRNA. The low amount of clones containing an insert can also partially be explained by the length of the inserts. Sometimes the mRNAs are very short which makes detection by agarosegels impossible since the fragments will be of the same length as primer-dimers and such. It might also be due to the clones ligating in an incorrect manner, which will generate no fragment even though the clone contains an insert.

4.2 Similarities with eosinophils

Interesting is that such large amounts of major basic protein and eosinophil peroxidase has been found. It is well known that basophils contain major basic protein but definitely not in such large amounts as I have found: 10% of the clones with insert corresponding to a protein. This might indicate that the cDNA library was made from basophil/eosinophil hybrid cells and might be supporting the theory that basophils and eosinophils have a common precursor. Despite this indication it should not be forgotten that the cells have been purified in a way that excludes eosinophils and that the library contains clones with the α-chain of IgE and β-tryptase. It is also noteworthy that in general the lineage specific proteins are found in more than one clone, indicating that their mRNA levels are much higher than for the housekeeping genes. Among the lineage specific proteins are the important granule proteins, the different proteoglycans, and the cytoplasmic Charcot-Leyden crystal protein that are characteristic for basophils.

4.3 Reasons for using a cDNA library

The method of choice was to screen a cDNA library in order to find basophil-specific proteins and hopefully generate a basophil-specific antibody, as a bonus. This method was chosen since three monoclonal antibodies already exists, for which we know little about the identity of their target proteins. A similar method has also been used earlier to identify novel eosinophil proteins[33]. Even more important is that information about new basophil proteins will be found, and thus finding out more about the basophil. Knowing the function of basophil specific proteins will give important clues about the function of basophils. Since this is such a large-scale research the use of microarrays has to be considered. However, microarrays cannot be used as we do not known what we are looking for. Another problem is that microarrays are often used to look at upregulated genes. It is expected that the protein that is searched for is not upregulated in basophils from healthy patients, and if the protein expression is high it does not automatically mean that the mRNA expression is high. In the search for novel proteins the use of 2D-gels can also be discussed. This method has been tried in my laboratory but so far no conclusive results have been obtained.

4.4 Primer design

After the primer design the primers used did not match their DNA in the 5' ends because of the added restriction site sequences that would enable the insertion in the GST-fusion protein vector. Despite this, six out of nine primer pairs gave only one PCR product (fig 2). When the lengths of the PCR products are estimated, it seems that they are of the expected size (Table 3). For the primer pairs that did not generate one single product the annealing temperature was above 59°C, which is much higher than the temperature used in the PCR reaction. This is probably the reason why they did not give one specific product, since a lower temperature allows non-specific binding. For the clone 715 two strong bands are seen, the larger about twice the size of the smaller. This could perhaps be solved by heating the solution to 94°C again which would break dsDNA and any attaching of dsDNA to each other, a phenomenon that sometimes occur in PCR reactions. When the solution cools down only the dsDNA would re-attach.

In conclusion, my project has now started the characterisation of the basophil protein profile that will continue until at least 500 inserts have been identified. A solid ground for producing GST-fusion proteins has been laid and now the exciting work starts to find out if the nine chosen clones correspond to real proteins and whether they are basophil-specific or not.

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