International Science Academies

San Francisco Academy 2003

Heidelberger Life-Science Lab

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Michael Breckwoldt — Sarah Claus — Christoph Fischer
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The SFA 2003 Academy Members at UC Berkeley

Good vibes at the lab of Kristin Scott at UC Berkeley

Foreword

"If you're going to San Francisco, be sure to wear some flowers in your hair"

San Francisco: In those days in the late 60s and 70s, the "Hippies" proclaimed the era of "peace, love and happiness" and "dancing in the streets" – the future looked bright. Things were coming our way, we were full of ideals – sure a bit crazy – with plenty of options. These kids were excited, determined, energetic, and bustling ... how to resist !?

When this group of fourteen young talented students (then high-school pre-graduates – "VorAbiturienten") approached me in January 2003 about assisting them as consultant for a "San Francisco Academy", I was reminded of those days of youth and idealism. What's more rewarding than helping a new generation make their dreams come true !? ... dreams of youth and becoming life scientists. Time was tight – and at first we were not convinced that time, fortune, and money would be on our side, but there was enough time, more fortune, and – less money! What mattered was that we made it happen!

San Francisco and the Bay Area are certainly among the most picturesque urban environments in the world. Since the days of the Gold Rush it has attracted the most unusual people who have created an atmosphere of livelihood and bizarreness coupled with innovative creativity and high-end productivity hardly surpassed by any other metropolitan area. The pleasant temperate coastal climate is the extra "spice", and placing your life on the fault-line, the extra "tickle". No wonder that "going to San Francisco" is still a dream of a myriad young people and quite many others. Nothing compares to walking the "Golden Gate". It's a great place – it's my home state.

Being a native Californian immigrant to Germany and "well-seasoned" biologist, I must have been perceived as the "right person to talk to" – and so it became a rewarding challenge to help the group with correspondence and paperwork, provide a sense for the American culture, lifestyle, and Californian-American language, and offer personal encouragement when needed – along with the necessary "grain of salt".

"San Francisco Academicians 2003": Congratulations to your great achievement and success! It was nice to have met you, and it was a pleasure working with you. We were a fine group and we actually made things happen, together! I still remember how proud and nervous I was when you finally were on your way to the airport – there you went! You had a dream, and you were going to make it come true. I'm proud of you – you're my idols: Anna-Lena, Laura, Sarah, Christoph, Felix, Florestan, Gunther, Jonas, Markus, Martin, Michael, Philipp, Samuel, Sebastian. Good luck, for the future!

Thanks, Claudia and Monika – you actually took on the largest share of responsibility by devoting yourselves to preparing, accompanying, and guiding the group as adult mentors; be assured of the gratitude and appreciation that all of the group members feel for your courage, confidence, and devotion. ... and special thanks to Dan Choon and Gerald Weber for their foresight!

Yours, Ted

Theodor C.H. Cole, Dipl.biol.
Consultant, San Francisco Academy 2003

Stanford University

Prologue

In November 1769, Captain Gaspar de Portola‘s expedition to find and fortify the port of Monterey for Spain found instead San Francisco Bay: they viewed it from the ridge of the mountains of San Mateo County. The party worked its way down the peninsula and camped on the banks of San Francisquito Creek near a giant California Coast Redwood that later travelers came to call El Palo Alto, or „the high tree“ in Spanish. From this campsite, on which one corner of the Stanford campus is now situated, Portola‘s reconnoitering parties explored the area. The old redwood, twin-trunked and well over 100 feet high, was visible for miles. The little town that started to grow across El Camino Real (the old Spanish „King‘s Road“) took the name Palo Alto. Today, El Palo Alto is rooted precariously on the east bank of San Francisquito Creek. In 1887, a winter flood rushing down the creek tore off one of the redwood‘s twin trunks, but half of the venerable tree lives on, a gaunt and time-scarred monument.

In 1876, former California Governor Leland Stanford purchased 650 acres for a country home and began the development of his famous Palo Alto Stock Farm for trotting horses. He later bought adjoining properties to bring his farm to more than 8,000 acres, land that became the Stanford campus.

From Stanford‘s beginning, the redwood tree has been the university‘s symbol and the centerpiece of its official seal.

“Die Luft der Freiheit weht” is Stanford’s unofficial motto. The phrase is a quote from Ulrich von Hutten, a 16th century humanist. Stanford’s first president embraced the critical spirit of von Hutten’s words and included them on his presidential seal.

Cardinal red has been the color of Stanford athletic teams since 1892, in 1972 Cardinal was adopted as the name of Stanford athletic teams.

The Founding of Stanford University

On October 1, 1891, Stanford University opened its doors after six years of planning and building. Jane and Leland Stanford established the university in memory of their only child, Leland jr., who died of typhoid fever at 15. Within weeks of his 1884 death, the Stanfords determined that, because they no longer could do anything for their own child, they would use their wealth to do something for other people’s children. “The children of California shall be our children.” Leland Stanford devoted to the university the fortune he had amassed, first by supplying provisions to the mining for California gold and later as one of the “Big Four”, whose Central Pacific Railroad laid tracks eastward to meet the Union Pacific and complete the transcontinental railway.
They settled on creating a great university, one that, from the outset, was untraditional: co-educational, in a time when most were all-male; non-denominational, when most were associated with a religious organization; avowedly practical, producing “cultured and useful citizens” when most were concerned only with the former.
Jane and Leland Stanford determined that the character of the Main Quadrangle and the Palm Drive main entrance would reflect a grand, formal style: California Mission-inspired buildings of local sandstone and red-tiled roofs surround a cloistered

quadrangle, the so-called Main Quad, with Memorial Church as its focal point.
The university‘s development has mainly conserved the original concept of quadrangles and rectangular connecting malls enlarging the campus to its today’s extension.
With more than 8,000 acres, more than 46 miles of roads, a 49-megawatt power plant, two separate water systems, a central heating and cooling plant, a post-office, its own fire and police service, the Marguerite shuttle bus system, the university is a self-sustaining community.
94 percent of undergraduates live on campus as do 58 percent of graduate students and 30 percent of faculty members. The housing system for students consists of residentials with traditional dormitories, apartments, suites and residences. Faculty and staff live in owner-occupied housing units or in rental units on the campus.

An aerial view of the Main Quad with the back of Memorial Church in the foreground and Hoover Tower and Palm Drive in the distance

As the architectural center of campus, the Main Quad contains the university‘s first buildings, constructed between 1887 and 1891

Stanford University is a Teaching and Searching University

In 2002, 6731 undergraduate and 7608 graduate students were inscribed, instructed by 1714 faculty. Three schools award undergraduate degrees: Humanities and Sciences, Earth Sciences, and Engineering. Working under the supervision of faculty, undergraduates may join laboratory teams on campus, research a topic through Stanford‘s extensive library or travel to field sites around the world to complete an independent project. Study opportunities are offered in overseas to participate in internships and research projects. Approximately 90 percent of undergraduates receive their degree within five years. A Stanford student needs a budget of 40,000$ a year (including tuition, costs for room and board, books and personal belongings). In order to allow admittance to qualified students without regard to their ability to pay, 74 percent of them get financial aid by the university.
Graduate students can pursue postbaccalaureate degrees in seven schools: Business, Earth Sciences, Education, Engineering, Humanities and Sciences, Law and Medicine. Exchange programs with the universities at Berkeley and San Francisco complement the courses.
Stanford researchers have contributed to many developments over the years, 25 percent of Stanford faculty have been awarded the Nobel Prize since the university’s founding.
Among the living Nobel laureates are:

- Arthur Kornberg, Nobel Prize in Physiology or Medicine for the discovery of the mechanisms in the biological synthesis of RNA and DNA,
- Paul Berg, Nobel Prize in Chemistry for his research on recombinant DNA,
- three physicists at the Stanford Linear Accelerator Center (SLAC) were honored for their work they conducted at SLAC on elementary particles,
- three professors in the Department of Physics,
- and eight are faculty in economics or business.

Infrastructure

Stanford's free public shuttle bus Marguerite runs through the campus and serves Caltrain station in Palo Alto and downtown Palo Alto.

Since its completion in the early 1960s, Tresidder Memorial Union is a meeting place for students. Tresidder Union includes a juice bar, a cafeteria, a Mexican restaurant and other snack bars that open to outdoor patios. Tresidder also offers a supermarket, a shop and a bank with ATM.

Nearby is the Stanford Bookstore. Here's where students get their course readers and textbooks and it's the place to stock up Stanford clothes and insignia goods.

Stanford maintains different sport facilities among them are a golf course, a basketball pavilion, a 14-court tennis stadium, a four-pool aquatic center and the largest privately owned football stadium.

Stanford for Visitors

Stanford is a popular holiday or weekend destination for families, parents, alumni and tourists allowing the visit of different kind of sights:

- The Memorial Church and the Main Quadrangle, the observation deck of the Hoover Tower having views of campus, the foothills and East Bay, an extensive collection of outdoor art throughout the campus, among them famous works by Auguste Rodin and the Cantor Center for Visual Arts showing Asian and Egyptian collections and modern art.
- The foothill habitat conservation area is open to people wanting to jog and hike on trails that pass oak chaparral, grazing land for cattle and beautiful panoramas.
- The Stanford Linear Accelerator Visitor Center nearby Stanford informs on research at SLAC and on the Stanford Synchrotron Radiation Laboratory.
- The Stanford Shopping Center at the northern end of the campus with 140 stores offers restaurants and cafes perfect for people-watching and avenues great for window-shopping.

The Hoover Tower is part of the "Hoover Institution on War, Revolution and Peace", a public policy research center, founded by Herbert Hoover. Hoover was a member of Stanford's pioneer class of 1895 and the 31st president of the United States.

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Monika Gessat
Academic Tutor for the Stanford Group

University of California at Berkeley

A Short Impression

UC Berkeley is looking back at 135 years of existence. Walking across the campus one finds many European-style buildings. This is due to plans made by Emile Bénard of Paris who won the building plan competition and brought Berkeley worldwide notoriety. The tallest building is the Campanile (1914), built after the one in Venice.

The most recent National Research Council study shows 35 of Berkeley's 36 graduate programs ranking in the Top 10 of their fields: biochemistry and molecular biology ranked 4th, mathematics 1st, and physics 3rd. This ranking is based on terms of faculty competence and achievement. The current faculty includes eight Nobel Laureates. In the history of Berkeley there have been ten more.

The Library provides 9 million print volumes, one of the finest research collections in the US. Of course the modern way of finding information by internet can be chosen by the students as well.

Extracurricular activities may include a visit to the Botanical Gardens, to a Symphony Concert at Hertz Hall, to museums (e.g., Paleontology, Natural History, Anthropology, Art) or to a "Golden Bears" basketball game.

We very much appreciate the generous offer of the Berkeley faculty of hosting us. It is an exciting experience to work with them and their staff. On top of this it should be kept in mind that students in a graduate program pay about $2830 per month in order to study at this outstanding University.
According to American standards this money is very well spent, because being a Berkeley graduate practically guarantees an opportunity for a successful professional career.

The Campanile – the center of campus

Sather Gate – main entrance as seen from Sproul Plaza

Nobel Laureates: Throughout the years many Nobel Prizes have been awarded to Berkeley faculty members. The first one to achieve a Nobel Prize (1939) was Ernest O. Lawrence, who developed the cyclotron. After having attended the Universiity of Minnesota and the University of Chicago, where he met famous physicists such as Niels Bohr, Lawrence completed his doctoral thesis at Yale University. In 1928 he moved to the University of California at Berkeley where he became professor in 1930 at the young age of 29. Lawrence was inspired by the suggestion of the astrophysicist A.S. Eddington that nuclear reactions might occur at very high energies, as in the stars. The linear accelerator available at the time was not powerful enough for light ions, so Lawrence evolved a scheme of acceleration in a spiral path. In his cyclotron he was able to produce energies of millions of electron volts, making possible the disintegration of atomic nuclei and the discovery of new elements. He set the pattern around the world for organizations such as CERN, Geneva. Today his name is commemorated in the Lawrence Berkeley National Laboratory, where two of our San Francisco Academy students attended their practicals. In cooperation with his brother John, a physician, Lawrence explored the medical uses of neutrons and found them to be more effective in destroying malignant tissue than x-rays.

Ernest Lawrence –
public commemoration

Five more Nobel Prizes have been awarded to Berkeley faculty for physics, seven for chemistry, four for economics and one for literature. The numbers clearly speak for a dedication to science. At one Berkeley laboratory the human polio virus was isolated.

Geography: The San Andreas vault runs right underneath the Berkeley stadium, so one might think it is not a very secure place. Fortunately there was no damage done to the campus by the big earthquake on October 17th, 1989 which at 5:04 p.m. shook the inhabitants of the Bay Area and tore down part of Oakland Bay Bridge.

Flora and Fauna: In order to relax from studying, the beauty of the Berkeley hills is very much enjoyable for an afternoon stroll or the athletic version: jogging. There are huge eucalyptus trees but also poison oak, which can cause burning skin rashes. Part of the Berkeley campus is built in the hills, so it is common to come across deer if one has worked long and is leaving at a late hour.

Further information at http://berkeley.edu

"*If you are bored with Berkeley, you are bored with life*"

Clark Kerr, former Chancellor, UC Berkeley

Claudia Bignion
Academic Tutor for the Berkeley Group

Hosting Professors

Stuart K. Kim

Professor
Department of Developmental Biology and Department of Genetics
Stanford University, School of Medicine

Global analysis of conserved genetic modules:DNA microarrays provide us with a first step towards uncovering gene function on a global scale. Functionally-related genes often exhibit expression patterns that are correlated under a large number of diverse conditions in DNA microarray experiments. Furthermore, gene interactions that are physiologically significant should be conserved through evolution, so that orthologous pairs of genes should show similar expression correlations in DNA microarray data from diverse organisms.

Molecular analysis of human aging: We propose to elucidate mechanisms of human aging at the molecular level. A powerful approach to understand molecular changes associated with age is to use DNA chips to profile gene expression changes during human lifetime across the entire genome. We are using the kidney to study human aging because this organ shows a strong decline in function with age.

http://cmgm.stanford.edu/~kimlab

W. James Nelson

Professor
Department of Molecular and Cellular Physiology
Stanford University, School of Medicine

Our research objectives are to understand the cellular mechanisms involved in the development and maintenance of epithelial cell polarity. Polarized epithelial cells play fundamental roles in the ontogeny and function of a variety of tissues and organs. Recent studies indicate that the development of epithelial cell polarity is a multistage process requiring instructive extracellular cues (e.g., cell-cell and cell-substratum contact) and the reorganization of proteins in the cytoplasm and on the plasma membrane. Once established, polarity is maintained by targeting and retention of proteins to functionally distinct apical and basal-lateral plasma membrane domains.

http://cmgm.stanford.edu/biochem

Suzanne R. Pfeffer

Professor and Chairman
Department of Biochemistry
Stanford University, School of Medicine

During intracellular transport, proteins destined for the plasma membrane, secretory vesicles and prelysosomes must be sorted from one another within the Golgi complex, and sent to their appropriate addresses. The long term goal of our research is to elucidate the molecular mechanisms by which proteins are targeted to specific and distinct compartments. We would like to understand how transport vesicles select their contents, bud off from an organelle, translocate through the cytoplasm to recognize their target, and then fuse with their target to deliver specific cargo molecules.

http://cmgm.stanford.edu

Alessandra Lanzara

Assistant Professor of Physics
Physics Department
University of California, Berkeley

The Lanzara Research Group conducts experiments in solid-state physics. The research interest is focused toward an understanding of the underlying physics in complex novel materials and nanostructures, there where the conventional picture for an electron does not hold anymore and the electrons are now dressed by the different degrees of freedom. Emphasis will be given to the electronic, magnetic and structural properties and the interplay between them in these systems. Examples of materials that will be investigated in Lanzara's group are strongly correlated electron systems (such as high temperature superconductors and colossal magneto-resistance manganites), organic materials (such as fullerenes and nanotubes), nanosphere and nanorods (gold, cobalt, CdSe etc.) and magnetic multilayers.

http://www.physics.berkeley.edu/research/faculty/lanzara.html

Hiroshi Nikaido

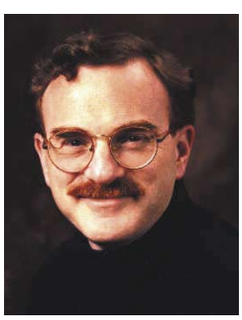
Professor of Biochemistry and Molecular Biology
Department of Molecular and Cellular Biology
University of California, Berkeley

We are interested in the biochemical and molecular genetic analysis of the structure and functions of bacterial membranes. Topics currently pursued include the specific and non-specific channel-forming proteins of the outer membrane, the diffusion of lipophilic compounds (inhibitors and antibiotics) across the unusually impermeable bilayer domain of the outer membrane, as well as the mechanism and regulation of multidrug efflux transport systems that pump out an incredibly wide range of compounds from bacterial cells. We are also studying the structure and functions of mycobacterial cell wall, which makes these bacteria intrinsically resistant to most drugs because it acts as an exceptionally efficient permeability barrier.

http://mcb.berkeley.edu/faculty/BMB/nikaidoh.html

Randy Schekman

Howard Hughes Investigator and Professor of Cell and Developmental Biology,
Affiliate, Division of Biochemistry and Molecular Biology
Department of Molecular and Cellular Biology
University of California, Berkeley



Our research is on the mechanism and control of intracellular protein transport in *Saccharomyces cerevisiae*. In particular we are studying the secretory process and its role in the assembly of cellular organelles. Enzymology, genetics, and electron microscopy are employed in this investigation. Protein translocation into the lumen of the endoplasmic reticulum represents the initial step in assembly of the eukaryotic cell surface. Subsequent stages in the secretory pathway involve protein sorting and transport from the endoplasmic reticulum to the Golgi body and from there to the cell surface. We are attempting to define the genetic and biochemical requirements for an unusual example of regulated transport in *Saccharomyces cerevisiae*.

http://mcb.berkeley.edu/faculty/CDB/schekmanr.html

Kristin Scott

Assistant Professor of Neurobiology
Department of Molecular and Cellular Biology
University of California, Berkeley

The aim of our research is to understand how sensory information is processed to produce specific behaviors. We study taste perception in the fruit fly, *Drosophila Melanogaster*, an excellent model organism with a simple gustatory system and robust gustatory behaviors. We are interested in defining taste neural circuits anatomically and functionally to examine how they operate, how they elicit behaviors and how they are modified by sensory experience.

To understand the function of different gustatory neurons, we are determining the ligands that different taste neurons recognize and the behaviors that they mediate. To determine the behaviors, inducible activators will be expressed in gustatory neurons, so that each neuron can be stimulated one by one to examine the fly's behavioral response. These studies will allow us to identify a sensory neuron by the stimulus that it recognizes and the response that it generates and will provide a starting point for dissecting taste circuits.

http://mcb.berkeley.edu/faculty/NEU/scottk.html

Mark Tanouye

Professor of Environmental Science, Policy and Management
(Division of Neurobiology)
Department of Molecular and Cellular Biology
University of California, Berkeley

We study *Drosophila* mutants that have behavioral abnormalities. By determining the genetic and molecular bases of these abnormalities, we are able to unravel new and basic features of nervous system structure and function.

Our approach is to use a combination of methodologies including classical and molecular genetics, behavior, electrophysiology, and neuroanatomy. We are especially interested in developing a *Drosophila* model system for examining human neuropathologies. Recently, we have been examining seizures, a serious problem in many neuropathologies. The long-term objectives of our research are to examine basic nervous system features underlying seizure-sensitivity and to discover novel treatments.

http://mcb.berkeley.edu/faculty/NEU/tanouyem.html

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Berkeley

Introduction

by Michael Breckwoldt
Student Coordinator, San Francisco Academy 2003

The first San Francisco Academy took place this summer from 14 July to 10 August and was a great success. Our four-week visit at the University of Stanford and University of California at Berkeley passed quite fast – unforgettable moments will remain: not only did we work on interesting scientific projects, but also got a taste of student life at two world-class universities.
To make our plans come true, a lot of work had to be done within the last three years. This science academy was mainly organized by students, which at times was really hard and there were often doubts whether we could manage it at all.

When the Life-Science Lab was founded in 2000, first ideas of a science academy in San Francisco were worded: the academy was to offer an opportunity for students to participate in world-class experimental research, within one of the most successful science communities worldwide – we were fascinated. A group of students started to develop a concept for the Academy, starting from scratch, looking for a concrete context. Of course, the group of students didn't have any experience in setting up such a project and we initially often got frustrated by the lack of progress and the seemingly "endless problems" which indeed did arise.

A new "boost" was felt after the opening weekend of the LSL 2002, when parents of a new participant encouraged us to request internships just by writing to professors. So we did and contacted 60 professors at the Department of Molecular and Cell Biology at UC Berkeley. This was the first big stride towards San Francisco since we received eight positive responses for internships.

In December we picked up pace when we found three adult mentors (was there a reason why all of them were physicians?): Philipp Gut, Jonas Tesarz, and Claudia Bignion became an important integral part of the group. In February, Monika Gessat and Theodor C.H. Cole (Ted) joined the group, and as the other mentors they did not only help us with the organization but also became real friends.

At this point we had the "manpower" to make this academy come true. But still problems had to be solved: financing was not clear – we tried to raise funds but especially in difficult economic times, fundraising is not an easy task - and as a result our participants hesitated to book flights. In these times I often "mumbled in my sleep" because I had to think of solving problems and finding perspectives. But people could be persuaded and convinced of the value of the program and in May the trip effectively was a "go" and participants booked their flights and dorms.
During group meetings in the following months we could feel the excitement: How would we be integrated in the lab between PhDs and postdocs? What would our scientific projects be and how would we perceive these top universities?

But in the end I hope it will become clear that this San Francisco Academy 2003 was a great experience and success for everybody. I would like to invite you to share our projects in the different papers following in this "documentation" and the presentations and reflections of the individual participants on the academy.

The diverse topics include papers by:

Samuel Bandara and Monika Gessat who worked on cellular protein trafficking and vesicular transport in the lab of Prof. Suzannne R. Pfeffer at Stanford.

Anna-Lena Beutel and Markus Schindler in their project at the lab of Prof. W. James Nelson at Stanford worked on aspects of cell cycle synchronization.

Laura Michel and Michael Breckwoldt – hosted by the lab of Prof. Randy Schekman – did research on unknown genes involved in the protein transport from the early endosome to the plasma membrane.

Sarah Claus and Jonas Tesarz worked at the lab of Prof. Hiroshi Nikaido on multidrug resistance in *Escherichia coli* and made interesting discoveries on antibiotics resistance and protein pumps.

Christoph Fischer and Florestan Ziem – our "physics department" at Lawrence Berkeley National Laboratory with Prof. Alessandra Lanzara – performed vibration measurements at the Advanced Light Source (ALS), the strongest light source in the United States.

Felix Gut and Gunther Schmitt, our first "Drosophila group", performed neurological studies at the lab of Prof. Mark Tanouye, determining the seizure threshold and behavioral characteristics of a new *stress sensitive* mutant of Drosophila.

Philipp Gut, at the laboratories of Prof. Stuart Kim at Stanford, used two different techniques to investigate the aging of the "lab worm" *Caenorhabditis elegans*.

Martin Schorb and Sebastian Urban, our second "Drosophila group", worked on taste perception in flies (they taste with their legs!) at the lab of Prof. Kristin Scott at Berkeley and visited a chocolate factory – together with their professor!

I wish you interesting hours reading the papers and hope that you enjoy learning about what puzzles the minds of devoted scientists – like us.

Michael Breckwoldt

Crosslinking Yip Family Peptides to the Blue Carrier Protein

Samuel Bandara, Laboratory of Suzanne R. Pfeffer,
Department of Biochemistry, Beckman Center, School of Medicine,
Stanford University

Abstract

Rab GTPases are important regulators in the process of vesicular trafficking. In the recent years, several membrane proteins were found to interact with Rab GTPases. They are referred to as the Yip protein family, and for some of these proteins an essential role in the process of vesicular trafficking was shown with intriguing results. To find out more about these proteins, specific antibodies will be raised. From each of twelve Yip family proteins, a characteristic peptide was synthesized previously. Six peptides were coupled to the immunogenic Blue Carrier protein by using the crosslinker sulfo-SMCC. Successful crosslinking was indicated by SDS-PAGE, and by a control experiment carried out with bovine serum albumin instead of Blue Carrier. For coupling the other six peptides to Blue Carrier, the crosslinking reagents EDC and sulfo-NHS were used, but sadly, these coupling reactions failed. However, peptide was successfully coupled to Blue Carrier, using varying amounts of EDC in small-scale reactions.

Introduction

Rab GTPases form a large family of important regulators in the process of vesicular trafficking. They all are prenylated at their carboxyl termini which make up lipophilic membrane anchors. Rab proteins attach to a variety of cellular membrane structures like the endoplasmic reticulum (ER), the Golgi apparatus, endosomes, and lysosomes, not only to large organelles, but also to small compartments like vesicles and transport intermediates. It is interesting to note that each type of membrane carries a distinct set of Rab GTPases. In their GDP-bound state, Rab GTPases can also be found in the cytosol, bound to a GDP dissociation inhibitor (GDI) protein. The delivery of Rab GTPases to various compartments by GDI is mediated by factors that reside on the membranes. On endosomal membranes, a GDI displacement factor (GDF) was shown to release those Rabs from GDI that are usually found on endosomes (Dirac-Svejstrup et al. 1997). As their carboxyl termini are prenylated and highly lipophilic, displacement of GDI makes Rab GTPases attach to the nearby membrane. In their GDP-bound state however, they can be extracted from the membrane by GDI again. For persistent membrane association, Rab GTPases are converted to their GTP-bound state by GDP-GTP exchange factors (GEFs), as theses were shown to increase the intrinsic nucleotide exchange rate of Rab proteins. As both the GDF and the GEF contribute to stable Rab localization, their combined specificity might cause each membrane structure to carry a distinct set of Rab GTPases which were shown to interact with a variety of other proteins implicated in the process of vesicular trafficking, dependent on bound GTP. The mannose 6-phosphate receptors (MPRs) are cargo-selecting membrane proteins recognizing acidic hydrolases for vesicular transport from the trans-Golgi network (TGN) to endosomes. After cargo is unloaded, the MPRs are returned to the Golgi for another round of transport. The recycling of MPRs requires Rab9 and a cytosolic factor called TIP47 which specifically binds to both Rab9 and to MPRs, forming a ternary complex. Interestingly, the binding of TIP47 to the cation-independent MPR was shown to be increased at least threefold in the presence of Rab9 which is predominantly found on late

endosomes (Carroll et al. 2001). This might be the reason for TIP47 to select MPRs into TGN-targeted transport vesicles only from late endosomes. By fluorescence microscopy, Rab9 was shown to stay on those vesicles until fusion with the TGN (Barbero et al. 2002). Arrived at the TGN, the Rab9 GTPase might hydrolyse its bound GTP for being extracted from the membrane by GDI, and for being delivered back to late endosomes. Rab GTPases not only serve as compartment identifiers in all stages of vesicular transport. As a variety of protein interactions determine the availability of active Rab GTPases on membrane subdomains, together with their intrinsic properties of GTPase function and nucleotide exchange, Rab GTPases also serve as important regulating factors in the process of vesicular trafficking.

In the recent years, several yeast two-hybrid screens revealed a number of proteins that interact with Rab GTPases. Screens using the Rab GTPases Ypt1 and Ypt31 as baits discovered an essential Golgi-localized protein named Ypt-interacting protein (Yip1p). Yip1 mutant cells show severe transport defects, as the periplasmic enzyme invertase is secreted in its immature underglycosylated form, resembling a defect that is known from Yrp1 mutants, and as proforms of both the vacuolar enzymes carboxypeptidase Y and alkaline phosphatase accumulate in the ER (Yang et al. 1998). These observations implicate an important role of Yip1p in the regulatory function of Rab GTPases. Yip1p was found to form a complex with a Yip1p-interacting factor (Yif1p) for which two-hybrid interactions with Ypt1 and Ypt31 were detected, too. Importantly, the transport defects shown by Yif1p mutant cells are similar to those of Yip1p mutants. In both mutant types, transport from the ER to the Golgi apparatus is blocked, and vesicles derived from the ER accumulate on their way to the Golgi, indicating a disability of those vesicles to fuse to their target (Matern et al. 2000). And yet another Yip one partner (Yop1p) was shown to interact with Yip1p. But Yop1p neither binds to Ypt1 nor to Ypt31 and it is dispensable for normal cell function. However, overexpression of Yop1p leads to large cells that also accumulate carboxypeptidase Y in the ER. Coexpression of Yip1p suppresses this phenotype. Without its N-terminal domain, Yop1p cannot bind directly to Yip1p and overexpression of the truncated protein does not block the transport of carboxypeptidase Y. However, it results in notably smaller cells, and this effect cannot be suppressed by coexpression of Yip1p. Surprisingly, multicopy overexpression of Yrp6 which is another Rab GTPase in yeast, restored cell size, while overexpression of neither Ypt1 nor Ypt31 suppressed the defect (Calero et al. 2001). The results presented above suggest an important but yet to be discovered role of Yip1p, Yif1p and Yop1p in mediating the fusion of ER-derived vesicles to the Golgi apparatus, which involves the interaction with several Rab GTPases. Other yeast two-hybrid screens found a novel binding partner of Rab3A and Rab1. It was named prenylated Rab acceptor (PRA1) and it was shown to bind to the vesicle-associated membrane protein VAMP2 which is part of a vesicular SNARE complex (Martincic et al. 1997). Interestingly, SNARE complexes are known to selectively drive the fusion of membrane structures. The binding of PRA1 to Rab3A or VAMP2 is mutually exclusive, and Rab3A can displace PRA1 from VAMP2. These findings implicate a connection of PRA1 and Rab GTPases with membrane fusion. PRA1 was shown to inhibit the extraction of GDP-bound Rab3A from membranes by GDI in vitro (Hutt et al. 2000), demonstrating the variety of interactions that influence Rab localization. Yip1p, Yif1p, Yop1p, PRA1 and a number of other proteins that were found to interact with Rab GTPases, all show high sequence similarity and share a common topology. Sequence-based database searches revealed even some more proteins to be promising candidates for Rab interaction. Because of close structural and putatively functional similarity, we will further relate to all of them as members of the Yip protein

family. The Yip protein family occupies an important role in the vesicular trafficking machinery, as genetic manipulation leads to severe transport defects.

In order to further investigate the function of Yip family proteins in detail, we decided to raise specific antibodies against them, by chemically crosslinking characteristic peptides to a strongly immunogenic carrier protein, for injection into rabbits. We used the heterobifunctional crosslinker sulfosuccinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate (sulfo-SMCC) and the crosslinking reagents 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC) and sulfo-N-hydroxysuccinimide (sulfo-NHS) to couple synthesized peptides from twelve Yip family proteins to Blue Carrier which is a strongly immunogenic protein obtained from the mollusk *Concholepas concholepas*, with a molecular weight of about 6 MDa. The synthetic peptides each contained 16 to 18 residues from Yip family proteins and they were acetylated and amidated as expected from posttranslational modification. On average, 25% of the residues differed from homologs in organisms like rat or mouse. Some additional amino acids were introduced terminally for coupling and spacing purposes. Most importantly, six peptides were equipped with a terminal cysteine residue. Cysteine contains a sulfhydryl group that can add to the double bond of the maleimidyl group of sulfo-SMCC. Coupling to the carrier protein takes place at the ester bond of sulfo-SMCC. Lysine residues of the protein contain free amino groups attacking the carboxylic carbon atom of sulfo-SMCC nucleophilically, as electron density is exceptionally reduced by the neighboring sulfo-succinimidyl group. Five of the other six peptides were equipped with lysine residues for coupling with EDC and sulfo-NHS. EDC is a carbodiimide, it contains two nitrogen atoms double-bonded to one carbon atom. Acidic residues of the carrier protein can add their hydroxyl group to one of those double bonds, resulting in an unstable, reactive intermediate for transesterification with sulfo-NHS. The introduced sulfo-succinimidyl group reduces the electron density of the carboxylic carbon atoms on the carrier protein. The N-terminal amino group of the backbone of all six peptides or the amino group located in the lysine residue of five peptides then nucleophilically attacks the carbon atom on the protein, finally forming the protein–peptide conjugate.

Results

As the peptides were not purified by the manufacturer after synthesis, we purified them on a gel filtration column directly before carrying out each coupling reaction, in order to get rid of reagents and uncoupled amino acids that remained from peptide synthesis. For the sulfo-SMCC reaction, the crosslinker was added to a solution of Blue Carrier protein, and the reaction was given half an hour at 37°C to complete. As described above, lysine residues of the protein nucleophilically attacked the carboxylic carbon atom of the crosslinker, making the sulfo-succinimidyl group leave as sulfo-NHS. Thereby, maleimidyl residues were introduced to the surface of the carrier protein. The activated carrier protein was purified on a gel filtration column, to remove sulfo-NHS and excess sulfo-SMCC. Then, activated Blue Carrier protein was added to the solution of each purified peptide. The sulfhydryl groups of the peptidic cysteine residues were given time to add to the double bond of the maleimidyl groups on the

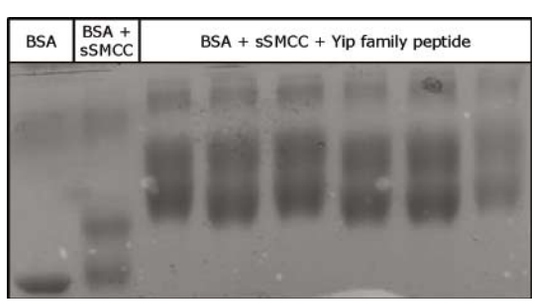

Fig. 1. Peptides were crosslinked to BSA, using sulfo-SMCC. The crosslinking reactions were carried out as described in ‚Methods'. Each an amount of 3 µg of plain BSA, of activated BSA, and of each protein-peptide conjugate was resolved on a 10% polyacrylamide gel

surface of the Blue Carrier protein at 4°C over night. After that, the protein-peptide conjugates were purified using gel filtration columns. In order to assess the crosslinking reaction, plain carrier protein, the activated carrier protein, and samples from the six protein-peptide conjugates were run on the same low-percentage polyacrylamide gels (data not shown). The plain carrier protein was resolved to large subunits of two different molecular weights. No protein was detected in the other lanes of the resolving gel. Because the protein concentrations of the samples were estimated using UV-photospectroscopy before loading, we conclude that sulfo-SMCC crosslinked the subunits of Blue Carrier to a conjugate that was unresolvable by the polyacrylamide gel, at least indicating that the crosslinker was active.

To find out more about the ability of sulfo-SMCC to crosslink Yip family peptides to a carrier protein, a control experiment was run, using bovine serum albumin (BSA) instead of the Blue Carrier protein. With a molecular weight of 66 kDa, BSA is much smaller than Blue Carrier, and therefore, protein-peptide conjugates of BSA are resolvable on a polyacrylamide gel. After the crosslinking reactions had been carried out, plain BSA, the activated BSA, and samples from the six conjugation reactions were run on the same polyacrylamide gel (Fig. 1). The bands of the protein-peptide conjugates were detected at a larger molecular weight than the band of plain BSA. Furthermore, the samples taken from the coupling reactions spread over two vast ranges in each lane, indicating that a variety of differently sized protein-peptide conjugates were formed, as not all molecules of BSA were crosslinked to exactly the same number of peptide molecules. The formation of two distinct ranges on each lane showed that not only the sulfhydryl groups of peptides, but also the ones of other BSA molecules were added to the maleimidyl groups on BSA, resulting in conjugates made up by presumably two carrier proteins. This finding was supported by the formation of two bands in the lane that was loaded with activated BSA alone.

For the EDC reaction, sulfo-NHS and EDC were added to a solution of Blue Carrier protein, and the reaction described above was given 15 minutes to label the surface of the protein with sulfo-succinimidyl residues by esterifying the carboxyls with sulfo-NHS. Remaining EDC was quenched by adding beta-mercaptoethanol. Then, activated Blue Carrier protein was added to the solution of each purified peptide. Either the amino group located on the lysine residue of the peptides or the amino group of their backbones was expected to nucleophilically attack the carbon atom of the esterified carboxyl groups on the surface of the carrier protein, as at the carbon atom, electron density was heavily reduced by the sulfo-succinimidyl group. Therefore, the sulfo-succinimidyl group was expected to leave as sulfo-NHS, and the peptide bond to be formed. This reaction was given two hours to complete at room temperature. Then, hydroxylamine was added in order to hydrolyse remaining sulfo-succinimidyl groups from the protein. Each protein-peptide conjugate was run on a gel filtration column in order to get rid of remaining hydroxylamine, of sulfo-NHS and of other byproducts of the crosslinking reaction which was assessed by running plain Blue Carrier protein, the activated Blue Carrier protein, and a sample from each of the six protein-peptide conjugates on the same low-percentage polyacrylamide gel (Fig. 2). No crosslinking took place, as the Blue Carrier protein was even resolvable to its subunits still, and as all bands were sharp. To confirm these results, a control experiment was run, using BSA instead of Blue Carrier. Plain BSA, the activated BSA, and samples taken from the reactions were run on a polyacrylamide gel (Fig. 2b). Again, all bands looked the same, indicating that no crosslinking reaction took place.

As EDC is a highly hygroscopic reagent that decomposes in water, the crosslinking experiment was repeated, using another batch of reagent. Instead

of forming sulfo-succinimidyl esters on the surface of the carrier protein as an activating step, we directly reacted the peptides with the intermediate that results from the addition of carboxyl groups to the carbodiimide, and which is far less stable than the sulfo-succinimidyl ester. Therefore, a one-step reaction was run, adding a solution of the plain Blue Carrier protein to each of the purified peptides before adding EDC. As a disadvantage, not only the protein, but also the peptide can be activated for binding to another peptide molecule. While it is recommended to run the reaction for two hours at room temperature, it accidentally was given two hours on ice and then one hour at room temperature to complete. Some of the reactions turned cloudy and one of them seemed to form a single polymer. Each protein–peptide conjugate was run on a gel filtration column, but for most filtrations, no fraction was found to contain any conjugate, indicating that it got stuck in the column (data not shown).

To confirm whether EDC alone can be used in a one-step reaction for coupling Yip family peptides to a carrier protein, crosslinking reactions were run, using BSA and different amounts of EDC, as described below in ‚Methods'. The reactions were given two hours to complete at room temperature.

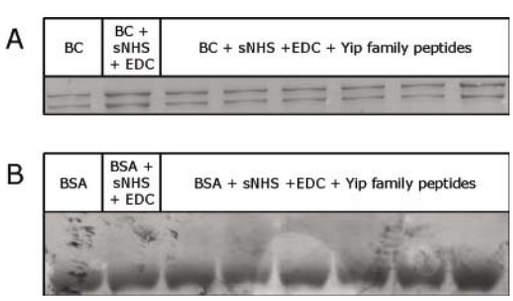

Fig. 2. EDC and sulfo-NHS failed crosslinking peptides to Blue Carrier or BSA. The crosslinking reactions were carried out as described in ‚Methods'. **A** Each an amount of 3 µg of plain Blue Carrier, of activated Blue Carrier, and of each putative protein-peptide conjugate purified, was resolved on a 7.5% polyacrylamide gel. **B** Each an amount of 3 µg of plain BSA, of activated BSA, and of each putative protein-peptide conjugate purified, was resolved on a 10% polyacrylamide gel

Then, samples from each reaction were run on a polyacrylamide gel (Fig. 3). The samples taken from the coupling reactions carried out with custom amounts of EDC (lane four to six), were spread over a large range, indicating a promising method for crosslinking Yip family peptides to BSA, using EDC without sulfo-NHS in a one step reaction.

For the EDC reaction, sulfo-NHS and EDC were added to a solution of Blue Carrier protein, and the reaction described above was given 15 minutes to label the surface of the protein with sulfo-succinimidyl residues by esterifying the carboxyls with sulfo-NHS. Remaining EDC was quenched by adding beta-mercaptoethanol. Then, activated Blue Carrier protein was added to the solution of each purified peptide. Either the amino group located on the lysine residue of the peptides or the amino group of their backbones was expected to nucleophilically attack the carbon atom of the esterified carboxyl groups on the surface of the carrier protein, as at the carbon atom, electron density was heavily reduced by the sulfo-succinimidyl group. Therefore, the sulfo-succinimidyl group was expected to leave as sulfo-NHS, and the peptide bond to be formed. This reaction was given two hours to complete at room temperature. Then, hydroxylamine was added in order to hydrolyse remaining sulfo-succinimidyl groups from the protein. Each protein-peptide conjugate was run on a gel filtration column in order to get rid of remaining hydroxylamine, of sulfo-NHS and of other byproducts of the crosslinking reaction which was assessed by running plain Blue Carrier protein, the activated Blue Carrier protein, and a sample from each of the six protein-peptide conjugates on the same low-percentage polyacrylamide gel (Fig. 2A). No crosslinking took place, as the Blue Carrier protein was even resolvable to its subunits still, and as all bands were sharp. To confirm these results, a control experiment was run, using BSA instead of Blue Carrier. Plain BSA, the activated BSA, and samples taken from the reactions were run on a

polyacrylamide gel (Fig. 2B). Again, all bands looked the same, indicating that no crosslinking reaction took place.

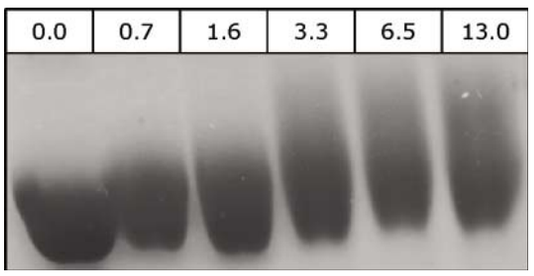

Fig. 3. Peptide was crosslinked to BSA, using varying amounts of EDC. In each reaction, the final concentration of BSA was 2.5 mg/ml, and the final concentration of peptide was 1.75 mg/ml. The final concentration of EDC (mM) is indicated on top of each lane. The reactions were run for 2 h at room temperature. Samples were resolved on a 12.5% polyacrylamide gel

As EDC is a highly hygroscopic reagent that decomposes in water, the crosslinking experiment was repeated, using another batch of reagent. Instead of forming sulfo-succinimidyl esters on the surface of the carrier protein as an activating step, we directly reacted the peptides with the intermediate that results from the addition of carboxyl groups to the carbodiimide, and which is far less stable than the sulfo-succinimidyl ester. Therefore, a one-step reaction was run, adding a solution of the plain Blue Carrier protein to each of the purified peptides before adding EDC. As a disadvantage, not only the protein, but also the peptide can be activated for binding to another peptide molecule. While it is recommended to run the reaction for two hours at room temperature, it accidentally was given two hours on ice and then one hour at room temperature to complete. Some of the reactions turned cloudy and one of them seemed to form a single polymer. Each protein–peptide conjugate was run on a gel filtration column, but for most filtrations, no fraction was found to contain any conjugate, indicating that it got stuck in the column (data not shown).

To confirm whether EDC alone can be used in a one-step reaction for coupling Yip family peptides to a carrier protein, crosslinking reactions were run, using BSA and different amounts of EDC, as described below in ‚Methods'. The reactions were given two hours to complete at room temperature. Then, samples from each reaction were run on a polyacrylamide gel (Fig. 3). The samples taken from the coupling reactions carried out with custom amounts of EDC (lane four to six), were spread over a large range, indicating a promising method for crosslinking Yip family peptides to BSA, using EDC without sulfo-NHS in a one step reaction.

Methods

Crosslinking of peptides to Blue Carrier, using sulfo-SMCC

Of each peptide, an amount of about 4 mg was weighed out. Each peptide was dissolved in PBS, to a concentration of 10 mg/ml, and each was run on a 4-ml sephadex G-10 gel filtration column. 12 fractions of 0.4 ml were taken each time, and the peptide was located using UV-photospectroscopy. On average, four fractions were pooled. Blue Carrier was diluted to a final concentration of 8 mg/ml in 1.75 ml of PBS. The solution was added to 3.5 mg of sulfo-SMCC, and the reaction took place at 37°C for 30 min. The reacted solution was centrifuged shortly, and the supernatant was run on an 8.3-ml PD-10 gel filtration column at 4°C. Ten fractions of 1.0 ml were taken, and the activated protein was located, using photospectroscopy. An amount of 10 mg of activated Blue Carrier was pooled from two fractions. A volume of 310 µl of the 5 mg/ml solution of activated protein was added to each peptide pool. The reaction took place at 4°C over night. Each conjugate was run on an 8.3-ml PD-10 gel filtration column at 4°C. Ten fractions of 1.0 ml were taken each time, and the protein-peptide conjugate was located, using UV-photospectroscopy. On average, two fractions were pooled. For SDS-PAGE, a sample was taken from each reaction. The pools were shock-frozen using liquid nitrogen.

Crosslinking of peptides to BSA, using sulfo-SMCC

Of each peptide, an amount of 1.5 mg was dissolved in PBS, to a concentration

of 10 mg/ml, and each peptide was purified as described for the Blue Carrier experiment. On average, two fractions were pooled. BSA was dissolved in PBS, to a concentration of 8 mg/ml. A portion of 1.0 ml of this solution was added to 2.0 mg of sulfo-SMCC, and the reaction took place at 37°C for 30 min. The activated protein was purified as described for the Blue Carrier experiment. The concentration of activated BSA was 3.9 mg/ml after purification, as it was measured by UV-photospectroscopy. A volume of 130 µl of this solution was added to each peptide pool. The reaction took place at 4°C over night. For SDS-PAGE, a sample was taken from each reaction. The protein-peptide conjugates were purified and stored as described for the Blue Carrier experiment.

Crosslinking of peptides to Blue Carrier, using EDC and sulfo-NHS

Of each peptide, an amount of about 4 mg was weighed out. Each peptide was dissolved in 0.1 M MES, 0.5 M NaCl, pH 6.0 (MES), to a concentration of 10 mg/ml, and each was purified using a 4-ml sephadex G-10 gel filtration column. 12 fractions of 0.4 ml were taken each time, and the peptide was located, using UV-photospectroscopy. On average, three fractions were pooled. Blue Carrier was diluted to a final concentration of 1.0 mg/ml in 10.5 ml of MES. An amount of 4.2 mg of EDC and an amount of 11.6 mg of sulfo-NHS were added, and the reaction was given 15 min at room temperature (RT). Then, a volume of 15 µl of 2-mercaptoethanol was added. From each solution of purified peptide, with an average concentration of 2 mg/ml, an amount of 1.5 mg of peptide was added to 1.5 ml of activated Blue Carrier, each. The reactions were given 2 h at RT. Then, about 20 µl of 1 M hydroxylamine was added. The putative protein-peptide conjugates were purified into PBS, using 8.3-ml PD-10 gel filtration columns. Ten fractions of 1.0 ml were taken each time, and protein was located using UV-photospectroscopy. After samples had been taken for SDS-PAGE, the pools were shock-frozen using liquid nitrogen.

Crosslinking of peptides to BSA, using EDC and sulfo-NHS

Purified peptides remaining from the Blue Carrier reactions were used. To a 1.0 mg/ml solution of BSA in 2.5 ml of 0.1 M MES, 0.5 M NaCl, pH 6.0 (MES) were added 1.0 mg of EDC and 2.8 mg of sulfo-NHS, and the reaction was given 15 min at RT. Then, 3.6 µl of 2-mercaptoethanol was added. From each solution of purified peptide, an amount of 0.3 mg of peptide was added to 0.3 ml of the solution of activated BSA, each. The reactions were given 2 h at RT. The putative protein-peptide conjugates were purified into PBS as described in the Blue Carrier experiment. Samples were taken for SDS-PAGE. The pools were shock-frozen using liquid nitrogen.

Crosslinking of peptides to Blue Carrier, using EDC alone in a one-step reaction

Of each peptide, an amount of 4 mg was dissolved in 0.1 M MES, pH 4.85 (MES), to a concentration of 10 mg/ml, and each was purified as described in the previous experiments, yielding an average concentration of about 2.9 mg/ml. An amount of 14 mg of Blue Carrier was diluted to a concentration of 10 mg/ml in MES. A volume of 200 µl of the protein solution was added to 500 µl of each peptide solution. Then, 50 µl of EDC, with a concentration of 10 mg/ml, was added to each, and the reactions were given 2 h on ice and 1 h at RT. Each protein-peptide conjugate was run on an 8.3-ml PD-10 gel filtration column with PBS, and fractions were screened, using UV-photospectroscopy.

Crosslinking of peptide molecules to BSA, using varying amounts of EDC

A volume of 100 µl of purified peptide with a concentration of 2.8 mg/ml in 0.1 M MES, pH 4.85 (MES) was added to 40 µl of a solution of BSA with a concentration of 10 mg/ml in MES. EDC was dissolved in MES, to a concentration of 20 mg/ml, and 2-fold, 4-fold, 8-fold, and 20-fold dilutions were rapidly prepared in MES. To volumes of 17.5 µl of the protein–peptide solution were added 2.5 µl of each solution of EDC.

The reactions were given 2 h at RT. Samples were taken for SDS-PAGE.

Discussion

The coupling experiment using EDC and sulfo-NHS to crosslink peptides to the Blue Carrier protein failed, as even the subunits of the protein were still resolvable by polyacrylamide gel electrophoresis, indicating that the crosslinking reagents were not active. This finding was confirmed by the control experiment using BSA instead of Blue Carrier. It was shown that alternatively, the peptides can successfully be crosslinked to a carrier protein, using EDC without sulfo-NHS in a one step reaction which has to be run for two hours at room temperature. Further, we confirmed the amounts of protein, of peptide and of EDC recommended for the crosslinking reaction. For coupling to BSA, ten parts of peptide at a concentration of at least 2 mg/ml, four parts of protein at a concentration of 10 mg/ml and two parts of EDC at a concentration of 10 mg/ml should successfully react to protein–peptide conjugates. One part of EDC should already be sufficient for coupling to the Blue Carrier protein, as it is much larger than BSA, and therefore exposes far less surface per mass.

The coupling reactions using sulfo-SMCC were assessed by polyacrylamide gel electrophoresis. The activity of sulfo-SMCC in the crosslinking reaction coupling Yip family peptides to the Blue Carrier protein was confirmed, as the subunits of the activated protein were no longer resolved on low-percentage polyacrylamide gels. In a control experiment, sulfo-SMCC was further shown to successfully crosslink these peptides to BSA. In the reactions crosslinking peptides to Blue Carrier, the absolute concentration of peptide was estimated to be larger, in average, than in the BSA reactions. Moreover, in the Blue Carrier reactions, the ratio of peptide to protein, too, was larger than in the BSA reactions. We therefore conclude successful crosslinking of the Yip family peptides to Blue Carrier protein, as the results of the control experiment are convincing. The Blue Carrier conjugates will be injected into rabbits for making them generate antibodies against Yip family proteins. The BSA conjugates in turn will be applied to purify the antibodies generated. These antibodies will help us finding out more about the interplay of Yip proteins together with Rab GTPases and other components of the vesicular transport machinery.

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Analysis of Cell Cycle Synchronization of Mammalian U-2 OS Cells for Investigating Localization of APC & EB1 to the Mother Centriole

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Introduction

Microtubules (MTs) are composed of the globular protein tubulin consisting of two subunits, α-tubulin and β-tubulin, both closely related 55-kD polypeptides. Due to the fact that MT arrays consist of a certain sequence of α- and β-tubulin, both ends of the array are clearly determined. The end which is located at the microtubule organizing center (MTOC), or centrosome, starts with β-tubulin and is called the minus end, while the other end starts with α-tubulin and is called the plus end of the MT (Fig. 1). MTs polymerize by β-tubulin binding to the α-tubulin binding site of the array. MTs are nucleated at the centrosome by a third type of

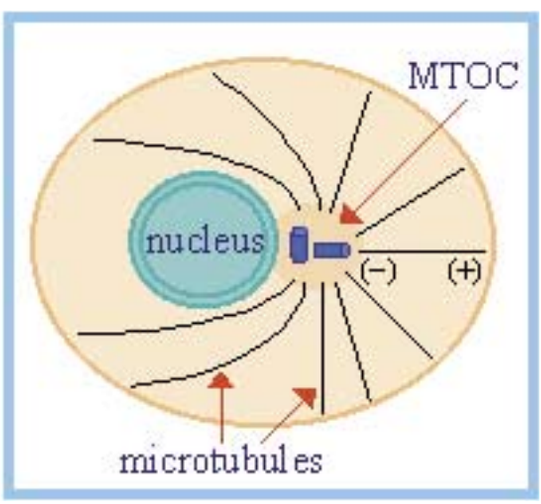

Fig. 1. MT and MTOC

tubulin, named γ-tubulin (Howard and Hyman 2003).
The bulk of the array is composed of tubulin dimers in which GTP bound to the β-tubulin is already hydrolyzed to GDP. This molecular structure is highly unstable and only the newly polymerized and not yet hydrolyzed section at the plus end, the GTP cap, prevents the array from depolymerizing (Fig. 2). If polymerization is slower than the hydrolysis of GTP, the GTP cap disappears and the GDP section of the MT is exposed. As a consequence, the MT depolymerizes. MTs stop shrinking

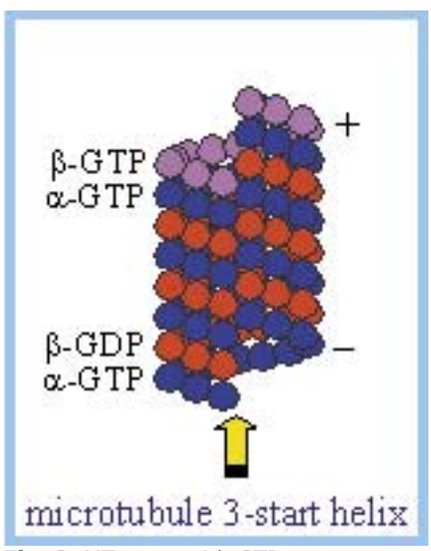

Fig. 2. MT array with GTP cap

when the concentration of tubulin is high enough for further growth, which means that the polymerization rate is higher than the GTP hydrolization rate.
MT plus ends are dynamically unstable, (Mitchison and Kirschner 1986): they undergo several rounds of growth and shrinkage that allow them to probe their environments. The transition from growth to shrinkage is called "catastrophe" and the transition from shrinkage to growth is called "rescue". Recently several proteins have been discovered that bind specifically to the plus ends and change the frequency of catastrophe and rescue. James Nelson's lab studies two such proteins: Adenomatous polyposis coli (APC) and, its C-terminal binding partner, EB1. In particular, it has been discovered that these plus-end MT-binding proteins localize to centrosomes, where the minus ends of MT reside.

Centrosomes comprise a pair of centrioles surrounded by a matrix of proteins named the pericentriolar material. Centrosomes nucleate and organize MTs into arrays important for different stages of the cell cycle. A G_1 centrosome has a pair of centrioles: one is the older centriole, leftover from at least two previous cell cycles; the other is the newer centriole, generated in the preceding cell cycle. The older centriole, which has distal and subdistal appendages, anchors MTs. The centrioles split between G_1 and S phase and duplicate during S phase. In G_2, the two pairs of centrioles have completely separated with associated pericentriolar material, and maturation occurs. At the onset of mitosis, mature centrosomes separate to the spindle poles where they will organize the mitotic spindle. The mother centriole anchors and focuses MTs at the different stages of the cell cycle. Recently, proteins have been found that specifically localize to the mother centriole throughout the cell cycle. It is believed that these proteins function in centriole duplication and microtubule organization.

Adenomatous polyposis coli (APC) is mutated in most colorectal cancers and is well-studied as a down regulator of the oncogene β-catenin. APC is also a regulator of the MT cytoskeleton indicated by its localization to cortical clusters and its ability to bundle MTs *in vitro.* End Binding 1 (EB1) binds the C-terminus of APC and microtubule plus ends. Louie et al. (2003) show the unexpected localization of APC and EB1 to the mother centriole independent of MTs. In particular, APC and EB1 localize to the mother centriole. To understand the function of these proteins at the mother centriole, we sought to determine whether their localization to the mother centriole is cell cycle dependent.

Long-term goal: Analysis of localization of APC and EB1 to the mother centriole in different stages of the cell cycle

Short-term goal: Analysis of cell cycle synchronization of U-2 OS cells by mitotic shake off method.

Eukaryotic Cell Cycle

Auto-reproduction is essential for cell life. During the cell cycle, growth and division of the cells lead to cell multiplication. Each mother cell gives rise to two identical daughter cells. According to the terminology introduced by Howard and Pelc in 1951, the eukaryotic cell cycle is divided into four coordinated processes: Gap1 (G_1), synthesis (S), Gap2 (G_2), and mitosis (M).

During mitosis, DNA is condensed, the nuclear envelope breaks down, and the mitotic spindle is formed. Chromosomes line up at the metaphase plate and are separated during anaphase to opposite poles of the cell. Cells complete division during cytokinesis. Mitosis is the shortest phase of the cell cycle, taking place in less than one hour. About 95% of the cell cycle is spent in interphase that is divided into the three subphases: G_1, S, and G_2.

G_1 and G_2 provide time for cells to grow and to monitor the internal environment (DNA damage, abnormal cellular structures, etc.) and the external surrounding (presence of growth factors, cell density, etc.) in order to check if the conditions are appropriate and the preparations such as protein synthesis are complete for entering the next phase.
During S phase, the cell is synthesizing and replicating its DNA. Additionally, the cell continues growing.

To accumulate a population of cells in each stage of the cell cycle for analysis, we attempted cell cycle synchronization of mammalian U-2 OS cells by the mitotic shake off method.

Experimental

U-2 OS Cell Cycle Synchronization by Mitotic Shake Off

The mitotic shake off method was chosen because Chang and Stearns showed that it was successful for synchronization of U-2 OS cells and because we did not want to add any drugs that would affect centrosome number, cell morphology, or DNA content (Chang and Stearns 2000). Mitotic shake off allows collection of cells only in mitosis, which means they are all in the same phase of the cell cycle and should grow in synchrony with each other throughout the next cell cycle. We let four T-flasks of U-2 OS cells grow until they covered 80% of the area they are plated on. Cells in mitosis adhere less to each other and to the plate. In order to concentrate cells in mitosis, we literally shook the mitotic cells off each plate by hitting the plate softly against the hood wall at one hour time intervals. Mitotic cells that were shaken off were plated onto polylysine-coated coverslips and allowed to grow

for 30', 1, 2, 4, 6, 8, 10, 12, 14, 16, and 18 hours before fixing and processing for immunofluorescence.
To determine whether or not cells were synchronized in G_1, S, and G_2 phase of the cell cycle, we stained cells from each time point with antibodies against BrdU, MT/centrin, and ε-tubulin/centrin.

Results

BrdU Labeling

During S-phase of the cell cycle DNA is duplicated. Cells incorporate BrdU in place of deoxy-thymidine during this duplication phase. BrdU incorporation is detected by immunofluorescence. –We processed cells at each time point for BrdU incorporation and analyzed when cells were in S-phase. We found that most cells were in S-phase at the 10-hour time point, and that the number of S-phase cells was very low at the 8-hour and 18-hour time points. Most cells seem to enter S-phase at the 6-hour time point (Figs. 3-5).

MT/Centrin/DNA Staining

To make sure we were only shaking off cells in mitosis, we fixed and stained cells 30 minutes after shaking them off. By looking at their DNA and cell morphology we were able to determine the number of mitotic vs. interphase cells shaken off. To truly synchronize cells, we do not want contamination of interphase cells that will not be in synchrony with the mitotic cells. We found that at most time points we shook off 60% of mitotic cells.

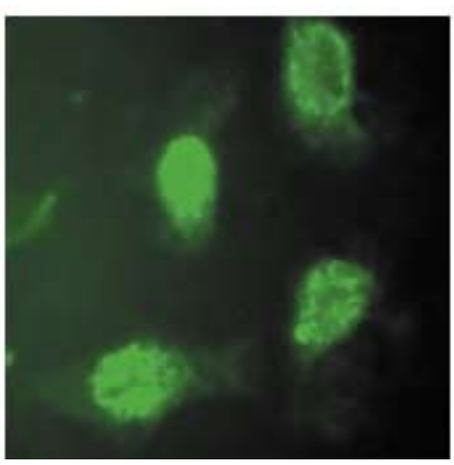

Fig. 3. Cells at 6-hour time point: all cells incorporated BrdU *(green)*

ε-Tubulin/Centrin

The number of the centrosomes and centrioles will also reveal if the shake off was successful. In G_1, cells have only one centrosome with two centrioles. After passing centrosome duplication in S phase, they have two centrosomes with four centrioles in G_2 phase. ε-Tubulin differentiates the old and new centrosomes because it localizes to the mother centriole in G_1, and begins to localize to the new mother centriole in S phase. ε-Tubulin localizes to both centrosomes equally in G_2 phase (Chang and Stearns 2000). We counted the number of cells with two to four centrioles at the earlier time points to determine whether our cells were synchronized. We expected that cells at the early time points are synchronized in G_1 phase and have two centrioles. Instead, only 30% of the cells had two centrioles.

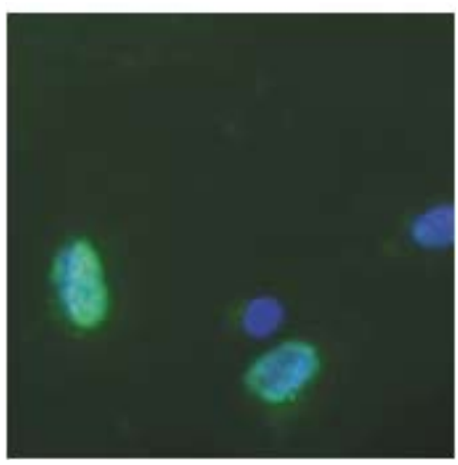

Fig. 4. Cells at 12-hour time point: 50% incorporated BrdU *(green)*

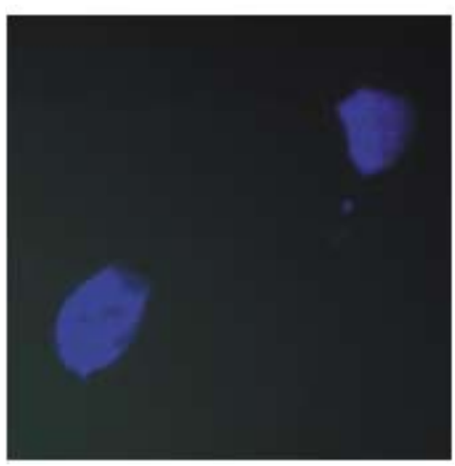

Fig. 5. Cells at 18-hour time point: all cells without BrdU *(blue)*

Conclusions

BrdU Labeling
If the cells were synchronized, we expected to observe a smooth transition into and out of S-phase. The duration of the cell cycle for U-2 OS cells is 12–18 hours. Therefore, the cells would go into S-phase (incorporate BrdU) by 6 hours and enter G_2 (no longer incorporate BrdU) sometime before 12–18 hours. (Figs. 3-6) Our results do not show a smooth transition into and out of S phase. Most cells entered S-phase together but there was a drop in S phase cells at 8 hours and an increase at 10 hours. Cells seem to remain in S-phase until 18 hours where there is a drop again.

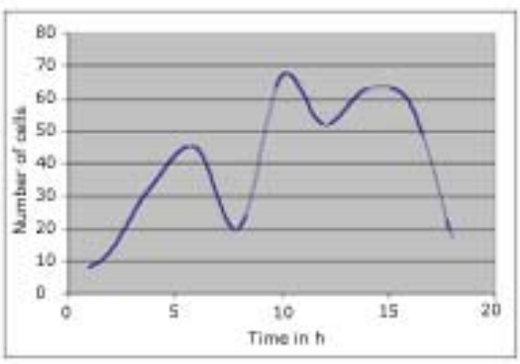

Fig. 6. Number of cells that incorporated BrdU

MT/Centrosome/DNA Staining
There was contamination of interphase cells at some time points that may have caused the lack of synchrony in the cells. The number of mitotic cells shaken off should have been over 85% at each time point to truly synchronize cells.

ε-Tubulin/Centrin
Analysis of centriole number shows that cells were not synchronized. This may be due to contamination of interphase cells and because U-2 OS cells are cancerous and may have defects in centriole duplication or structure. Because centriole number did not clearly define cell cycle stage, we were unable to show the cell cycle dependent localization of ε-tubulin, which was a positive control for this experiment.

Outlook

Cell cycle synchronization of human U-2 OS cells by mitotic shake off requires improvement as shown by BrdU incorporation, DNA and cell morphology 30 minutes after shake off, and centriole number at each time point. Because U-2 OS cells are cancerous they have defects in DNA content, cell cycle progression, and centriole duplication and/or structure. To eliminate these

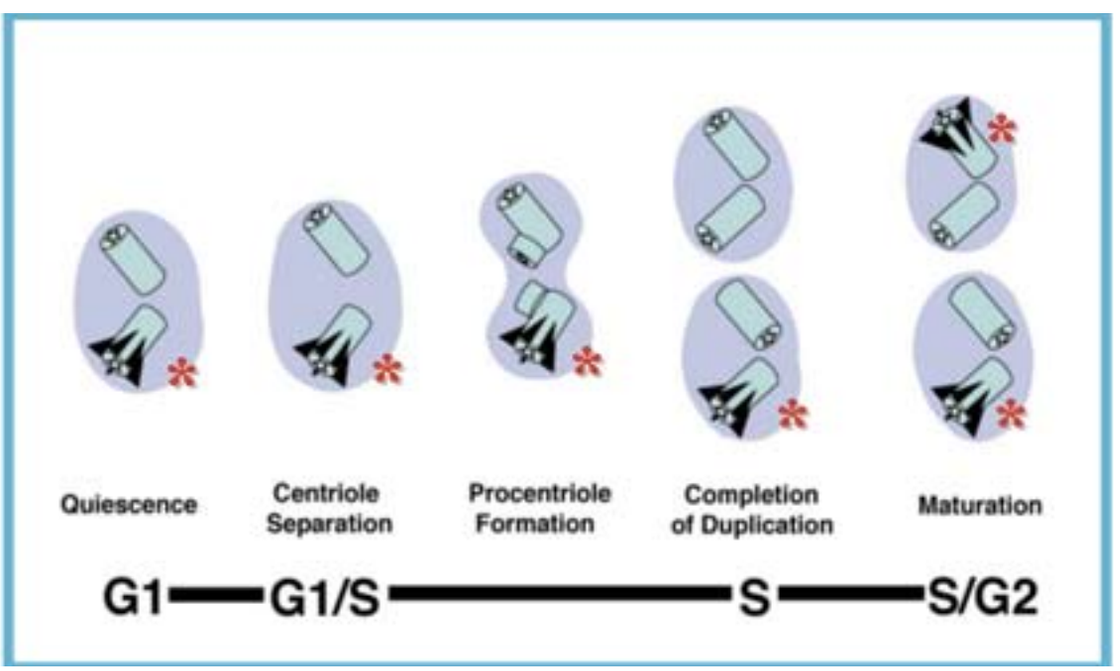

Fig. 7. Centriole duplication during the cell cycle

problems BHK-21 cells could be used instead for synchronization. These Hamster Ovary cells are wild-type diploid cells with a short cell cycle period. However, human antibodies we used for immunofluorescence may not react in this cell type. In addition, they may have poorly defined centrosome morphology. Another possibility is to isolate diploid U-2 OS cells using FACS by sorting out cells with high DNA content. However, these cells can only be used for a few generation times before the abnormal cells will have to be sorted out again.

Acknowledgements

Our sincere appreciation is expressed to Prof. James Nelson who made this internship possible; we thank all members of the Nelson Lab and especially Shirin Bahmanyar and Frauke Drees for their support and acceptance – an invaluable experience which significantly will influence our careers.

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Figure Credits

Fig. 1: http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/microtub.htm

Fig. 2: http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/microtub.htm

Fig. 6: Louie et al. (in press)

Immunoblot – A Tool for Identifying Proteins and Calculating Protein Concentrations in Cells

Monika Gessat, Laboratory of Suzanne R. Pfeffer,
Department of Biochemistry, Beckman Center, School of Medicine, Stanford University

In the days of my studies at university in the 1970s the activity and the concentration of enzymes were often determined by reactions that could be pursued by optical measurements – provided a suitable enzymatic reaction was available. In-vitro or in-vivo synthesized proteins could be labelled with tritium or other radioactive isotope and identified by autoradiographic methods after SDS-PAGE; gels were larger than nowadays, the verification of a specific protein was difficult. The use of antibodies, especially monoclonal antibodies, has changed the job. The specificity of the antigen–antibody reaction allows the identification of almost any protein either in vitro or in living cells: immunoblotting and immunofluorescence microscopy are fascinating tools of modern biochemical research.

Introduction

Cells synthesize, modify, and degrade molecules at the same time, by separating individual reactions into membrane-enclosed compartments. Compartmentation, on the other hand, requires a tightly regulated transport of enzymes, other proteins and lipids between different membrane-bounded organelles. The transport between organelles occurs through vesicles that bud from the donor compartment carrying proteins and lipids as cargo and then moving to the acceptor compartment, where fusion of the membranes delivers the cargo into the acceptor compartment.
Trafficking by vesicles requires several signals that manage:

- selection of the cargo by receptors situated in the membrane of the budding vesicle
- transport of the vesicle
- fusion of the vesicle with the correct acceptor compartment by reciprocal recognition
- transport of the cargo-receptor back to the donor compartment.

A major goal of the research of Professor Suzanne R. Pfeffer and her group is to understand the molecular basis of receptor trafficking in mammalian cells. To study these processes, the vesicular transport of mannose 6-phosphate receptors (MPRs) from late endosomes back to the trans-Golgi network (TGN) is under investigation. MPRs are the cargo-receptors for hydrolases transporting them from the TGN to prelysosomes. The acidic pH within prelysosomes triggers the release of the hydrolases; the empty MPRs are returned in vesicles to the Golgi for another round of transport.

Among others, two proteins are required for MPR transport. The cytosolic protein TIP47 recognizes and binds directly to the cytoplasmic domains of the two cellular MPRs (Diaz and Pfeffer 1998; Orsel et al. 2000). TIP47 function requires interaction with the Rab9 GTPase. Although MPRs are located in the Golgi complex, at the cell surface and in late endosomes, TIP47 shows preferential binding to MPRs that are present in Rab9-containing late endosomes by increasing the affinity of TIP47 for these MPRs. Thus, late endosomes form a vesicle carrying the collected MPRs from endosomes back to the TGN (Caroll et al. 2001; Pfeffer 2001). The interaction between TIP47 and Rab9 in cells is one of the questions under investigation in the lab of Professor Pfeffer.

Results and Discussion

For many reasons it is interesting to know the approximate concentration of proteins in cells, especially when looking for interactions between proteins.
The first approach of my project was to estimate the relation of TIP47 and Rab9 in cells (Figs. 1 and 2). Lysates of HeLaS3 cells (Fig. 1 A, lane 1 and 2) were separated on SDS-PAGE together with purified Rab9 (Fig. 1A,

lane 3–5). Detection by immunoblot showed a specific, singular band at 22 kDa, the known molecular weight of Rab9, consistent with the fact, that monoclonal antibodies were used.
TIP 47 has a molecular weight of 47 kDa. In lysates of the HeLaS3 cells (Fig. 2 A, lane 1 and 2) polyclonal antibodies detected two protein

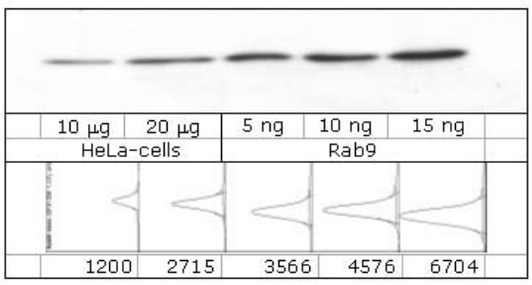

Fig. 1. Lysate of HeLaS3-cells contain less than 5 ng of Rab9/ 20 µg protein. Samples containing indicated amount of protein were run on SDS-PAGE; blots were analyzed by monoclonal anti-Rab9 antibodies. The amount of Rab9 was calculated by densitometric scanning of the blot

fractions, a lighter protein at 47 kDa which represents TIP47 and an additional unspecific staining at higher molecular weight. Additionally, some degradation of the purified TIP47 occurred as can be seen by faint stainings of lower molecular weight. The slight increase of molecular weight for the purified TIP47 is due to the tags of the used recombinant TIP47 (Fig. 2A, lanes 3–5).
The amount of Rab9 and TIP47 in both blots was determined by densitometric scanning (Figs.1 B and 2 B). For the Rab9 only a vague estimation is possible, but at least 2.5 ng of Rab9 can be assumed in 20 µg of protein

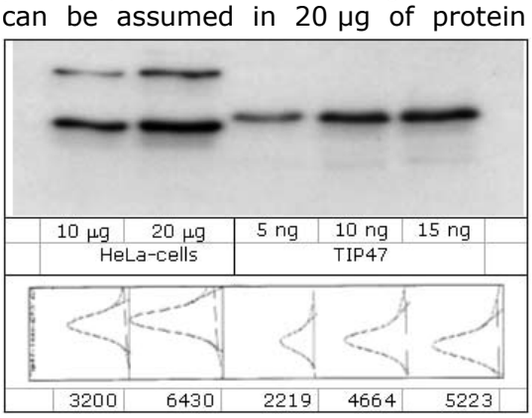

Fig. 2. Lysate of HeLaS3-cells contain 7.5 ng of TIP47/10 µg protein samples containing indicated amount of protein were run on SDS-PAGE;blots were analyzed with polyclonal anti-TIP47 antibodies. The amount of TIP47 was calculated by densitometric scanning of the blot

from HeLaS3 lysates. Considering the molecular weight of Rab9, about 3.4×10^9 molecules of Rab9 per µL lysate can be detected.
For the TIP47 the data allow a more precise calculation. 10 µg of protein from lysates of HeLa contain about 7.5 ng of TIP47. Considering the doubled molecular weight of TIP47 the number of protein molecules is in the range of 9.6×10^9 molecules per µL lysate.
In HeLaS3 cells the concentration of TIP47 molecules is two to three times higher than the concentration of Rab9 molecules. This may suggest a regulatory role of Rab9 for the action of TIP47.

Since 1998, siRNA has become a perfect tool for probing gene expression (Hannon 2002). A further current project in the lab is to investigate the effect of suppression of Rab9 expression by small doubled-stranded RNA (siRNA) on MPR retrieval and on the function of TIP47. The synthetic oligonucleotides of the Rab9siRNA are introduced in cells by incubating HelaS3 cells with a liposome–siRNA mixture. The siRNA employed is a construct of just 21 nucleotides which is complementary to Rab9 mRNA between position 282–302 of a 1080-nucleotide-long sequence.
I tested the efficiency of the siRNA treatment. Figure 3 shows the nearly complete depletion of Rab9 in siRNA-treated HeLaS3 cells compared to buffer-treated cells. The small amount of staining can be due to cells that did not take up the siRNA.
It is not clear whether and how cells survive the siRNA treatment. According to our knowledge absence of Rab9 should produce a severe disruption of traffic in the cell. siRNA-treated,cultured cells cease with mitosis for about 3–4

Fig. 3. siRNA inhibits expression of Rab9 lysates of HeLaS3-cells containing 20 µg protein were run on SDS-PAGE; blots were analyzed with monoclonal anti-Rab9 antibodies

days, then the cell number increases again. It is likely that a small number of non-transfected cells survive because they did not take up the siRNA. Current and further investigation in the lab shall give answers to these questions.

Methods

SDS-PAGE was performed on gels with 12.5 or 11% acrylamide/bis, 1% SDS at pH 8.8; samples were heated in loading buffer/SDS/mercaptoethanol to 100°C for two minutes before loading, gels were run at 100 V. Proteins were blotted at 250 mA/gel for 1 h on a nitrocellulose membrane. Concentration of primary anti-TIP47 polyclonal antibodies was 1:2000 in 6% milk protein/buffer; for the Rab9 monoclonal antibodies from supernatant of mouse-hybridoma culture were used as primary antibody source; the concentrations of secondary antibodies coupled with horse radish peroxidase were 1:4000 in 6% milk protein/buffer; incubation time with primary and secondary antibodies was 45 min; the luminol–H_2O_2-chemoluminescence reaction was used to detect the protein-antibody complexes. The exposition time to X-ray films varied between 1-5 sec.

Acknowledgements

I would like to thank Professor Suzanne R. Pfeffer for providing the opportunity of an internship in her lab and to Ian Ganley who surveyed my experiments.

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"Working with the Worm" – Genetic Pathways in the Aging Worm and Intestine-Specific Gene Regulation

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Beckman Center, School of Medicine, Stanford University

Indroduction

The nematode worm *Caenorhabditis elegans* (Fig.1) is one of the most intensively studied animal models in molecular biological research. Being as small as about 1 mm in lengths, the worm normally inhabits soil and feeds primarily on bacteria. The life cycle lasts 3 days with a usual life span of 14

Fig. 1. *C. elegans*

days, both strongly depending on the conditions in the worm's environment. There are two sexes, a hermaphrodite that produces both oocytes and sperm and a male that only produces sperm cells and occurs at low frequency. By self-fertilization the unmated hermaphrodite can lay 300 eggs during its reproductive life span. The short reproduction cycle and the possibility to grow *C. elegans* on agar plates together with its anatomic and genetic simplicity makes it a perfect model for scientific investigations in many aspects of modern biology.

The work with the worm started to spread in the late 1960s after Sydney Brenner`s pioneer work on *C. elegans*. The worm's transparency allowed him to determine the entire cell lineage (Fig. 2) and by that predict the fate of every single cell out of 959 somatic cells that finally build up an adult hermaphroditic worm. The entire genome has now been sequenced, giving biologists the opportunity to connect the genotype or the gene expression profile of a worm with its phenotype. As a powerful tool for determining gene expression in a worm under special conditions or in a specific tissue, the microarray technology can provide large sets of data. These data can be used to solve scientific questions on diverse issues in biology, such as genetic pathways in development or aging.

The "Kim Lab" at Stanford University is one of the leading research groups worldwide, using the microarray technique to investigate worm genetics in development and aging of *C. elegans*.

Project I Description

Aging in C. elegans

For decades, aging was considered as the deterioration of the body in the

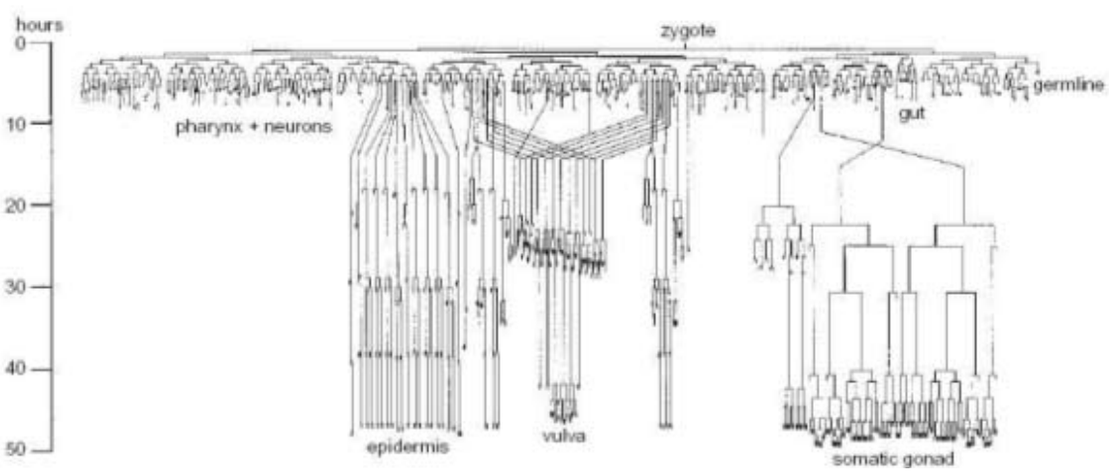

Fig. 2. Cell lineage of *C. elegans*

post-reproductive phase that does not underlie natural selection. The findings of genetic mutations that could shorten or prolong – in the case of the insulin/ IGF-1 receptor DAF-2 even double – the life span of an organism abolished this historic theory of aging and suggested genetic pathways that could cause or fight the aging process. Like DAF-2, most of the genes that are believed to influence aging are linked to metabolic pathways, confirming the importance of reactive oxygen species, like hydrogen peroxide, in the aging process. In many cases, the higher the metabolic rate of an animal, the shorter is its life span, which contributes to the production of reactive oxygen species in metabolism that can harm cellular components and thereby trigger the aging process. This, however, must be regarded as an oversimplification, because animals that have a high metabolic rate, but nevertheless are long-living, like birds and primates, show that the way a cell handles intracellular stress is even more important for a "forever young" phenotype. Additionally, reactive oxygen species have physiological functions in body homeostasis as they trigger signaling pathways, for example, smooth muscle relaxation by NO, or in host defense by granulocytes. For normal growth and metabolism it is necessary for a cell to maintain a balance between reactive oxygen species production and elimination. Multiple mechanisms that are used by a cell to keep homeostasis have to be fine-tuned by genes, in many cases genes that will correlate with genes that influence the aging process.

Microarray experiments of different time courses in *C. elegans* suggested several genes that are either up- or downregulated during the aging process of the worm.

One way to prove and understand the role of these genes in aging is to locate gene products, showing where, as well as how long, they are expressed during development, adulthood, and aging of the worm. By the technique of microinjection, DNA constructs can be injected directly into the gonads to yield worm strains containing GFP (green fluorescent protein)-labeled gene products (Fig. 3).

The GFP makes the gene product visible under a fluorescent microscope and helps to get an insight into its specific location and its role in aging.

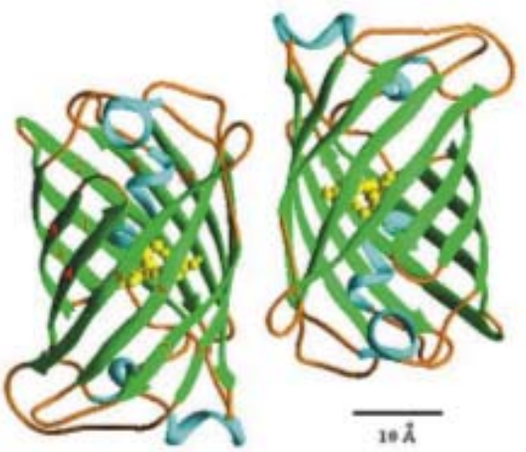

Fig. 3. Green Fluorescent Protein (GFP)

Project II Description

Tissue-specific expression of genes and their regulation by GATA motifs

Every somatic cell that builds up a complex organism contains the whole set of genes. A muscle cell, for example, has the same genome like a neuron, even if properties and function differ largely from each other. This specificity of cells that determines tissue function can be explained by epigenetic processes like imprinting or by different signal molecules that alter cellular gene expression. The tissue-specific gene expression pattern is regulated by transcription factors that bind to DNA motifs in the promoter region and thereby regulate the activity of a gene. By tissue-specific RNA isolation, the *Kim Lab* was able to produce DNA microarrays that show which genes, for example, in gut or muscle cells, are upregulated and are active in this special kind of tissue. By bioinformatic approaches the tissue-specific genes could be divided into several groups, each influenced by different regulatory units. To show which role a GATA motif, a special DNA regulatory binding site, plays on gene expression in the intestine, we chose genes out of the intestine-specific gene list that are regulated by a GATA motif and are either clustered or non-clustered. For these genes we constructed GFP

reporter genes that will help to visualize the location of the gene product under a fluorescent microscope.
The next step will be to disturb GATA motif regulation on gene expression by site-directed mutagenesis and to observe the influence on the phenotype of the worm. These experiments will lead to a better understanding of how gene expression is orchestrated, creating cell diversity of a multicellular organism.

Methods and Results

Although my project during the internship did not involve microarray experiments, all projects in the *Kim Lab* are based on the technique; a short overview of the procedure will help to understand the experimental settings. The idea of DNA microarrays is based on base-pairing of homologous sequences. A gene that is active in a tissue at a certain time will transcribe its gene sequence into a mRNA strand in order to produce a certain gene product. To detect gene expression on a microarray, the first step is mRNA isolation from an organism or a special tissue. By a reverse transcriptase the mRNA sequence can be transcribed into a cDNA strand and by chemical coupling the cDNA samples get labeled with the fluorescence markers Cy3 or Cy5, usually a wild type with Cy3 and a mutant probe with Cy5. Two labeled cDNA probes can now be simultaneously hybridized to a DNA microarray which contains thousands of 200-µm-small DNA dots of a genome. By base-pairing, the isolated cDNA samples bind to the genes, indicating the active genes. The ratio between Cy3 and Cy5 can be determined via quantification of emission values and gene expression patterns can be compared between the probes by bioinformatic analysis (Fig. 4).

1. Methods Project I - Aging in *C. elegans*

a) Microinjection
DNA transformation assays in a whole organism provide experimental links between molecular structure and phenotype. Experiments with transgenic *Caenorhabditis elegans* start in general with the injection of DNA into the adult gonad, which is built up as a syncytium and consists of two U-shaped arms (Fig. 5).

Extrachromosomal transformation is the most likely heritable DNA transformation after DNA injection.
These extrachromosomal DNAs can be integrated into the genome using radiation, yielding a worm strain that transmits the injected gene from one generation to the next without loosing the extrachromosomal DNA in the majority of progenies.

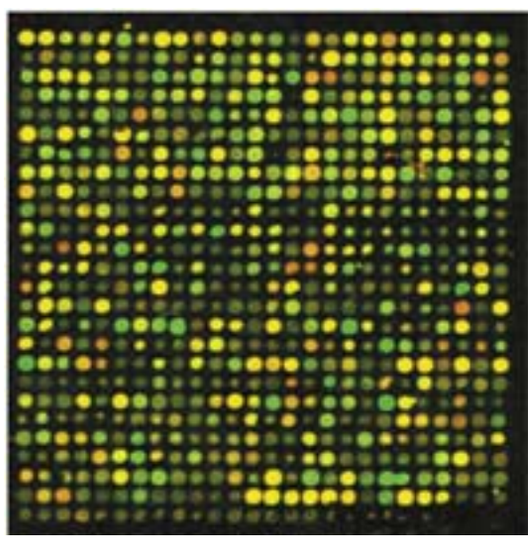

Fig. 4. DNA Microarray

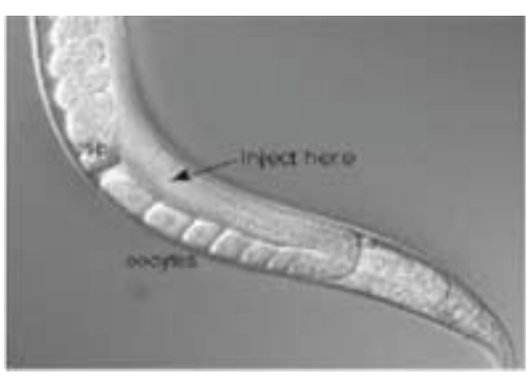

Fig. 5. DNA injection site

b) DNA constructs
DNA constructs of a GFP reporter gene, each including one of the genes that are believed to be aging related, were injected into the gonads of *unc*-119/ fer-15 double mutants.
C. elegans animals which are mutant for the *unc-119* gene exhibit abnormalities

in movement, sensory, and behavioral traits that can be detected by light microscopy.
The injected DNA construct additionally contains a *unc-119* rescue sequence, so that animals with successfully integrated plasmids can easily be separated from animals without the aging gene by normal movements. f*er*-15 mutants are temperature-dependent and fail to have gonad organogenesis at a temperature higher than 25°C. Initially these mutants were used to isolate mRNA for the microarray experiments. The infertile phenotype restricted the isolated mRNA to gene expression products from the adult worm without including potential gene expression by eggs inside the gonad.

c) Microinjection procedure
Step 1: Microinjection needles were produced from glass capillaries with a needle puller and filled with 1-1.5 µl DNA. The tip of the needle gets sufficiently filled by capillary forces. The optimal needle should be stiff enough to allow penetration of the animal‘s cuticle without bending and wide enough to not get clogged by the DNA, but narrow enough to not cause the animal to hemorrhage after withdrawal. As the tip is welded shut after melting and pulling, it has to be broken to the perfect tip size. After placing the loaded needle to the collar of the instrument holder, a broken object slice on a worm pad is used to gently crush the tip under microscopic observation.

Step 2: Worms move by swimming on a layer of moisture located between their body and the substrate. To immobilize them we used injection pads with a thin layer of dried 1.5% agarose, which deprived them of the layer of moisture and made them stick to the pad. After spotting a drop of oil on the pad, the worms could be transferred from the petri dish to the injection pad with a platinum „worm pick“ and, by gently rubbing them into the agarose, they could be fixed.

Step 3: The needle is under controlled nitrogen pressure. By increasing the pressure, the DNA flows through the tip of the needle. After bringing the worm and the tip into focus under 10-fold magnification, the tip can be moved to the close proximity to the gonad of the worm. Now the objective lens can be switched to the 40-fold magnification to position the tip in the middle of the distal gonad. By a gentle tap the tip can penetrate the cuticle and the membrane of the gonad. Pressure is then applied to the needle, causing DNA solution to infiltrate the gonad syncytium. After withdrawal of the needle the pressure can be turned off and the second gonad arm can be injected. Since mounted worms will slowly desiccate, which reduces their viability, it is important to transfer them soon after injection to the agar plate for recovery. A drop of M9 buffer on the worm pad around the injected animals can free them and with a wide-opening pipet the worms can be moved to the new culture plate.

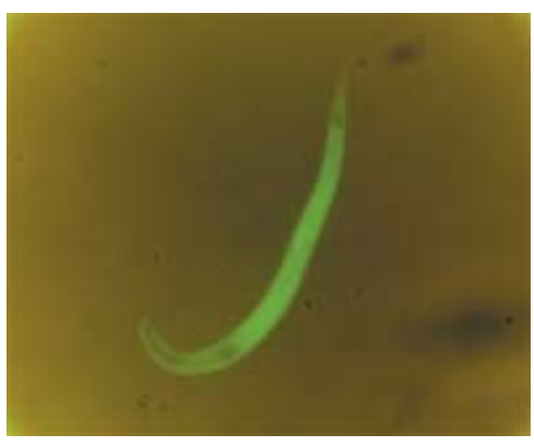

Fig. 6. Young worm (L1).
Gene product expression in gut cells

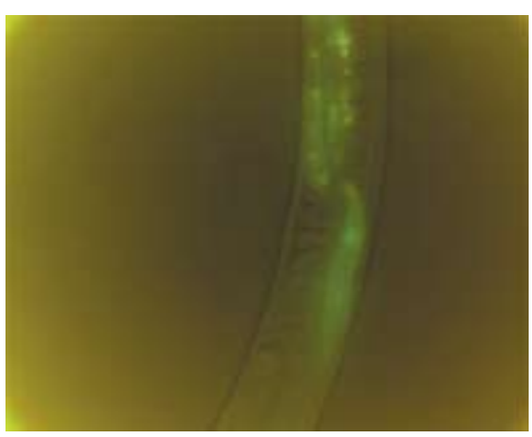

Fig. 7. Adult worm.
Gene product expression in gut cells

Results Project I

We obtained a *C. elegans* strain, injected with F35E12.5. Gene products of this gene are expressed in the intestine (Figs. 6 and 7) and in the muscle cells of young worms, but not in the cuticle cells or nerve cells. Furthermore, gene expression seems to decrease during aging of the worm, but this cannot be verified until worms are selectively observed during their whole life span. The expression pattern in the gut and in muscle cells - both cell types that have a high metabolic rate, but show decreased activity in elderly animals – could explain the gene's impact on aging.

2. Methods Project II

Intestine-specific expression of genes and their regulation by GATA motifs:

a) Vector for tagging of C. elegans exons with GFP. The microarray experiments suggest a list of 635 genes that are intestine-specific in *C. elegans*. 208 of these genes seem to be regulated by GATA motifs, without being part of an operon. We chose three genes coding for transmembrane proteins out of 12 possible genes and one trypsine-inhibiting enzyme to insert them into the GFP vector pPD114.35.
The GFP vector pPD114.35 consist of 3963 base pairs overall, 852 base pairs coding for GFP leaving 3111 base pairs of non-coding region. The GFP site is flanked by multiple cloning sites, containing a variety of rare-cutting restriction sites.
The exon insertion of the gene will take place with one part upstream of the GFP coding region and one part downstream of the GFP coding region. This will lead to a gene product that contains the 238 amino acids of GFP, in many cases without disturbing protein function and location.
We created a vector digestion with four different restriction enzymes leading to four different restriction sites that guarantee a proper insertion of the gene parts. As an example the construction of one of the four genes is described below.

b) GFP reporter gene for C25E10.8 (trypsine inhibitor-precursor).
We digested pPD114.35 with Sac II, Kpn-1, Eag-1, and Hind III leading to a vector 3‘ Sac II RS (restriction site),

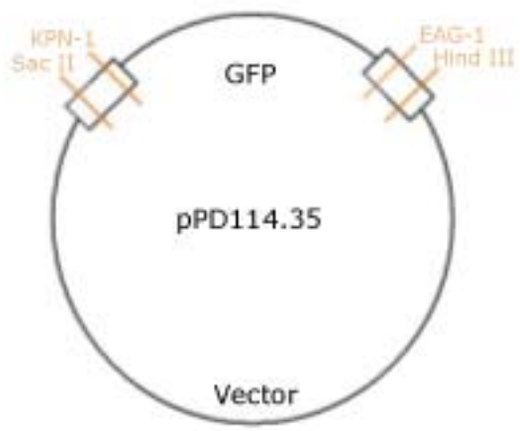

Fig. 8. Restriction sites

a GFP 5‘ Kpn-1 RS, a GFP 3‘ Eag-1 RS, and a vector 5‘ Hind III RS (Fig. 8).
To insert the trypsine inhibitor precursor gene into the multiple cloning sites, we created four primers that contain the proper restriction enzyme cutting sequence, specifying the insertion site of the gene part. PCR DNA amplification using these primers will lead to two strands of the original gene, divided in Intron 2. In a one-step procedure the PCR products can be inserted into the vector.

The trypsine inhibitor-precursor gene consists of a 644-bp sequence, with 414 coding base pairs, separated by 3 introns and a 300-bp upstream regulatory region.

The primers were modeled with the program ‚Oligo - Primer Analysis Software, Version 6.13".
The complete gene sequences were from the ‘Wormbase’ data base.

Primer I is 26-bp long with a
5’ TCTCCGCGG restriction site (RS) sequence for Sac II: TCTCCGCGGGTGT GATGAAGGAAACTGGAGTTGTG

Primer II is 34-bp long with a
5’ GGGGTACC RS sequence for Kpn I and a G insertion for inframe reading:

GGGGTACCGCAAATCCTTTCTTGCAGTC
GCATGC

Primer III is 33-bp long with a
5’ CCCGGCCG RS sequence for Eag-1 and a CC insertion for inframe reading: CCCGGCCGCCTCTGCTCAATGTATTGAA
AACGC

Primer IV is 36-bp long with a 5’CCCCGGAAGCTT RS sequence for Hind III: CCCCGGGAAGCTTGGCAGAAC
CTTTCTGACGCGTACC

Results Project II

In computer modeling the primer seemed to work sufficiently. The GFP reporter gene has not been injected into worms until now.

Acknowledgements

I would like to thank Prof. Stuart Kim, the members of the *Kim Lab* and especially Andreas Eizinger for the great time at the Beckman Center and for everything I learned during the internship.

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Genes Involved in the Transport of the Chitin Synthase Chs3p in *Saccharomyces cerevisiae*

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Abstract

We report here about three different techniques for detecting genes involved in the transport of chitin synthase III in *S. cerevisiae*. Genetic analyses suggest that ylr*112w*, a gene on chromosome 12 of the yeast genome, is not involved in the transport of Chs3p. Furthermore, we show that none of the sorting nexin mutants we tested affect the trafficking of Chs3p.

Introduction

Chitin synthase III (Chs3p) is an enzyme that synthesizes chitin in the cell wall of *Saccharomyces cerevisiae*. In the wild-type (WT) cell, it achieves steady-state localization through cycling between the trans-Golgi network (TGN) and the early endosome (EE). During cell division Chs3p is transported to the cell surface where it polymerizes chitin, a part of the cell wall (Shaw et al. 1991).

Mutations in the Chs3p transport can be identified by the use of the chitin-binding drug calcofluor (see Materials and Methods).

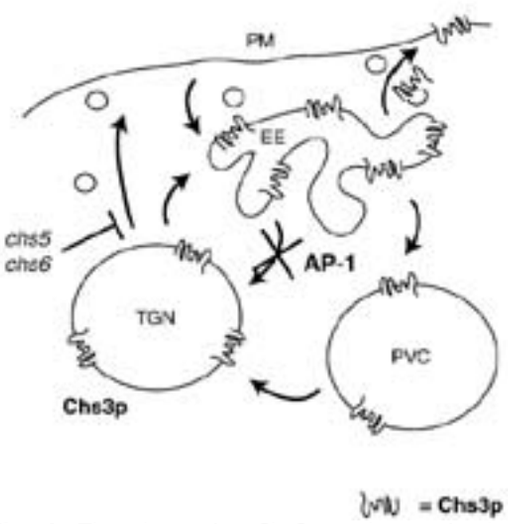

Fig. 1. The alternative Chs3p pathway via the early endosome in a AP-1 Δ

The Chs3p transport is investigated because it is an example of a regulated protein transport in yeast. Thus, Chs3p is taken as a model protein for the secretory pathway in eukaryotic cells.

In the WT cells Chs3p is transported from the TGN to the plasma membrane (PM). This pathway is mediated by Chs5p and Chs6p. In a *chs6*Δ cell, Chs3p is no longer transported to the PM but cycles between the EE and the TGN. An interesting phenotype appears with an additional mutation in a subunit of the clathrin vesicle (e.g., *apm1*). In the *chs6*Δ*, apm1*Δ double-mutant, Chs3p is again transported to the PM due to an alternative pathway which is opened via the EE (*Fig. 1, Valdivia et al. 2002: The alternative Chs3 transport in a AP1Δ).

So far seven mutations – all in genes that encode components of the clathrin vesicles – which open this pathway have been described. Here we try to identify more mutations that restore the transport of Chs3p to PM in *chs6* cells.

It was suggested that *ylr112w*, a gene on chromosome 12 of the yeast genome might be a co-protein like Chs6p which mediates the pathway via the EE.

To develop a strain with a certain genetic background mutants were crossed, sporulated and tetrads were dissected. We use this genetic approach for a knockout of *chs6, apm1,* and *ylr112w* to get an understanding of the correlation between these genes. Another way we used was cloning by complementation in order to identify further genes involved in the alternative pathway.

Materials and Methods

Strains and Plasmids
Plasmids (p366) from a genome library containing a –leucin (–leu) marker were used to transform TLS56 cells.

Strains used in this study:

Name	Genotype
TLS56	
YRV19	*Mata chs6Δ::HIS3 ade2-101oc his3-Δ200 leu2Δ trp1 ura 3-52*
YRV95	*YRV19 apm1Δ::URA3*
	Mata Chs6Δ::HIS3, apm1Δ:: URA3
	Mata Chs6Δ::TRP1, apm1Δ:: ura3
	YLR112WΔ::G418
BY4742	*MATα his3 leu2 lys2 ura3*
imh1	*BY4742 imh1::G418*
YKR078w	*BY4742 ykr078w::G418*
YPR 097w	*BY4742 ypr097w::G418*
YHR 105w	*BY4742 yhr105w::G418*
BY4741	*MATa his3 leu2 met15 ura3*
snx4	*BY4742 snx4::G418*
snx41	
snx42	
mvp1	
grd19	
vps5	

Cell Fractionation and Gradient Analysis
A gradient density centrifugation protocol was used: "One-step fractionation of total yeast membranes on step sucrose gradients" (Valdivia 2002). The strains YRV19, YRV95, and TLS56 were fractionated using eight OD_{600} of cells.
The cells were treated with 350 µl TEA lysis buffer (20 mM triethanolamine, pH 7.2, 1 mM EDTA, 5% sucrose) and protease inhibitor (2 mM PMSF) and opened with 100 µl of glass beads in the bustulator at 4°C for 10 min. 2400 µl of lysate containing the membranes were loaded on a sucrose gradient of 35% (400 µl), 45% (500 µl), and 55% (200 µl) sucrose and centrifuged in 7/16x 13/8 Beckman tubes at 55K for 2.5 hours. 200-µl fractions were collected from the top and loaded on a sodium dodecylsulfate (SDS) polyacrylamide gel, blotted on a nitro-cellulose membrane using antibodies against Chs3, Sec12 (Golgi marker), and Tlg1 (ER marker). The exposure time was 10 min.

Lithium Acetate Transformation
TLS56 cells were transformed by a lithium acetate protocol for bringing in the plasmid through a single-strain carrier-DNA. Cells were pelleted and re-suspended in LiCl 0.1 M. The cells were centrifuged and LiCl was removed. 240 µl of PEG, 36 µl of LiCl 1M, 10 µl single-strain carrier-DNA (prepared as follows: 5 min boiled and immediately placed on ice), 3 µl of the gapped vector and 3 µl of the insert were added. Ingredients were mixed by vortexing and applied for 30 min at 30°C and for 15 min at 42°C to transform the cells. Cells were centrifuged and the supernatant was removed. 250 µl H_20 was added to the pellet and 50 µl were plated on –leu plates.

Isolation of the Plasmid (Miniprep)
"Gene elude plasmid miniprep kit" by Sigma was used for the plasmid isolation from the *E. coli* cells and the TLS56 strain (lab protocol differed in some points from the original Sigma protocol since we used the kit designed for *E. coli* for yeast, as well).

Transformation of E. coli
Transformation of *E. coli* (xl blue ultra competent cells) with the plasmid was done through a heat shock. 50 µl of the eluted plasmid DNA and of the *E. coli* cells were incubated for 30 min. on ice and then put into a water bath of 42°C for 45 s. Transformed *E. coli* cells were afterwards plated on AmpLB plates (ampicillin) and grown up in the incubator at 36°C for 12 hours.

Primer for Sequencing
Primers were constructed by a service company; these were used to sequence the unknown insert.
Tetrad Dissection and Cell Preparation
In order to optain haploid spores, cells were transferred from rich YPD media into poor liquid media where the diploid cells tend to sporulate. To prepare the four haploid spores for the dissection

100 µl of a liquid culture were pelleted, afterwards treated with 50 µl of sorbitol to prevent cell stress and finally treated with 2.5 µl of the enzyme zymolyol to lyse the cell wall of the tetrads.
After an incubation period of 5 min at room temperature (rt), 500 µl of H_2O were added and 65 µl were plated on a tetrad dissection plate. (YPD plate)
Using the light microscope each of the four spores was transferred with a micro-needle to different parts of the plate and placed into the 37°C incubator for 24 hours.

Tetrad Experiments
The spores were replica plated to the different marker plates −his3, −ura3, G418 (an antibiotic).
Additionally, we performed a mating test to determine their mating type.
Finally, the spores were tested on CF plates towards CF sensitivity.

Selection Methods
Calcofluor- and ostmotic shock plates (1/2 YPD) were used to select mutations in the Chs3p transport. Chitin which is synthesized by Chs3p makes the cell wall more solid. An osmotic shock can not harm the cell. The toxin calcofluor can bind to chitin at the cell surface and kills the cell if chitin is synthesized in the cell wall.

Calcofluor Sensitivity Test
Cells were diluted down with H_2O to 100% , 10%, 1%, 0.1%, 0.01%, and 0.001% and tested on YPD, CF100 (100% CF), CF20, and CF10 plates.

Cells were grown for 3 days at 30°C. Cultures were grown overnight in YPD liquid media (OD below 1). The cells were washed with H_2O and 1×TE. LiAc, 5 µl of the *chs6::HIS3* fragment (amplified by PCR), 10 µl of single-strain carrier-DNA, and 600 µl of 40% PEG in 1xTE+LiAC were added.
The cells were incubated while rotating at room temperature for 30 min and placed into a 42°C water bath for 25 min.
The transformed cells were pelleted and re-suspended in 100 µl H_2O, placed on SC^- HIS plates and grown for 2 days at 30°C.

Mutant Test on CF Plates
To test the mutants we streaked out 6/8 colonies of each strain on CF plates and let them grow for 3 days at 30°C.

Results

Cloning by Complementation
Chs3p is not transported to the PM in TLS56 – the one-step fractionation of the TLS56 strain (*chs6*Δ*, apm1*Δ*,* unknown mutation) and the two control strains YRV95 (*chs6*Δ, *apm1*Δ) and YRV19 (*chs6*Δ) showed that Chs3p is not transported to the PM in YRV19 and TLS56 but stays as an intracellular pool (ER, Golgi). In contrast, in YRV95 Chs3p can be seen not only in the various organelles of the secretory pathway but also at the PM.
Density centrifugation produced positive results and was checked by the two

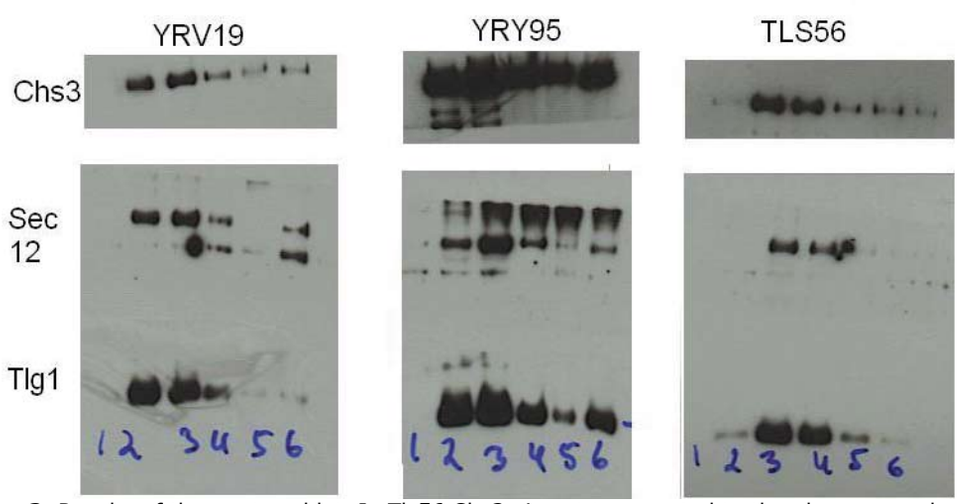

Fig. 2. Results of the western blot. In Tls56 Chs3p is not transported to the plasma membrane

markers Sec12 (ER) and Tlg1 (Golgi; Fig. 2: result of the western blot).

Yet TLS56 is Osmotic Shock Stable
The TLS56 cells, which were transformed by lithium acetate transformation were plated on 1/2 YPD. All cells grew making the selection for osmotic-resistant cells impossible.
About 3160 colonies could be counted on the plates. The colonies were replica plated on YPD and on CF plates. Six colonies grew on YPD but not on CF plates and seemed to be CF sensitive.
These colonies were picked from the YPD plates and put into –leu liquid media.

MB5 is CF Sensitive
Having grown the six colonies (MB1, MB2, MB3 etc.) they were streaked on CF plates to check their sensitivity towards CF again (unpublished data). MB3 and MB5 grew less well than the other colonies. To check this again, we tested MB3 and MB5 through a serial dilution on CF, which underlined that especially MB5 is CF sensitive (Fig. 3).

We continued to work with MB3 and MB5 and re-isolated the plasmid. The transformation into *E. coli* was successful. About ten *E. coli* colonies grew on each AmpLb plates. Clearly, the *chs6* gene was not on the plasmid as proved by PCR (unpublished results).

The *E. coli* cells were placed into liquid media for 12 hours and minipreped. The plasmid MB5 was sequenced twice but sequencing was not successful. MB5 and MB3 were run on an agarose gel but DNA was not found in the samples (unpublished data).

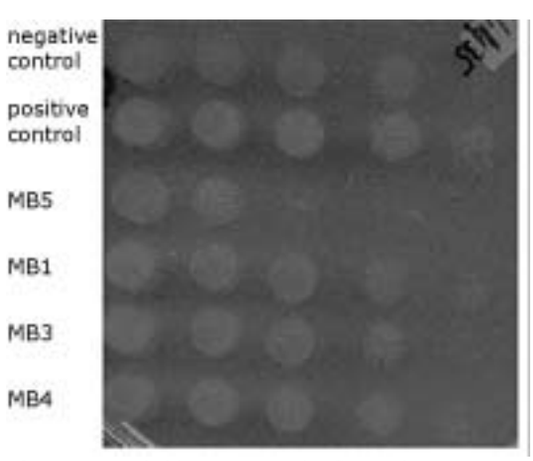

Fig. 3. Serial dilution on calcofluor plates MB5 is CF sensitive. The plasmid which was transformed seems to affect the Chs3 transport

Tetrad Dissection
In a second approach we checked the impact of a *ylr112w* delete in a *chs6Δ, apm1Δ* cell by using tetrad dissection. We picked eight times four haploid spore lines. Two lines grew up completely (all of the four spores grew). On the selection plates –histidin, –uracil, G418, line 1 grew up with an expected 2:2 proportion and one colony (1d) could be seen on every plate.
Colony 1d was haploid since it mated with a mata strain. Consequently, 1d is a matα. In a serial dilution on CF plates, 1d did not grow (see Fig. 4).

Search for chs6Δ Suppressors
Single mutant strains from the lab were tested on YPD and different CF plates in various concentrations. All strains grew on YPD plates but were sensitive towards CF (except the control strains) (see Fig. 5: Calcofluor sensitivity test).

On HIS-plates cells which were transformed with the *chs6::HIS3* fragment grew very differently: numbers of colonies varied on each plate from about 10 colonies on the *vps5×chs6::HIS3* plate to about 100 colonies on the *snx4×chs6::HIS3* plate.
Then we tested the transformants on CF plates to see if the second mutation affects the Chs3p trafficking or not and only two strains did not grow at all (*ykr078w×chs6::HIS3* and *imh1Δ×chs6::HIS3*) (Fig. 7). In

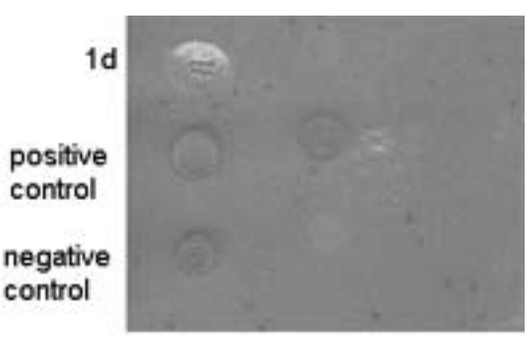

Fig. 4. Serial dilution on calcofluor. The triple mutant 1d does not grow. Consequently, ylr112w does not affect the transport of Chs3p

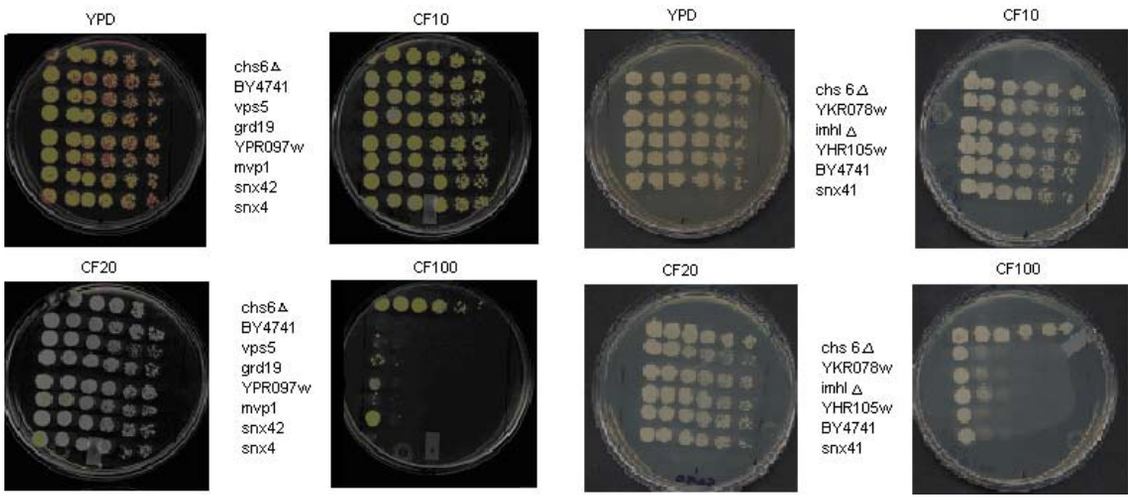

Fig. 5. Calcofluor test of various strains before transformation

contrast, the second check showed that also these two strains were able to grow (Fig. 6).
A PCR attempt of amplifying the transformed *chs6::HIS3* fragment was unsuccessful.

Analysis and Discussion

Cloning by Complementation
The western blot showed that Chs3p is no longer effectively transported to the plasma membrane in TLS56. The fact that a small band of Chs3p could still be detected at the cell surface might be an explanation for the unexpected

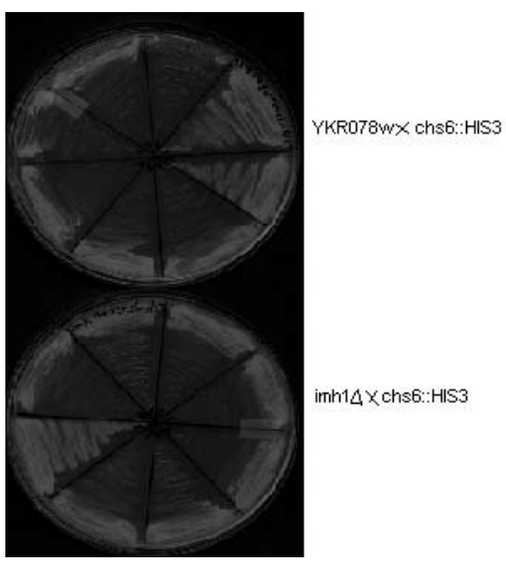

Fig. 6. THe second test on CF plates shows that *ykr078* and *imh1* do not affect Chs3p trafficking. We suggest that there are so many cells that do not grow because the transformation was not successful

osmotic shock stability of the cells. That is why we had to select the transformed cells through CF plates. The amount of cells (3160 colonies) was not satisfying since the proposed optimum is 10,000 colonies.
After the isolation of the plasmid we omitted the re-transformation in yeast as a matter of time and proceeded with the transformation in *E. coli* as a way to multiply the plasmid. This second check of the accomplishment capability could have proved that the phenotype was really dependant on the transformed plasmid.
Especially MB5 seemed to be CF sensitive checked by the serial dilution. The PCR excluded that *chs6* had been the reason for this CF sensitivity.
The re-transformation of the plasmid into *E. coli* should have worked since colonies grew quite well on AmpLb plates, which is only possible if the transformation was successful (–leu markers on the plasmid). Yet there were problems to grow these colonies in AmpLB liquid media, but several tests showed a contamination in the media.
Finally, a culture grew using a new liquid media but sequencing was unsuccessful which was probably due to the fact that the Sigma kit - used to miniprep the cells – had not worked. All samples were devoid of DNA. It is also possible that the isolation of the plasmid from yeast had not worked and that the colonies which grew on the LB amp plates were contaminated.

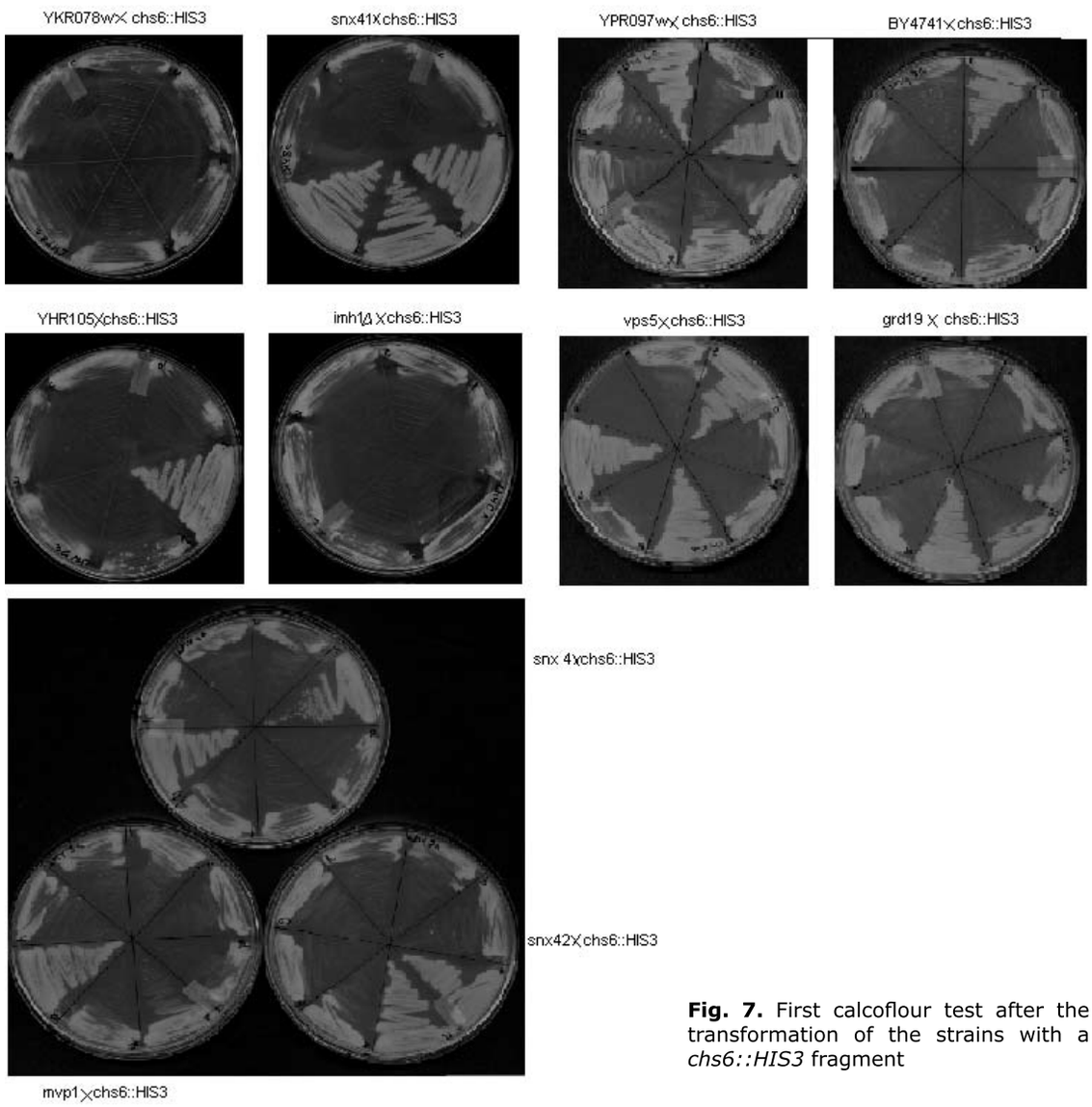

Fig. 7. First calcoflour test after the transformation of the strains with a *chs6::HIS3* fragment

Tetrad Dissection
1d grew on every marker plate and the mating test showed that 1d was a haploid spore since it could mate with another haploid spore. Consequently, 1d is the triple mutant we were looking for.
On the CF plates it could be seen that 1d is CF sensitive. So, Chs3p is transported to the cell surface, which suggests that *ylr112w* has no impact on the Chs3p transport since *chs6Δ, apm1Δ* cells show the same phenotype.

Search for chs6Δ Suppressors
Our first tests on CF suggested that *ykr078w* and *imh1Δ* might be suppressors for a *chs6Δ* because the double mutant could not grow on CF. In contrast, the second test showed that this assumption was incorrect and *ykr078w ×chs6::HIS3* and *imh1Δ×chs6::HIS3* were able to grow on CF.
It is most likely that the cells did not grow on the first plates because the transformation had not worked properly and the amount of double mutants was too low.

Perspectives

A transposon technique (genome priming systems) was recently used to knock out different genes on a plasmid which had accomplished (same experiments as described above). *ylr111w*, which is located right next to *ylr112w* on chromosome 12 of the yeast genome, seems to be more likely a gene of interest. On CF plates the

triple mutant *apm1*Δ*, chs6*Δ*, ylr111w*Δ does grow. *ylr111w* seems to play a major role in Chs3p transport from the EE to the PM. This result will have to be confirmed by further experiments.
In case these are successful, a description of the protein - which might be another co-protein for the protein secretion like Chs5p or Chs6p will be possible. *ylr111w* could also transcribe a new kind of coat-protein complex like clathrin or coatomer since there has been no proof of the vesicle which transports Chs3p from the EE to the PM.

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*Fig. 1 was used from Validivia R. et al. (2002)

Localization of Transporter Domains Involved in the Substrate Specificity of the RND-Type Multidrug Efflux Pumps AcrB and AcrD of *Escherichia coli*

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Abstract

The incidence of multidrug resistance is a serious problem in cancer chemotherapy and treatment of bacterial infections. Multidrug efflux is one of the major mechanisms of drug resistance in both prokaryotic and eukaryotic cells and is often associated with the enhanced expression of multidrug transport proteins.
AcrB and AcrD are multidrug export proteins which belong to the RND superfamily and share a similar topology. In comparison to other families of multidrug efflux pumps, the topology of the RND-type transporters is very unusual. This makes it very interesting and important to locate the transporter domains that are involved in the substrate specificity.
In this study, we utilized the strong sequence similarity between AcrB and AcrD transporters of *Escherichia coli* as well as their substantially different substrate ranges and searched for changing inhibitory concentrations of various chimeras between AcrB and AcrD. The chimeras of which we exchanged the pn22 region, a domain located in the periplasmic vestibule, showed that this region must be at least partially responsible for the recognition and presumably binding of the drug SDS.

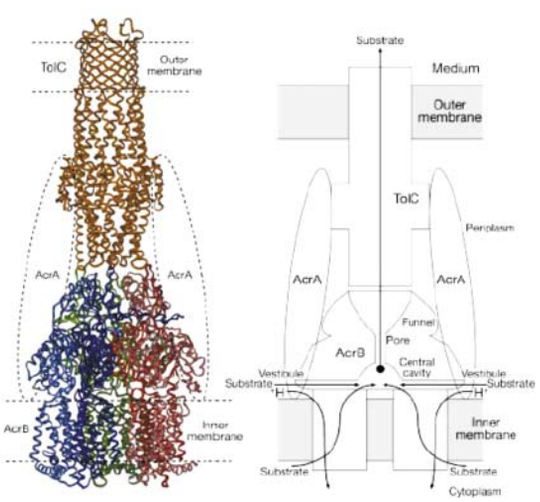

Fig. 1. Proposed model of the AcrAB-TolC complex and the schematic machanism of multidrug export (Murakami, Nakashima, Yamashita and Yamaguchi 2002)

Introduction

Bacteria have evolved several different resistance mechanisms to be able to survive in a pharmacologically hostile environment. One of the major resistance mechanisms is the multidrug efflux pumps (Zgurskaya and Nikaido 2000). Efflux pumps export drugs as soon as they diffuse through the membrane. Thereby the concentration of drugs in the cell is kept at such a low level that they are not able to cause harm.
The AcrAB-TolC system (Fig. 1) is the major multidrug resistance mechanism in *E. coli*. It is composed of a cytoplasmic membrane protein AcrB of the resistance nodulation-cell division (RND) family, a membrane fusion protein (MFP) AcrA, and a multifunctional outer membrane channel TolC. The substrate specificity of the AcrAB system is unusually broad. It extrudes cationic, neutral, and anionic substances including dyes, detergents, and most lipophilic antibiotics. The natural function of AcrAB is the pumping out of bile salts to help *E. coli* to survive in its natural habitat, the gut.
The active part of the AcrAB-TolC complex is the AcrB transporter. It is composed of three identical protomers which organize together in the shape of a jellyfish. The protomers are subdivided into a transmembrane region and a protruding headpiece in the periplasm. There they form a pore that opens like a funnel where TolC might directly dock into AcrB. At the bottom of the headpiece, the pore

connects to a central cavity where the substrates are collected before they are actively transported out through the tunnel and TolC. Beside the substrate entrances in the cell interior through the transmembrane region, there are also vestibules which connect the central cavity with the periplasm. This explains why the pump also causes resistance to antibiotics like penicillin which attack within the periplasm (Murakami, Nakashima, Yamashita and Yamaguchi 2002).

Another multidrug efflux pump in *E. coli* is AcrD which also forms an efflux transporter complex with TolC, but does not form an operon with an MFP gene. AcrD belongs also to the RND superfamily and provides resistance to a variety of aminoglycosides as well as to a limited range of amphiphilic agents, such as SDS, bile acids, novobiocin, deoxycholate, and fusidic acid. Both efflux pumps, AcrB and AcrD, share a similar topology and a strong sequence similarity (Elkins and Nikaido 2002).

Our aim is to understand more about how the pumps perform and which domains are responsible for drug specificity.

Christopher Elkins and Hiroshi Nikaido detected that the two large periplasmic loops are predominantly responsible for substrate specificity of AcrB by comparing AcrB and AcrD chimeras where the two periplasmic loops have been exchanged. The suggestion of Hiroshi Nikaido that the vestibules (Fig. 2) might play a significant role, formed the basis of our research in which we attempted to locate transporter domains involved in the substrate specificity. Using the strong sequence similarity between AcrB and AcrD we constructed chimeras by exchanging a domain or just single amino acids. The mutated DNA was constructed by a PCR-based method and then transformed into *E. coli* cells. To check the correctness of the mutation we isolated the DNA again to prepare it for a sequence analysis. The correct mutated DNA was then transformed again into *E.* coli cells and spread out onto drug gradient plates to test them for changes in the minimum inhibitory concentration.

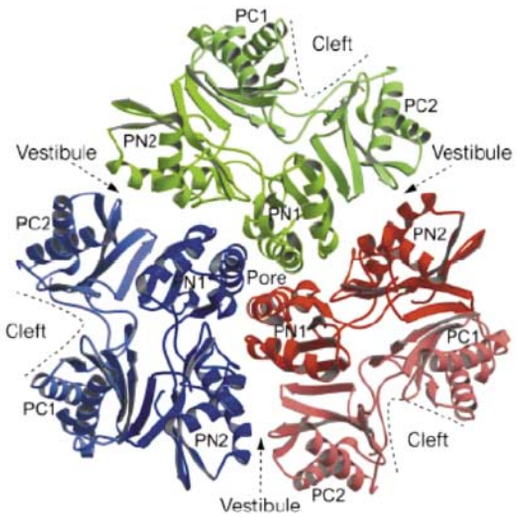

Fig. 2. Cut view of the pore domains from the boundary between the pore and the TolC docking domains. At each vestibule you can see the PN2 domain from which the PN22 region is a part of (from: Murakami, Nakashima, Yamashita, and Yamaguchi 2002).

Materials and Methods

Culture Techniques and Strains

All *E. coli* used in this study were maintained at −80°C in 15% (vol/vol) glycerol for cryoprotection. These strains were grown at 37°C in Luria-Bertani (LB) medium (1% tryptone, 1% yeast extract, and 0.5% NaCl), or on LB agar (1.5%) plates, made by using Difco components (Becton Dickinson).

The *E. coli* cells used in this study are DH5α, a standard host strain for cloning and HNCE1a strains which lack *acrB* and *acrD.* We used two vectors, pSportI, a high-copy vector and pHSG575, a low-copy vector. The high-copy vector has the advantage that less bacteria produce a very large amount of DNA material. The disadvantage of this vector is that it results in an overproduction of the efflux pumps causing a weak bacterial membrane and therefore unstable results.

Oligonucleotide Primers and Miscellaneous Chemicals

Oligonucleotide primers used for cloning and mutagenesis techniques were obtained from Genemed Biotechnologies, Inc. (San Francisco, CA). Miscellaneous chemicals, including antimicrobial agents, were obtained from Sigma Chemical Co.

Construction of the Chimeric Pumps

Chimeras in which the pn22 regions were replaced precisely by those of the homologous gene were constructed by a novel PCR-based mutagenesis protocol (Fig. 3; Geiser et al. 2001).

In the first step, DNA encoding for AcrB, was PCR-amplified by using long primers that contain the pn22 region of *acrB*. In the second step, the PCR amplicons were then used as primers in a second PCR with a plasmid containing the wild-type *acrD* as template, so that the entire plasmid became amplified with the precise replacement of the pn22 region. Replacement of *acrD* domains with *acrB* followed a similar procedure with appropriate changes in the templates. Digesting with DpnI, which recognizes methylated DNA, eliminated template DNA encoding native pump proteins in the final PCR product. Newly synthesized plasmids encoding chimeric proteins were transformed into DH5α cells and were selected on LB agar medium containing either 100 µg of ampicillin/ml for bacteria containing the pSportI vector or 20 µg of chloramphenicol/ml for bacteria containing the pHSG575 vector.

Construction of the Point-Mutated Pumps

Mutants were constructed by replacing single amino acids by those encoded by the homologous gene using a novel PCR-based mutagenesis protocol.

The DNA fragment is amplified with two conventionally designed primers. The primers contain the code for the changed amino acid and are elongated at both ends for 15 or more nucleotides corresponding to the DNA sequence found upstream and downstream and end finally with a G or C residue. During the PCR process, the primers are annealed to the methylated template plasmid DNA and each strand is elongated with the PfuUltra DNA polymerase. After elongation the newly created DNA carries the mutated base and is unmethylated. Upon DpnI digestion, the methylated template DNA is destroyed. Thus, after transformation into DH5α, only the mutated plasmid DNA will be recovered from the bacteria. The cells were spread onto LB agar medium containing either 100 µg of ampicillin/ml for bacteria containing the pSportI vector or 20 µg of chloramphenicol/ml for bacteria containing the pHSG575 vector for selection.

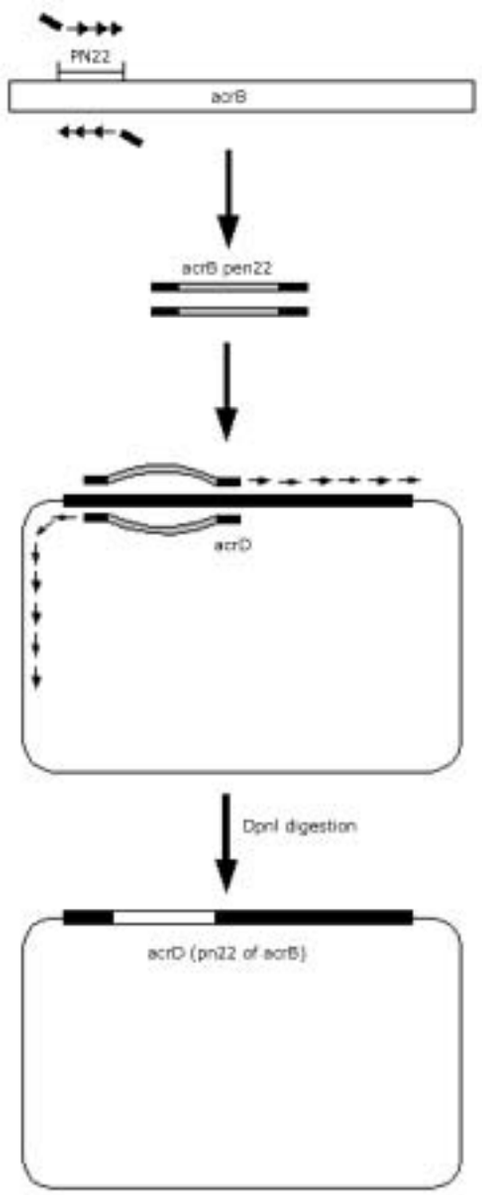

Fig. 3. PCR mutagenesis of *acrD*. The first step shows the PCR amplification of the DNA coding for the PN22 region of *acrB*. In the second step these amplicons were then used as the primers in a second PCR to produce the entire plasmid sequence containing the chimeric gene *acrD* (pn22 of *acrB*). The final DNA product was then digested with DpnI to destroy the methylated template DNA

DNA Sequencing and Analysis
Plasmid constructs created in this study were sequenced to check the mutation by using an automated method. Standard T7 Forward and SP6 primers and synthesized walking primers for DNA sequencing were used (Elim Biopharmaceuticals Inc., Hayward, CA).

Drug Susceptibilities
To determine changes in the resistance of the mutants compared to the wild type, the plasmids were transformed into HNCE1a cells and spread out twice (↑↓) with an inoculating loop onto drug gradient plates (Fig. 4). The bacteria suspension (OD_{650} = 1.0) was diluted 10^3-fold with saline before spreading out. The results were photographed after 24 hours and again after 48 hours. This test is just a screening to determine resistance changes. In case of good results, the next step would have been to figure out the MICs (minimum inhibitory concentration) of the mutants. This is desirable, because with gradient plates it is not possible to give precise data about the caused resistance. In addition to that the possibility to test bacteria with drug gradient plates is so far limited, that you can compare the behavior of different bacteria with one another just on the same plate and not over several plates. Furthermore, it is not possible to give a statement about the minimum inhibitory concentration of some bacteria on one plate, if the minimum inhibitory concentration of these bacteria is higher than the maximum drug concentration of the plate.

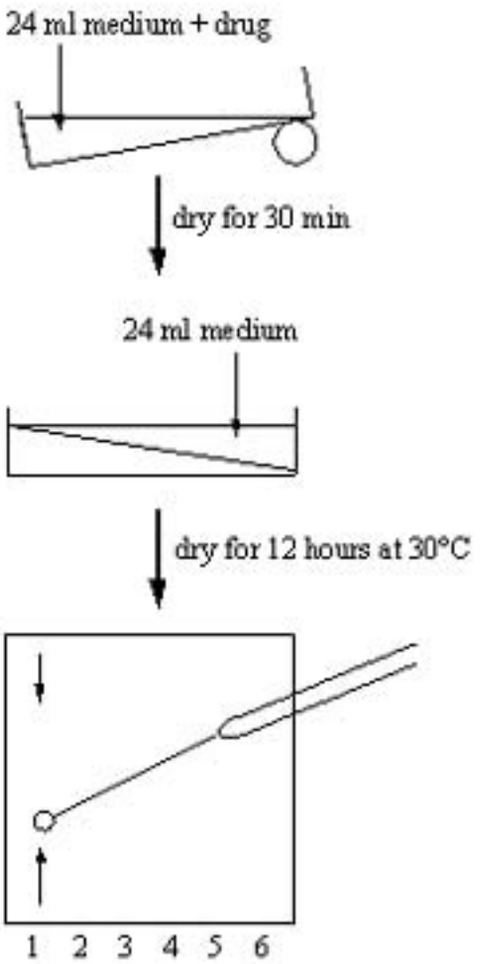

Fig. 4. Construction of gradient plates. First the plates were filled with 24 ml of LB medium containing a drug and placed on a pipet for a slant position to create the gradient. After a drying period of 30 min. the plates were filled up with another 24 ml of plain LB medium. Before spreading out bacteria, the plates have to dry completely for about 12 hours at 30°C

Results

We cloned *acrB* into two different expression vectors: pSport1 and pHSG575. In these plasmids we mutated the gene *acrB* in several ways. On the one hand, we exchanged the pn22 region of the *acrB* gene with the pn22 region of the *acrD* gene and otherwise the *acrD* gene with the pn22 region of the *acrB* gene. After transformation in *E. coli* HNCE1a we performed a resistance screening of drug susceptibility with drug gradient plates (Table 1). In regard to the drug SDS we discovered a change in the minimum inhibitory concentration. AcrB with the pn22 region of *acrD* caused less resistance to SDS than the AcrB wild type. In the converse experiment, the AcrD with the pn22 region of *acrB* caused a higher resistance to SDS than the AcrD wild type (Fig. 5).
We discovered a similar reaction with the drugs carbenicillin, ethidium bromide, and novobiocin. The minimum inhibitory concentration of the mutant AcrD with pn22 of *acrB* to carbenicillin decreased compared to the wild type, but the activity of the mutant AcrB with pn22 of *acrD* remained about the same. Considering the drugs ethidium bromide and novobiocin, the minimum inhibitory

Table 1. Gradient plate analysis of *E. coli* HNCE1a cells expressing AcrB, AcrD, or AcrBD chimeras in pHSG575. Length of growth zone (mm)[a] of HNCE1a/pHSG575 containing:

Drug	n.c[b].	AcrB	AcrB (pn22 of acrD)	AcrD	AcrD (pn22 of acrB)
Carbenicillin (5 µg/ml)	13	42	37	> 80	> 80
Carbenicillin (30 µg/ml)	0	8	8	**57**[c]	**40**
Chloramphenicol (200 µg/ml)	11	> 80	> 80	24	23
Erythromycin (50 µg/ml)	0	73	67	0	0
Ethidium bromide (10 µg/ml)	47	> 80	> 80	~ 80	~ 80
Ethidium bromide (200 µg/ml)	0	**~80**	**53**	0	0
Gentamicin (4 µg/ml)	29	40	37	40	39
Gentamicin (8 µg/ml)	16	27	24	24	24
Novobiocin (20 µg/ml)	17	> 80	> 80	52	55
Novobiocin (200 µg/ml)	0	**53**	**27**	7	7
Sodium dodecylsulfate (200 µg/ml)	8	> 80	> 80	**37**	**48**
Sodium dodecylsulfate (2000 µg/ml)	0	**>80**	**> 80** [b]	0	2
Tetracycline (8 µg/ml)	0	40	33	10	12
Taurocholic acid (6 500 µg/ml)	15	71	70	67	63

[a] The length of the plate was 80 mm. Thus, the value of 80 means complete resistance up to the highest concentration in the plate, and that the MIC may be any value higher than that.
[b] The negative controle is a strain containing the vector plasmid only.
[c] Values shown in boldface represent interesting cases with a change in the inhibitor concentration
[d] Another SDS gradient plate with HNCE1a harboring pSportI-derived constructs with AcrB and AcrB with pn22 of *acrD* showed that the MIC of AcrB is much higher than the AcrB chimera

concentration of the mutant AcrB with pn22 of *acrD* decreased, while the resistance caused by the corresponding mutant remained about the same. Regarding the drugs chloramphenicol, gentamicin, erythromycin, tetracycline, and taurocholic acid we were not able to find changes in the minimum inhibitory concentration of the mutants in comparison with the wild type.
On the other hand, we introduced point mutations in the *acrB* gene by PCR.

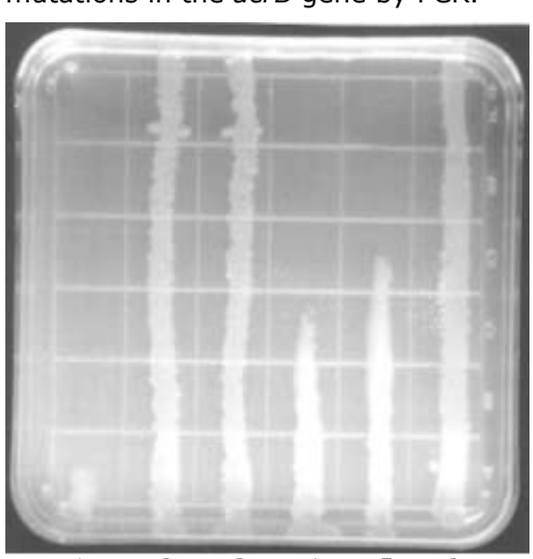

Fig. 5. SDS gradient plate with a maximum drug concentration of 200 µg/ml.
1: pHasg575; 2: pHasg575 – acrB; 3: pHasg575 – acrB (pn22 of acr D); 4: pHasg575 – acrD; 5: pHasg575 – acrD (pn22 of acr B); 6: pHasg575 – acrB (A311D, K312E)

We exchanged single amino acids of the AcrB pump with the corresponding ones of the AcrD pump as well as two to four amino acids. First we exchanged the amino acid number 311 from alanine to aspartic acid (A311D) and the amino acid number 312 from lysine to glutamic acid (K312E). There was no change in comparison with the wild-type AcrB (data not shown). Therefore, we mutated these double mutants again and exchanged the amino acids

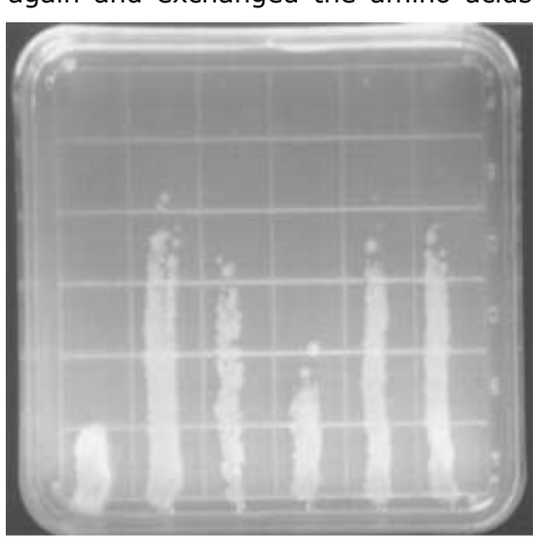

Fig. 6. Carbenicillin gradient plate with a maximum drug concentration of 5 µg/ml. 1: pHasg575; 2: pHasg575 – acrB (A311D, K312E); 3: pHasg575 – acrB (E95K, A311D, K312E); 4: pHasg575 – acrB (A299E, A311D, K312E); 5: pHasg575 – acrB (A304E, A311D, K312E); 6: pHasg575 – acrB (A309R, A311D, K312E)

Table 2. Gradient plate analysis of *E. coli* HNCE1a cells expressing AcrB mutants in pHSG575. Length of growth zone (mm)[a] of HNCE1a/pHSG575 containing:

Drug	n.c.	AcrB (A311D, K312E)	AcrB (E95K, A311D, K312E)	AcrB (A299E, A311D, K312E)	AcrB (A304E, A311D, K312E)	AcrB (E309R, A311D, K312E)	AcrB (E314A, A311D, K312E)	AcrB (A311D, K312E, M313L, E314A)	AcrB (A311D, K312E, K322E)	AcrB (A311D, K312E, D858E)
Carbenicillin (5 µg/ml)	13	51	**44**[b]	**24**	46	48	53	**43**	53	**43**
Chloram-phenicol (200 µg/ml)	11	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80
Erythromycin (50 µg/ml)	0	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80
Ethidium bromide (200 µg/ml)	0	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80
Gentamicin (4 µg/ml)	29	31	31	31	33	33	30	30	28	28
Gentamicin (8 µg/ml)	16	17	17	16	16	17	18	17	16	16
Novobiocin (200 µg/ml)	0	58	58	**40**	58	55	55	**53**	55	**54**
Sodium dodecyl-sulfate (2000 µg/ml)	0	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80
Tetracycline (8 µg/ml)	0	47	47	**27**	47	45	47	45	47	45
Taurocholic acid (6 500 µg/ml)	15	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80

[a] The length of the plate was 80 mm. Thus, the value of 80 means complete resistance up to the highest concentration in the plate, and that the MIC may be any value higher than that.
[b] Values shown in boldface represent interesting cases with a change in the inhibitor concentration

number 95, 299, 304, 309, 313, 314, 322, and 858 each (Table 2).

The carbenicillin resistance caused by the mutant in which we exchanged in addition to the amino acids number 311 and 312, also the amino acid number 95 from glutamic acid to lysine (E95K), decreased slightly compared to the AcrB (A311D, K312E) mutant (Fig. 6). Approximately similar results were obtained with the mutant in which we additionally exchanged the amino acid number 304 from alanine to glutamic acid (A304E) and the mutant of which we exchanged additionally the amino acid number 313 from methionine to leucine and the amino acid number 314 from glutamic acid to alanine (M313L, E314A) as well as the mutant in which we exchanged additionally the amino acid number 858 from aspartic acid to glutamic acid (D858E) (Fig. 7).

The mutant in which we exchanged additionally the amino acid number 309 from glutamic acid to arginine (E309R), the mutant of which we exchanged additionally the amino acid number 314 from glutamic acid to alanine (E314A), as well as the mutant of which we exchanged additionally the amino acid number 322 from lysine to glutamic acid (K322E) remained at the same carbenicillin resistance level as the AcrB (A311D, K312E) mutant.

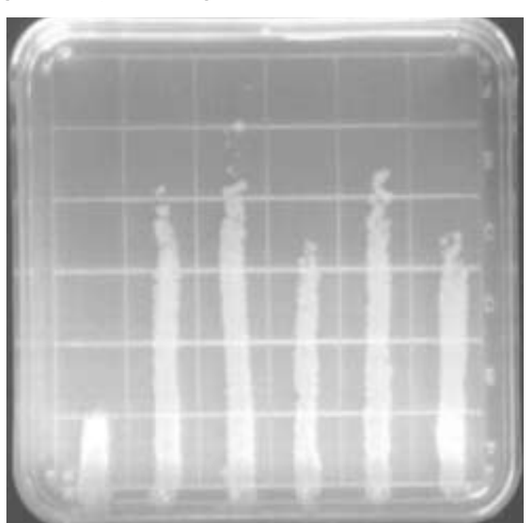

Fig. 7. Carbenicillin gradient plate with a maximum drug concentration of 5 µg/ml.
1: pHasg575; 2: pHasg575 – acrB (A311D, K312E); 3: pHasg575 – acrB (A311D, K312E, E314A); 4: pHasg575 – acrB (A311D, K312E, M313L, E314A); 5: pHasg575 – acrB (A311D, K312E, K322E); 6: pHasg575 – acrB (A311D, K312E, D858E)

We discovered a higher decrease of resistance to carbenicillin, novobiocin, and tetracycline of the mutant in which we exchanged additionally the amino acid number 299 from alanine to glutamic acid (A299E). With the other mutants no changes in the resistence to novobiocin and tetracycline were observed. All mutants as well as the negative controle show the same resistance to the drug gentamicin.
Regarding the drugs chloramphenicol, ethidium bromide, erythromycin, SDS, and taurocholic acid no changes were observed in the minimum inhibitory concentration of the mutants in comparison with the AcrB (A311D, K312E) mutant under the conditions of our drug susceptibility test (see: Materials and Methods). Further tests with higher concentrated gradient plates might be needed.

Discussion

The results show that the pn22 region must be important in the recognition and presumably binding of the drug SDS. It probably is not the only responsible domain because the resistance of the AcrB chimera with pn22 of *acrD* merely approached the resistance caused by the AcrD wild type, not absolutely agreeing with it. The converse experiment showed the same and thus confirmed our hypothesis. The activity of AcrD with pn22 of *acrB* increased and the chimera approached the resistance caused by the AcrB wild type. This result is also comprehensible regarding the charge of this region. SDS is a negatively charged drug and the pn22 region of AcrD consists of many glutamic acids and aspartic acids and is therefore also mainly negatively charged. In this case the charge could be an explanation for the previously reported phenomenon.
There is a slight but significant difference with the drugs novobiocin, ethidium bromide, and carbenicillin. AcrB wild type causes a much higher resistance to novobiocin and ethidium bromide as the AcrD wild type. The minimum inhibitory concentration of the AcrB chimera with pn22 of *acrD* decreased as expected, but the minimum inhibitory concentration of the AcrD chimera with pn22 of *acrB* remained about the same in comparison with the AcrD wild type and did not increase. With this result we are not certain whether the change of resistance was caused because of a mere change in the conformation or because the region is jointly responsible for the recognition and presumably binding of the two drugs. We can suggest that the region has at least some importance, because if not, most likely nothing would have changed in this experiment. The same appeared with the drug carbenicillin. The minimum inhibitory concentration to carbenicillin of AcrD wild type is much higher than the minimum inhibitory concentration of AcrB wild type. As expected, the minimum inhibitory concentration of the AcrD chimera with pn22 of *acrB* decreased, but there was no change in the minimum inhibitory concentration of the AcrB chimera compared to the AcrB wild type.
That implies for us in the future, that we will concentrate more on experiments where we can expect increasing resistance, in order to be sure that the reason of the change is caused by the exchange of regions or single amino acids.
With the point mutations we reached another problem. We hoped and expected to detect an increasing resistance to carbenicillin of the AcrB mutants, because as said before, AcrD is known to be a better pump for carbenicillin than AcrB. Contrary to this we found that the mutants, especially AcrB (A299E, A311D, K312E) but also AcrB (E95K, A311D, K312E), AcrB (A311D, K312E, M313L, E314A), and AcrB (A311D, K312E, D858E) caused a decreasing resistance. Accordingly, the exchange of single amino acids may have evoked a change in the conformation with a decrease of activity. Therefore these specific amino acids are considered to play a role, but probably they are not directly involved in the recognition and binding of the drug carbenicillin. Another attempt for explaining this phenomenon could be that these amino acids are in fact jointly responsible for recognizing and presumably binding carbenicillin, but

because they are just one among many they are not exerting higher activity and the effect of the conformation change prevails. The activity of the mutant AcrB (A299E, A311D, K312E) decreased compared to the wild type with the drugs novobiocin and tetracycline, too. This result is so far expected because AcrB is known to be a better pump for novobiocin and tetracycline than AcrD, but the previous results led us to the suggestion, that this decrease can also be evoked by a mere conformation change and probably not be due to the exchange of the amino acids. This implies, as indicated earlier, to focus only on results showing increased resistance.
Another plan for future experiments would be to perform randomized mutagenesis – first, mutating the plasmid DNA randomly by either a PCR process or by chemical mutagens, and then testing the mutants for changing minimum inhibitory concentrations. For effective mutants, the base sequence of the according genetic domains would then be determined.

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Vibration Measurements at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory

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Advanced Light Source, Lawrence Berkeley National Laboratory (LBL)

Introduction

The "Advanced Light Source" (ALS), America's brightest source for light from ultraviolet through soft x-rays, is synchrotron located at Ernest Orlando Lawrence Berkeley National Laboratory, the oldest national laboratory in the United States. It is an institution of the United States Department of Energy, managed by the University of California Berkeley.
At the ALS, electrons are accelerated to 99.999994% of the speed of light (299,792,000 meters per second) using different types of accelerators. The first stage is done by a linear accelerator (linac); after that the electrons are given into a booster ring, in which they reach their target energy. With that energy of about 1.9 GeV they are injected into the storage ring where they circulate for hours.
During their circulation within the strong magnetic field, the electrons are emitting energy in form of photons due to their acceleration. These photons, called synchrotron radiation, are focused in thin beams and steered away from the sample into the target sample inside an experiment chamber (endstation).
For most applications the beamlines are equipped with monochromators to filter certain wavelengths.
The size of one photon beam is about 0.20 mm×0.02 mm. Currently there are 27 operating beamlines at the ALS.
When designing a new beamline, it is of high importance to know the influence of the surrounding experiments, especially of the vibration caused by machines, which will displace the beam.

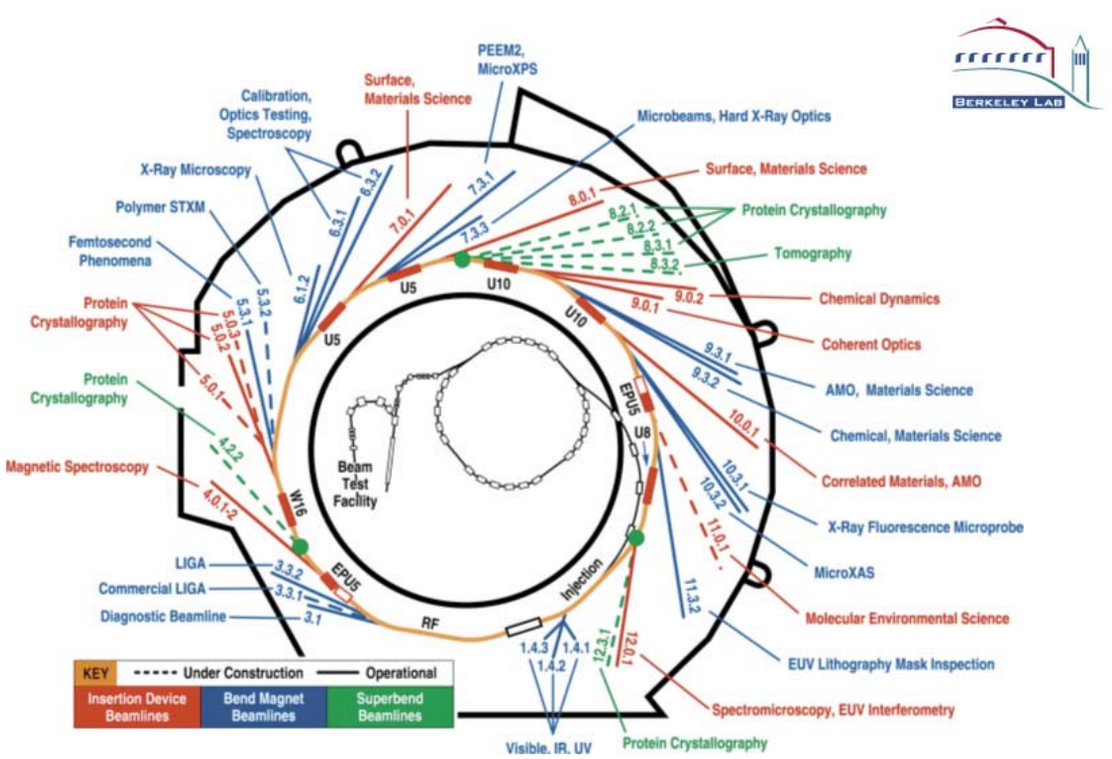

Fig. 1. Scheme of the ALS

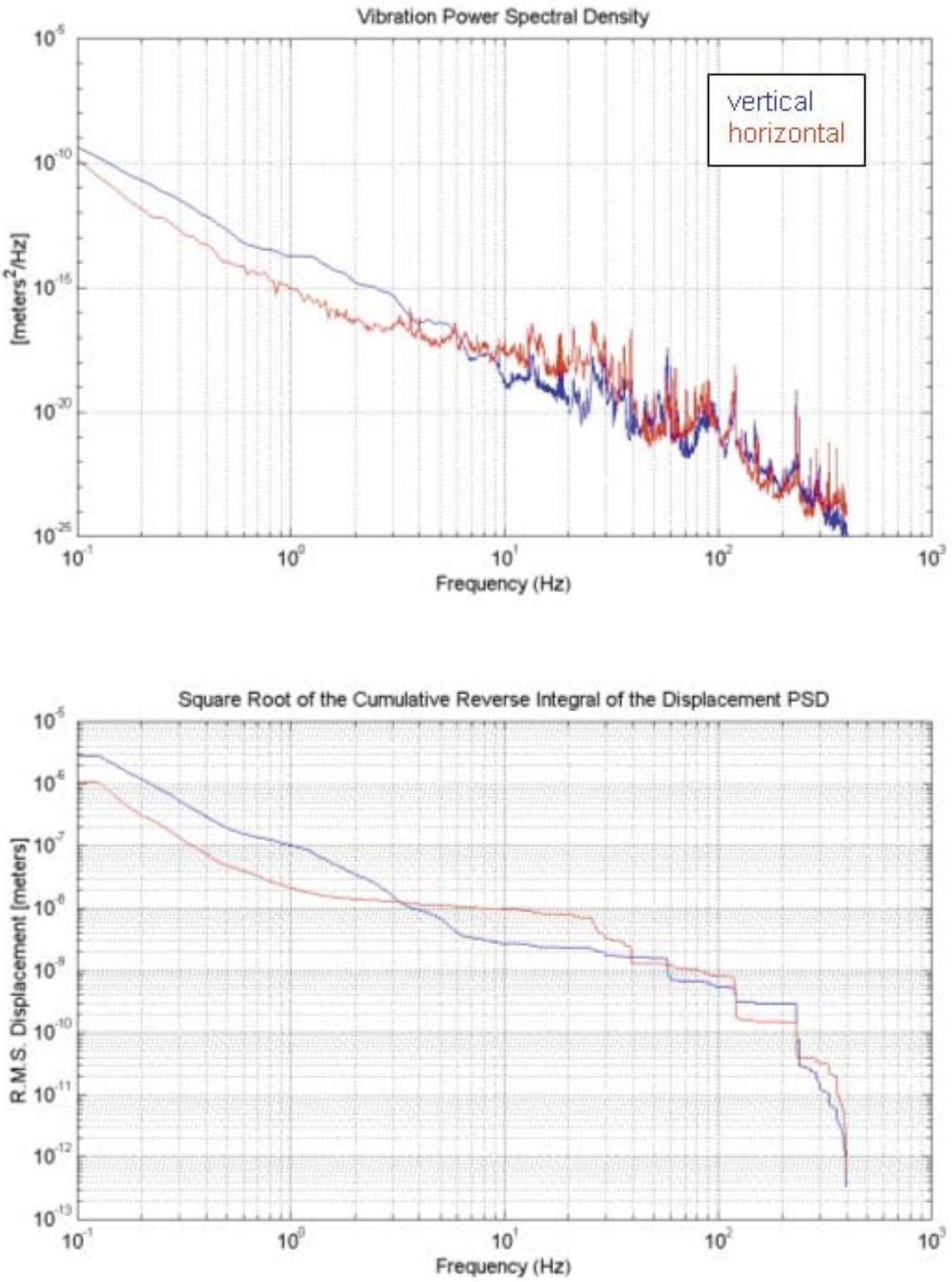

Fig. 2. Measurement on an experimental endstation podest. The displacement ranges from 10^{-12} to 10^{-5} meters. These are generally acceptable values

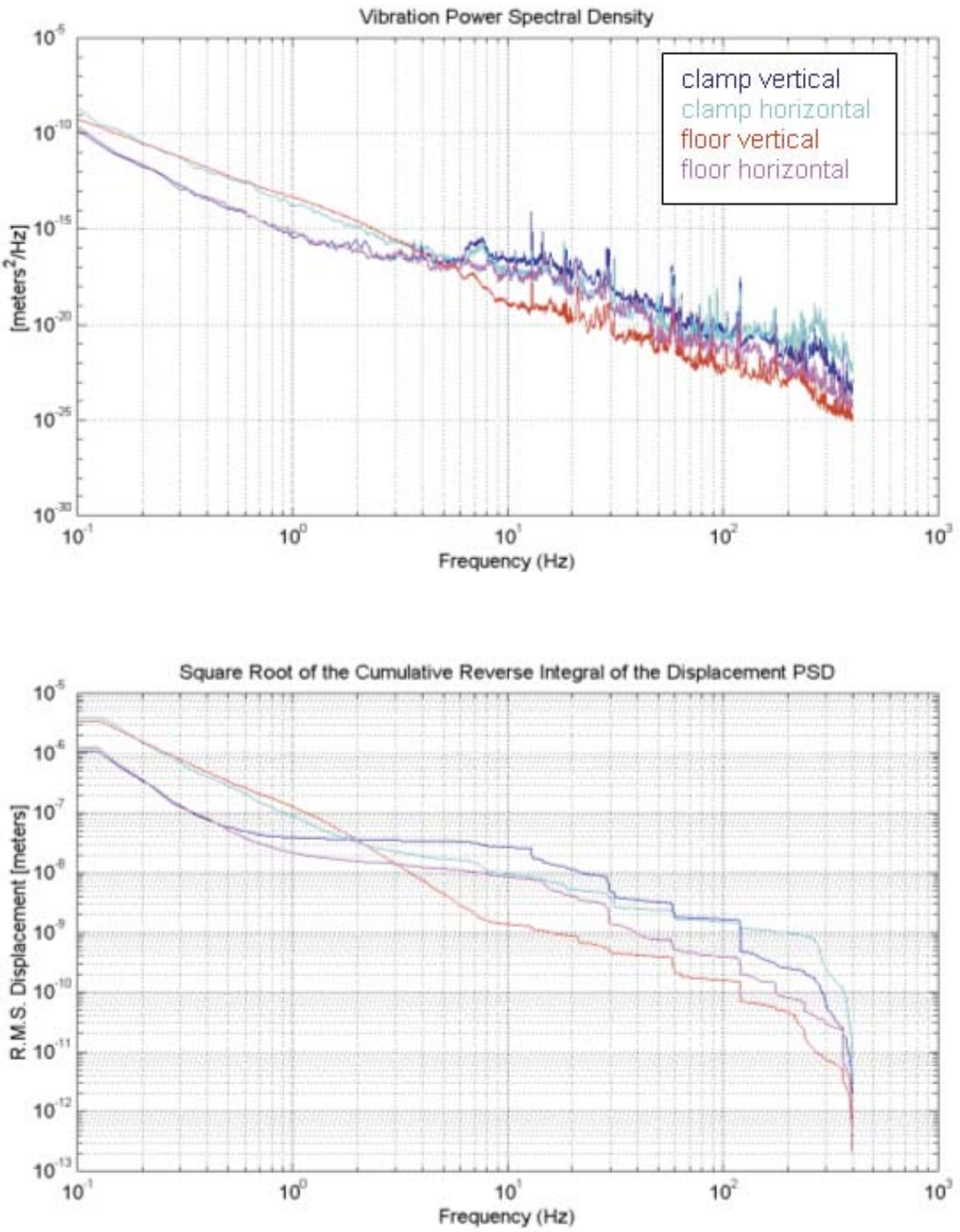

Fig. 3. Comparison of two measurements. As can be seen in both cases acceleration and thus displacement hardly differ

Method

To find sources of displacement, seismic accelerometers (model 731A) and power amplifiers (model P31) from Wilcoxon Research, connected to a spectrum analyzer (HP-3563A) were used. The analysis was performed using Matlab.
The bandwidth and noise specifications for the accelerometers, as provided by the manufacturer, are the following:

Bandwidth	0.1 – 300 Hz
Noise power spectrum (g being the gravitational acceleration)	0.03 μg/√Hz at 2 Hz
	0.01 μg/√Hz at 10 Hz
	0.004 μg/√Hz at 100 Hz

1% coupling between horizontal and vertical motion (not including mounting)

During the measurements the accelerometers were put on the floor with a holder which was pinned down with lead bricks or clamped up to frames which were bolted to the floor.
The frequency range for each measurement was from the lowest possible frequencies of about 0.3 Hz up to 400 Hz. To get a higher resolution of the power spectrum data, three data sets were taken for each measurement – one from 0–20 Hz, one from 0–100 Hz and one from 0–400 Hz. To reduce the variance of the estimated spectrum 25 power spectra were averaged for each measurement (by the HP-analyzer). For all the data taken, a Hanning window was used. The HP-analyzer uses a 2048 FFT (1024 are kept in the power spectrum). Anti-alias filtering is already performed inside the HP-analyzer.
The data read from the HP-analyzer was post-processed inside Matlab. Integration of the acceleration power spectral density provided displacement information and ultimately a cumulative RMS-amplitude (Parseval's theorem).
Sensitivity of the accelerometers was set to 1000 V/g for all measurements. The low pass filter in the amplifier of the accelerometers was set to 450 Hz.

Analysis

Accelerometers produce a voltage signal proportional to acceleration. In order to convert this to displacement, the signal must be integrated twice. Integration can be done in both time and frequency (Fourier) domains. As the accelerometers are band-limited to a range of 0.1–300 Hz and exhibit slow low-frequency variations below 0.1Hz, numerical integration is distorted. The method to use on actual accelerometer data is to convert the signal into the frequency domain before integrating. This is obtained via discrete Fourier transformation (DFT). To avoid sidelobes due to the DFT, the data is windowed with a Hanning Window.
In addition to providing the necessary RMS information via Parseval's theorem, Fourier analysis yields useful spectral information on which frequencies the dominant vibrations are occurring. Additionally, it allows easier averaging of multiple samples and convenient integration from acceleration to displacement via properties of Fourier transformation.

Results

Figures 2 and 3 are processed plots of two of our measurements. Each figure contains the power spectral density as the upper plot and the cumulative RMS-amplitude of the vibration as the lower plot. During all measurements there were no exceptional vibrations seen, that would have had an impact on the new experiment.

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Wilcoxon Research Operating Guide: Model 731 Seismic Accelerometers, Model P31 Power Unit / Amplifier

Seizure Susceptibility in *stress sensitive B* Mutants of *Drosophila melanogaster*

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Abstract

We report here the seizure susceptibility characteristics of *sesB*, a stress sensitive mutant of *Drosophila melanogaster*. Electrophysiological analyses have shown that this mutant is comparable to the wild type because it has a similar seizure threshold and behavior.

Research Interest

Research on *Drosophila melanogaster* has proved the fruitfly to serve as an ideal model system for a better understanding of neuronal systems. By examining so-called *bang sensitive* mutants, like *eas*, *bss,* or *sda*, scientists expect to gain insight also into human neuronal functions and neuropathologies, especially epilepsy. There are several different mechanisms that cause epilepsy or seizure sensitivity, but the exact way how this works is still unknown. The according genes in the *Drosophila* genome have been localized, but the function of their proteins is only partially known. There are genes that cause and others that suppress seizures. The first step to make progress is to find mutants, identify their genes, and characterize them. After a detailed examination of the function of their genes, new and more seizure-resistant mutants can be created by crossbreeding. The objective is to transfer this knowledge to humans in order to find a therapy or cure for human epilepsy.

Introduction

This research in Drosophila sets its focus on *bang sensitive* (BS) mutants. These mutants show similar symptoms to human epilepsy after a mechanical bang or electrical shock as they react with hyperactive behavior and paralysis. All of these mutants run through the same five distinguishable phases: an initial seizure, paralysis, recovery seizure, refractory period, and complete recovery. In the refractory period the flies cannot get a second seizure, because they suffer from synaptic failure and no action potential can be built up. These phases are typical for all BS mutants but the duration varies from type to type.

The wild type of *D. melanogaster* can tolerate 30 V before getting a seizure, while BS mutants have a lower threshold due to genetic mutations and are more susceptible to seizures (at 3-6 Volts).

Examples of these types of mutants are the *slamdance* mutant (*sda*) – this name was given because of their "dance" after shock – *easily shocked* (*eas*), the *bang senseless* (*bss*), and the *technical knockout* mutant (*tko*).

Their behavior under normal circumstances is comparable to that of the wild type (Zhang et al. 1999, 2002). The results of experiments with these mutants have been described in detail (Tanouye 2000).

We worked with the new *stress sensitive* mutant (*sesB*). Generally, *sesB* flies also show normal behavior. Thus, it is interesting to determine all the behavioral characteristics and the seizure threshold of these *stress sensitive* flies.

Materials and Methods

Flystocks. Stocks were maintained on standard cornmeal-molasses medium at 22°C. Wild-type flies were the Canton Special (CS) strain. Seven *sesB* mutants were used: $sesB^{41}$ (yl(1)9Ed[G16] vf/FM6), $sesB^{42}$ (yl(1)9Ed[M27] vf/FM6), $sesB^{48}$ (l(1)9Ed[G16] v/FM7), $sesB^{58}$

(yl(1)9Ed[Q10] v f/ln(1)FM6, y[31d] sc[8] dm B), *sesB*^{74} (l(1)9Ed[DC701]/ FM7), *sesB*^{85} (l(1)9Ed[A17], yvf/FM6), *sesB*^{86} (l(1)9Ed[H22], yvf/FM6). These mutants were obtained from John Roote (Department of Genetics, University of Cambridge). *Bang sensitive* flies were the *easily shocked* (*eas*^{PC80}*f*) strain.

Gene Mapping. *D. melanogaster* are dipteres, the group of flies with two wings and not four. Dipteres have giant chromosomes. These are chromosomes in the salivary gland which are not separating from each other during mitosis and which are not despiralizing; so they grow larger with each mitosis. With the help of a microscope several characteristic bands can be seen. If one band is missing it can be definitively told which genes are missing. Every one of these bands has a number and some additional letters as a smaller unit. With these details, the location of every gene is unambiguous. The *sesB* is located at band No 9 Ed.
The *sesB* mutants we used are all lethal on chromosome No 1. This is the meaning of l(1). The v and the f are abbreviations for special markers. The letters and numbers in square brackets indicate which bases are mutated. FM 7 and FM 6 are complementary alleles to *sesB*, which are sterile if they are homozygous. Homozygous FM 6 can be recognized by their white eyes. FM 7 flies have very small eyes, while all heterozygous flies just have a small nudge. Males cannot be used because all males with *sesB* are lethal.
A stock consists of heterozygous females, which are used for research, sterile homozygous FM 6 or 7 females and FM 6 or 7 males. This guarantees the lineage of the stock.

Behavioral Testing. Testing for BS paralysis was performed on flies which were rested for > 4 hr after exposure to CO_2 anesthesia before testing. These flies were vortexed on a VWR vortex at maximum setting for 10 sec.

Electrophysiology. Electrophysiology was performed using methods already described to stimulate and record giant fiber (GF)-driven muscle potentials and seizures (Kuebler and Tanouye 2000; Pavlidis and Tanouye 1995).
It is performed under a microscope as the flies are about two millimeters small (Fig. 1). The fly is taken from a vial by sucking onto its head with a 23-gauge needle attached to a vacuum line. A second needle is attached onto its abdomen (backpart). The fly is stretched by pulling the needles apart to put a wire with glue (cyanoacrylate adhesive) between its head and thorax.

Fig. 1. Workstation for electrophysiology

Fig. 2. Mounted Drosophila

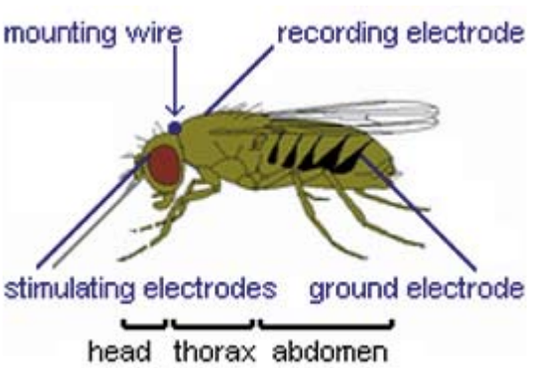

Fig. 3. Electrophysiology in Drosophila

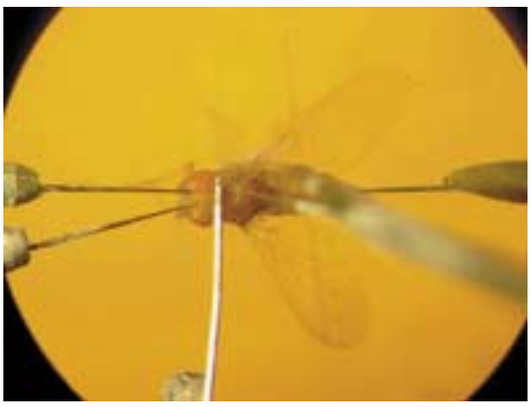

Fig. 4. Drosophila with electrodes

After the glue has dried the fly is fully immobilized and fixed to the wire ("mounted") and the other needles can be removed (Fig. 2).

The ground electrode has to be inserted into the abdomen by lifting up a piece of the hard chitin layer and put the electrode underneath (Figs. 3 and 4). The two stimulating electrodes are inserted on the margin of the eyes or under the antennas of the fly. The recording electrode has to be inserted into the dorsal longitudinal flight muscles (DLMs) on the top of the thorax (Fig. 5).

Thereafter, single-pulse stimuli (0.2-ms duration at 0.5 Hz) are given to the fly to drive the giant fiber (GF). The lowest voltage possible is used to determine the threshold at which the short latency GF pathway responds with an action potential (GF threshold, Fig. 6). During the course of each experiment, the GF was stimulated continuously to assess GF system circuit function. To elicit seizures, short wavetrains of HF electrical stimuli (0.5-ms duration, 200 Hz for 300 ms) were delivered to the brain and the intensity (voltage) of the HF stimulus was varied as noted (Table 1). Seizures consist of high-frequency activity in at least seven different muscle groups and >30 muscle fibers in the thorax (Kuebler and Tanouye 2000).

The recorded muscles are flight muscles. There are just a few direct muscles for flight. Most of the muscles are indirect. The fibers of the indirect flight muscles whose contractions cause vibrations in the body to move the wings have no junction to the wings. There are wing depressors (DLM) and wing elevators (DVM). They contract alternately; this causes the up and down movement of the wings. To start, insects use the targo trochante muscles (TTM). The contraction of the TTM leads to a jump and the first stroke (Fig. 5).

Muscles consist of actin and myosin. Myosin is an ATPase and constitutes the tail of the muscle. Actin is a globular protein which constitutes the head. The heads form a filament; the tails are clinging together. ATP induces the contraction of the fibers.

The contraction is regulated by two proteins. Tropomyosin binds to the binding site at the actin so that there is no contraction. Troponin is regulated

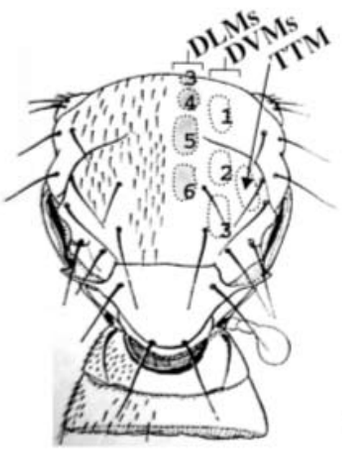

Fig. 5. Flight muscles of Drosophila

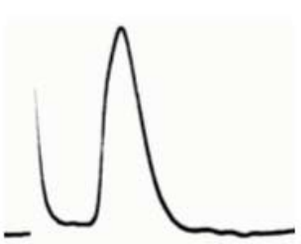

Fig. 6. A recorded AP indicates the GF threshold

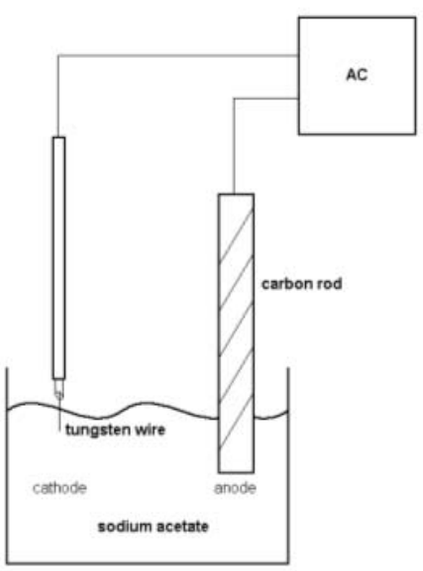

Fig. 7. Sharpening of electrodes

by Ca^{2+}. If Ca^{2+} is present, troponin binds to tropomyosin and impedes the binding to the active center of myosin. This triggers the contraction of the muscle. Ca^{2+} is the release transmitter of synapses. Within insects Ca^{2+} is in the extraplasmatic space. It floods into the cell when the canal proteins get opened by acetylcholine, the transmitter of most synapses. With Ca^{2+} the cell is able to contract, but it does not. What gives the start signal is not known yet; this is the project of another scientist in Prof. Tanouye's workgroup. The electrical impulses given by us cause the release of Ca^{2+} and leads to contraction.

Manufacture of Electrodes. The four electrodes inserted in Drosophila are made of tungsten wire with a diameter of 0.2 mm. As the body of *Drosophila melanogaster* is very small and should not be injured severely, the top of the wire has to be very thin and sharp. For the sharpening of the electrodes the mechanism of electrolysis is used. A voltage of 10 volts is given to a carbon rod that has been placed into an electrolyte ($CH_3COOHNa$). When the tungsten wire enters the solution, the circuit is closed, it reacts with the sodium acetate, and gets thinner. The wire has to be moved in and out quickly so that the top rests in the solution a little longer than the rest and the wire becomes tapered (Figs. 6 and 7).
This sharp tungsten wire is bent before it is inserted into a needle to ensure the contact between the materials.

Results

The *sesB* Behavior. The behavioral testing has shown that there is no paralysis following a mechanical bang. The *eas^{pc80f}* had an initial seizure and paralysis as already described by Kuebler and Tanouye (2000).

The *sesB* Electrophysiology. *SesB* flies have similar threshold as the wildtype. It has been shown that the threshold is about 23 V (Fig. 8), while the bang-sensitive mutants have a lower threshold at 6 V.

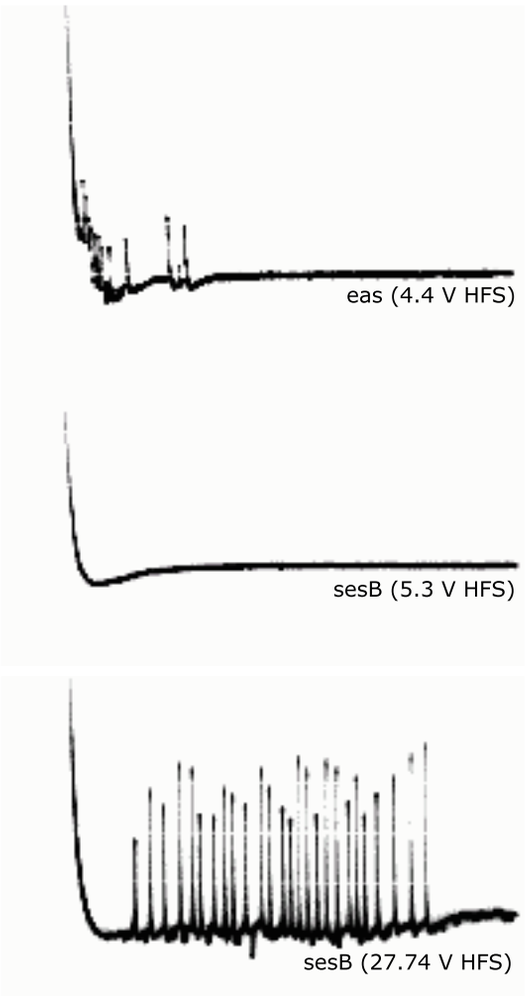

Fig. 8. A easPC80f has a seizure after a HF low strength stimulus of 4.4 V; B sesB shows no reaction to a HF stimulus of 5.3 V; C 27.7 V cause a seizure in sesB

Table 1. Seizure thresholds for the mutants tested[a]

Genotype (n)	Seizure threshold, V
eas^{pc80}f (2)	3.8 ± 2.4
sesB^{41} (10)	25.5 ± 7.9
sesB^{42} (8)	26.7 ± 7.3
sesB^{48} (3)	35.5 ± 2.7
sesB^{58} (5)	27.5 ± 1
sesB^{74} (16)	23.2 ± 4.5
sesB^{85} (3)	21.5 ± 3.5
sesB^{86} (5)	32.8 ± 5.2
cs^{WT001} (2)	32.6 (± 0)

[a]Short wavetrains of high-frequency (HF) electrical stimuli (0.5-ms pulses at 200 Hz for 300 ms) are delivered to the brain at various voltages. Seizures are elicited in an all-or-nothing manner with the value of the threshold voltage indicated. Seizure activation is monitored by electrodes that record HF activity in the dorsal longitudinal muscle (DLM) reflecting aberrant firing of the DLM motoneuron. The *bang sensitive* (BS) mutant *eas* has a seizure threshold that is substantially lower than the wild-type strain Canton Special (CS). These results are representative in spite of the small number tested (cf. Kuebler, Zhang, Ren and Tanouye 2001). The threshold of *sesB* is comparable to that of the wild type

Discussion

On the one hand, the threshold values are more similar to that of the wild type; on the other hand, we have only used heterozygous flies. It can be said, that *sesB* is not dominant over the FM alleles but there is an influence of the thresholds. The FM allele is definitively dominant over *sesB* referring to the behavior after an electrical or mechanical shock.

Acknowledgements

We thank Prof. M. Tanouye for the daily lessons and the generosity of inviting us to his lab. We also thank Daria Hekmat-Skafe, Edward Glascock, Jeff Tan, and Diana Ho for introducing us to the techniques, and for their support and openness.

Our stay at Berkeley University was an enrichment in many ways: We could work on our own project from beginning to end. We were challenged to obtain good results and representative data. It was our duty to process the data, draw the necessary conclusions and present them in a paper. Prof. Tanouye provided us with the necessary background by teaching us in daily lessons. In the first place, he introduced us to the techniques; later, we were taught the physical, neurobiological, and historical facts in order to understand all the correlations in this field of research.
In order to achieve the objective of a "real" paper, we worked eight hours every day making 107 recorded experiments, of which 46 gave no usable results. The first week, we practiced on more than 80 flies – some escaped, but some could be retrieved. At first we were slow, but later we were able to work individually on different experiments.

Of course we often needed advice or a helping hand, but we did the main work on ourselves. So the laboratory staff respected us and treated us more as scientists than as students. This was the greatest experience!

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Figure Credits

1. MCB Berkeley, http://mcb.berkeley.edu/faculty/NEU/tanouyem.html

2. Dickinson's Lab, University of California, Berkeley; http://istsocrates.berkeley.edu/~flymanmd/flies&flight.html (edited and described by Gunther Schmitt)

3. Flybase; http://flybase.bio.indiana.edu/.bin/fbimage?FBim5410622 (edited by Tanouye Lab, University of California, Berkeley and Gunther Schmitt)

Subcloning of *Mycobacterium smegmetis* by the Transposon EZ::Tn <kan2> for Searching New Resistance-Genes

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Abstract

The re-emergence of tuberculosis worldwide has produced a serious public health problem. Tuberculosis kills approximately 2 million people each year and the global epidemic is becoming more and more dangerous: the WHO estimates that between 2002 and 2020 approximately 1000 million people will be infected! (WHO Geneva 2002). The breakdown in health services, migrations, and especially the spread of multidrug-resistant TB strains has been worsening the situation in recent time so far, that in 1993 the WHO declared TBC as a global emergency. Although the complete DNA sequences of a large number of microbial genomes are now available, a big part of the putative genes lack any identifiable function. Unfortunately, there is also a serious deficit of understanding gene function, regulation, and especially the development of resistance mechanisms in mycobacteria. In our work, we try to establish the needed methods for genetic analyses of this less-characterized organism and to discover new resistance genes in mycobacteria.

Introduction

As mentioned, the increase of multidrug-resistant strains disables therapy and therapy-success seriously. Tuberculosis and other mycobacterial diseases (mycobacteria is the name of the superfamily of the group of bacteria responsible for the tuberculosis disease) is difficult to treat because mycobacteria possess a high intrinsic resistance to most common antibiotics. This problem has stimulated an intense research for new antimycobacterial agents. But progress in research is slow, since mycobacteria, especially because of their slow growth, are very difficult to cultivate. Another problem is, that up to now most biotechnological procedures have been developed for "standard" microorganisms like *E. coli* or *Saccharomyces cerevisiae*; hence there are many problems in using classical methods for mycobacteria.
The aim of our recent work was to discover new resistance genes in mycobacteria. For this work, we chose the following strategy characterized by three principal steps: 1) producing mutants by mutagenesis, 2) selection of suitable mutants, and 3) search for homologies.

Methods

Before setting out, we needed to search for a suitable subspecies of *Mycobacterium* as wild type: *M. smegmetis* mc 155 became our organism of choice, because the strain is fast-growing, nonpathogenic, and DNA can be introduced into it by electroporation. We also worked with a special mutant-strain of *M. smegmetis* called ERB 1, which possess a high resistance to ethidium bromide. Responsible for its resistance is a special repressor gene, which regulates the expression of a multidrug efflux pump. A knockout of the *erb1* gene enhances the synthesis of that pump and results in a high resistance to ethidium bromide. As such, we used the two strains as genetic testing ground for its more pathogenic cousin *M. tuberculosis*. Thus, we could compare the phenotype of our new mutants either with the wild type or the resistant one. A "key-step" in our work was mutagenesis. We had to develop an efficient and functional method for producing a high number of mutants. At first we used "normal" chemical electroporation – a standard

method for producing mutants in working with common bacteria. But its efficacy in mycobacteria had been too low, so we decided to employ a special electroporation kit, called EZ: :Tn <kan2> transposome, for our mutagenesis. Recently, a simple and elegant in vivo transposon delivery and transposition system was described that has become the transposon mutagenesis method of choice for such organisms (Goryshin et al. 2000). To find and develop well-functioning conditions and methods to obtain a sufficient amount of mutants for further research had been our basic problem, but by using the transposon kit we found an adequate solution! The EZ::TN <kan2> Tnp transposome is a stable complex formed between a transposase enzyme and a transposon. The transposon itself contains a kanamycin resistance gene, flanked by mosaic ends, which serves as transposase recognition sequences. The transposome can be electroporated into living cells, where the transposase is activated by Mg^{2+} in the host's cellular environment resulting in random insertion of the EZ::TN transposon into the host genomic DNA (Fig. 1; Derbyshire, Tacacs, Huang 2000). So, by electroporation we integrated our transposon in the bacterial genome

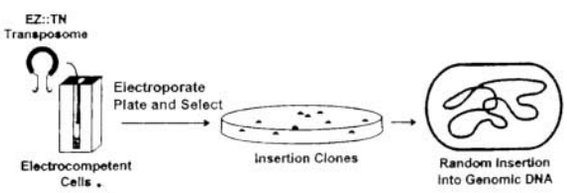

Fig. 1. Steps of mutagenesis (Derbyshire, Tacacs, Huang 2000)

and this way knocked out randomly different genes.

As a next step, we grew up new cultures of our mutants. Since our inserted transposon contained a kanamycin-resistance gene, we were able to select "successfully electroporated mutants" from "failed-electroporated" cells by adding kanamycin (a well-known antibiotic drug of the group of aminoglycosides). Only transformed cells contain the resistance gene and therefore are able to survive on the selection-media. To confirm that there were only bone-fide insertions, we digested the DNA and compared the number and size of our fragments. Finally, we crossed them out on special gradiant plates directly next to a wild-type and an ERB1-line (Fig. 2). On these gradient plates, an ascending gradient of ethidium bromide is produced. By comparing the growth directly to wild-type and to resistant ERB1-lines, we were able to screen for lines with a changed phenotype in an easy and elegant way (Fig. 3). A changed phenotype means that they show changed growth abilities in regard to ethidium bromide resistance. If there had been new resistance abilities, the genes we had knocked out by electroporation must have had any function in regard to the resistance mechanisms.

The next projected steps have the aim of identifying the knocked out genes. A further trick in identification is based on the transposon. The transposon not only contains the kanamycin resistance gene, but also includes – next to several restriction sequences – two primers (a forward and a reverse primer) by which we are able to amplify and finally to sequence our gene of interest! By this method, we have a useful tool for obtaining more information about the gene which is responsible for the phenotype change in mycobacteria; but we still have no knowledge of the gene itself in terms of product, function, or regulation of this "candidate" gene. The next step is determining the gene's sequence, and most importantly its

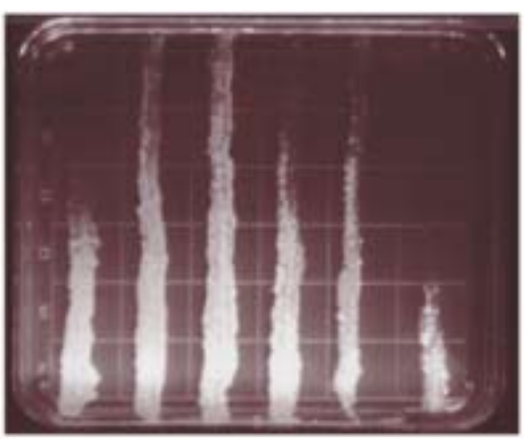

Fig. 2. Ethidium bromide gradient-plates. *From left to right*: wildtype, ERB1, high-resistant mutant, no change, no change, high-sensitive mutant

function. By comparing the sequence to sequences of genes in other bacteria, whose products and functions are better known, it may be possible to get a hint. For example, if the gene in question shows similarity to a membrane-transporter in *E. coli*, one can postulate, that the gene encodes a specific kind of membrane-transporter in mycobacteria, responsible for ethidium bromide-resistance. This may suggest further strategies in research on this pump system. Finally, the hypothesis would need to be confirmed by further tests. Eventually, one may have accumulated sufficient information for developing a specific drug against that efflux pump.

Results and Discussion

As mentioned above, the key step in identifying resistance genes in mycobacteria is mutagenesis. Such "classical methods" as chemical electroporation (a technique in which the permeability of the cell membrane is enhanced in order to import DNA by chemicals) are ineffective in mycobacteria. So it was necessary to search for an alternate route. Application of the new transposon system seems to be a useful alternative for mutagenesis in mycobacteria:
We carried out two attemps: first we used 3 µl of DNA (transposome kit) on 500 µl cell suspension and second we used 9 µl of DNA on about 1000 µl.
The electroporation of the first (second) template resulted in 504 (701) kanamycin-resistant mutants from which we obtained 35 (12) ethidium bromide-sensitive lines; in 6 (0) cases the phenotype changed to a more sensitive and in 13 (12) to a more resistant one. In our opinion, probably different temperatures of the electroporated cell suspensions are responsible for the difference in efficacy between the first and the second trial. There had been some delay between preparation and electroporation, so it could be possible that the solution in the vials had warmed up!
But all in all, we can say that the efficacy by using the EZ::TN <kan2> Tnp transposome Kit is now high enough for further application. But next to the improved efficacy, there is an additional advantage: being its simplicity! The

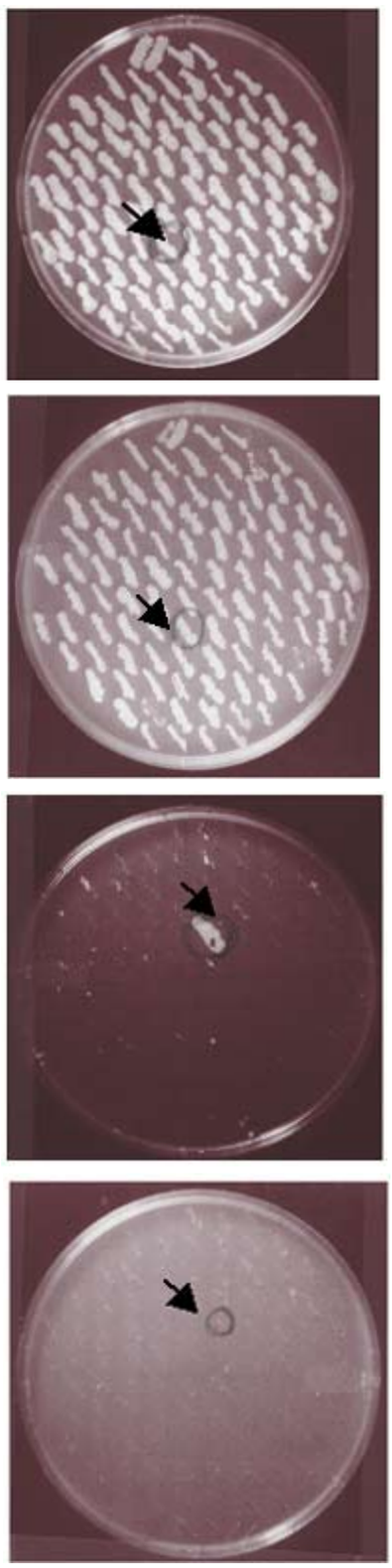

Fig. 3. Plates of different ethidium bromide concentration (vol/vol): *From top to bottom*: 1, 4, 16, and 32 µl/liter; growth of a single high-resistant

insertions can be selected directly after electroporation, and all insertions are independent. This system does not require the difficult construction and development of conditional replication vectors. Therefore, we are now able to produce a high number of suitable mutants (i.e., mutants whose phenotypes are changed). Especially because of the low growth rate and difficult mutagenesis, the subcloning of mycobacteria is a very difficult and lengthy process. But in our opinion, by using the transposome EZ::TN <kan2>, the process is speeded up significantly. So we hope that we will soon be able to find some important resistance genes; with this perspective we may have achieved a small step forward in regard to fighting tuberculosis and multidrug-resistance.

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Taste Recognition in *Drosophila melanogaster*

Martin Schorb and Sebastian Urban, Scott Lab, Department of Molecular and Cellular Biology, University of California at Berkeley

Introduction

The gustatory system (taste) – in collaboration with other sensory systems – must distinguish nutritive and beneficial substances from those that are potentially harmful or toxic.

Taste receptor cells detect a wide range of chemicals, everything from simple ions, such as sodium or chloride, to very complex compounds, including numerous bitter and sweet molecules. The major role of taste receptor cells is to recognize these chemical signals and translate this information into the language of the cell. This includes changes in membrane potential or intracellular free calcium concentration that lead to signaling this information to the brain via gustatory afferent nerve fibers. There are many chemical compounds that stimulate the gustatory system and the transduction mechanisms found in taste receptor cells are numerous and varied.

One of the most important issues in taste transduction is to understand which receptors recognize which substances.

The gustatory receptor protein complexes are located in the cell membrane of taste neurons. Examining the role of these receptors requires a simple model organism with a small number of neurons and a simple genome, such as *Drosophila melanogaster.*

Approximately 60 gustatory receptor (GR) proteins were discovered by searching for transmembrane proteins in a genome database and examining their expression in taste cells.

The 'Scott Lab' created several transgenic lines of Drosophila to study the taste system. In one, specific receptor cells are fluorescently labeled. In another one, there is a toxin expressed along with the GR. This means if the receptor is expressed, the diphtheria toxin (dti) is also expressed and those cells die.

Method — Larva Assays

To make conclusions about the connection between certain receptors and specific tastes, we used a method called larvae assay. We continued existing experiments with the six mutant receptor-knockout (dti) GR-lines and a wild type of another Drosophila. The aim was to determine whether larvae lacking specific receptors no longer recognize specific tastes.

The assay consists of two major parts: (1) Placing larvae of one fly line on a surface partially containing the substance (the so-called tastant), and (2) observing and analyzing the behavior.

Drosophila is cultured in plastic bottles that are filled with a nutrition mixture, mainly containing sucrose, where they lay eggs and the larvae grow. It takes one week for them to lay eggs and grow larvae. To collect the larvae, you float the larvae out of their food using a sucrose solution, segregate them from pupae, clean them, so that there is no more sucrose left sticking to their bodies, and let them starve for about an hour.

You prepare a dish filled half with 1% agarose solution and half with the substance dissolved in 1% agarose solution. To guarantee an as randomized as possible position, the gel parts are cut and placed as seen in Fig. 1.

The starved and, of course, hungry larvae are placed out in the marked middle line.

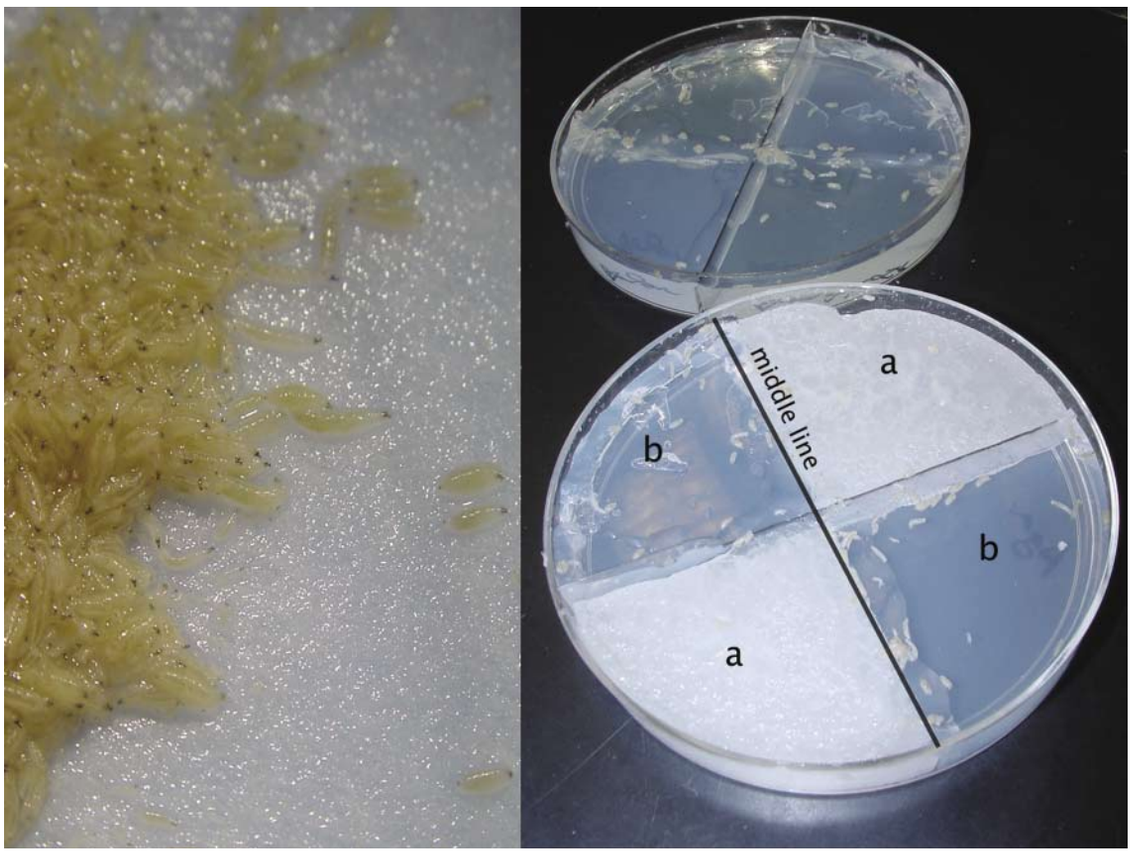

Fig. 1. *On the left:* Larvae spreading and starving on filter paper. *On the right:* Experimental setup, agarose-filled dish. Section (a) contains tastant (here: caffeine), section (b) contains only agarose

You let them spread for a few minutes and then count the ones on the substance as well as those on the agar. The larvae that did not move at all or are trying to escape at the edges of the dish are not counted.
If larvae do not taste a difference between the substance in agar and the (tasteless) agar, they are supposed to spread all over the plate randomly. According to this we calculate the percentage of larvae on substance and substract 50% (the random case). So we can make out three groups of results: values around 0% mean, that the larvae do not taste a difference, values obviously larger than 0% (at least 15%) show an attractant behavior, while values obviously smaller than 0% (fewer than -15%) suggest an avoiding behavior.
The GR22B1 and GR47A1 receptor is not expressed in larvae at all. So we expect the lines that have knocked out these receptors to behave similarly on all substances, and that we use them as a wild-type-like standard in comparison with the four mutant lines left and the W1118 wild type that might behave different than the others.
If now one of the wild types is showing a definite behavior towards a certain substance and one of the mutants seems not to care about the same substance, it should be possible to state that the receptor knocked out in this mutation is responsible for tasting that substance.
The goal of the experiment is to determine the specific taste function of certain receptors.

There is in fact quite a number of difficulties: To have enough data to give reliable results, you have to run the assay many times, because the results of the assays vary enormously.
Another problem in the assay is that there are all different ages of larvae present and larvae at different ages may show different taste preferences. The variability of the assay may be reduced by using larvae of the same age. This is presently being tested.

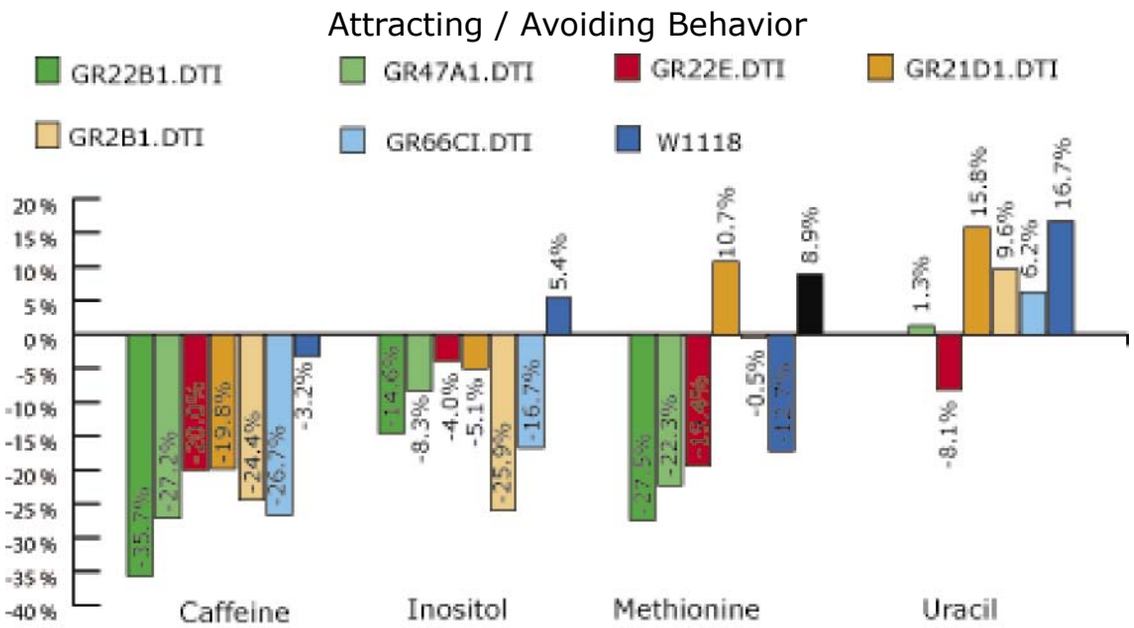

Fig. 2. The graph lists four sample substances on the *bottom*. While each color represents one fly line, positive values show attracted behavior, negative values illustrate avoiding behavior. Caffeine shows avoiding behavior for most fly lines. For inositol the result is not that clear, it shows more the difference between the GR-lines and the W1118. Methionine has an interesting result: GR21D1.dti and GR2B1.dti show different behavior than the wild types and even the two other GR-lines. Uracil is just shown as an example how sometimes strange results arise

Our major task was to develop and introduce mathematical approaches to correlate, sort, and analyze the resulted data to determine more specific goals – and then to run these assays.

Method — Tissue Culture

Another method that is just being evaluated in order to discover receptor responses to chemicals is tissue culture. The idea is that you can monitor single receptor cells reacting to certain substances. A calcium-sensitive GFP is used to monitor changes in activity. If the receptor recognizes a substance there should be a change in fluorescence seen under the confocal microscope.

Experiment Development

We merely applied the first steps in this method: extracting the cells out of pupae proboscises and culturing them. We used an old protocol in the lab and improved it more and more adapting them to our aims. First you have to unpeel the pupae out of their cases and decapitate them. Pupae are used because their taste neurons are fresh and unused but all are fully developed. While cutting the proboscises it is important not to cut the maxillary palps, because there could also be some labeled neurons in it. After cutting them, which requires a lot of patience, the proboscises are washed in a salty solution (HBSS) and treated with the enzyme papain to extract single cells. These cells attach to a special coated coverslip and then grow within L-15 growing medium. To set up one culture, about 60 proboscises are needed, because every proboscis contains only about 100 gustatory neurons. There is a lot of cutting work to do.

Since our experiments were set up for the first time, there were numerous problems with contamination and often only few cells survived the first days of growth. So we attempted to refine the procedure. But with the modifications in the protocol and increased attention to sterile working conditions, this method is really one step closer to application.

Personal Impressions

Both of our experiments gave us the possibility to really get involved in fundamental science. We got used to the daily work in the lab and learned

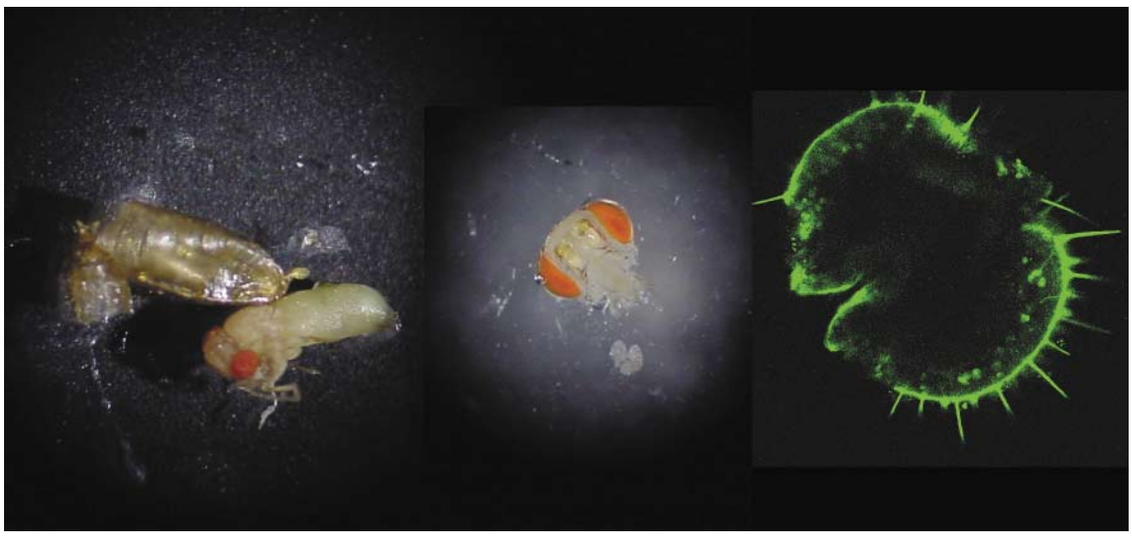

Fig. 3. *Left:* Unpeeling a pupa out of its pupa case; *Middle:* Head with the proboscis cut off right under the maxillary palps; *Right:* Confocal microscopy picture of a proboscis with GFP-labeled neurons *(green dots)*

considerable know-how by doing all experiments ourselves. Self-contained working and learning is significant in the Scott Lab. We were taught always to have a second, distanced look on our results. Well, in the Scott Lab you have to think and taste for yourself.
Looking back at four intense weeks, there were really experiences: setting up an experiment, including own ideas and improving the methods, or speaking English as the normal language for conversation even with other German LSL group members or with another German intern in our Lab. What made our time in the Scott Lab so unforgettable is the incredible atmosphere — the international flair with roots in India, China and Iran and even from the East Coast. Visiting a chocolate factory, going to an open air festival and a baseball game together, even watching videos together in the evening, with the Scott lab staff nearly anything is possible.
Of course, the taste assays are not only limited to flies. Maybe the results of the human assays, such as having breakfast at 'Bongo Burger' or in the Lab, or lunch at 'Grégoire's', are much more interesting. It is easy to prove that humans are totally attracted to sugar, such as original German "Gummi"-Goldbears or the customar doughnuts and bagels during the weekly journal-clubs. And of course, it's scientifically proven: chocolate is extremely attracting for the human gustatory system.

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The Academy Participants

Samuel Bandara

was born in November of 1982. During my time in high school, I worked for a leading speech technology company as a software developer and programmer. At the *Heidelberg Life-Science Lab*, I participated in the workgroups on bioinformatics and pharmacy. After graduating from high school in 2002, I began my studies in Molecular Biotechnology at the University of Heidelberg. Presently, I tutor students of the *Heidelberg Life-Science Lab* in "Business@School" – a competition carried out by The Boston Consulting Group.

My hobby is playing the violoncello with the Symphonic Orchestra of Neckargemünd, which is a beautiful town upstream the Neckar river.

In the laboratory of Suzanne Pfeffer at Stanford, I was able to learn basic techniques in biochemistry, and got enthusiastic about membrane trafficking. I will never forget the weeks at Suzanne Pfeffer's lab; this experience will contribute to my studies in Heidelberg in a most valuable way.

Anna-Lena Beutel

born on 14th of February 1984, I received my secondary education from the Edith-Stein-Gymnasium in Bretten. During this time my interest grew in science, management, and cultural studies. After a five-month scholarship at the French secondary school "Lycée de Grand Air", I joined the *Heidelberg Life-Science Lab* in 2001. There I joined the neurobiology, business, and molecular biology workshops where I enjoyed working with motivated people - equally as during my time at the "Deutsche Schüler Akademie" (DSA). The International Science Academy Portugal in 2002 and my engagement in several weekend workshops contributed to finding my way.

Sometimes people ask me what I have learned while participating in the San Francisco Academy. One aspect and probably the most important one is best expressed by Sir Philip Sidney: *"Either I will find a way or I will make one".* At the beginning my assignment to establish internships at Stanford University appeared to be almost impossible. But as I knew how interesting and sophisticating an abroad internship can be, I didn't cease my efforts to promote the San Francisco Academy. I enjoyed the oppurtunity to be a member of a great team!

Claudia Bignion

Studienrätin at Christiane Herzog Schule, Heilbronn; Doctor for Emergency Medicine, Bachelor of Science Degree in Dental Hygiene, University of Colorado, School of Dentistry – Since January 2003, Tutor for the San Francisco Academy of the *Heidelberg Life Science Lab*.

Hobbies: golf, ballroom dancing, ballet, swimming laps, visits to Württembergisches Kammerorchester, Heilbronn, skiing, sailing, travelling, seeing friends, enjoying the beauty of nature and life in general.

Impressions of the San Francisco Academy 2003: My main feeling at the end of our journey to San Francisco was gratitude and relief. The internships and spare-time activities worked out very well. The students were independent, reliable, and very communicative. All in all, this made our stay a full success.

Thanks to all the participants of the San Francisco Academy for a pleasant and enjoyable stay in California. May life treat you well and please keep in touch!

Michael Breckwoldt

Student coordinator of the San Francisco Academy 2003.
Born on August 6, 1983 in Heidelberg. Having graduated (Abitur) with honors from the English Institute, Heidelberg in early summer, I enrolled at medical school in Munich commencing my university studies.
The San Francisco Academy 2003 grew out of my initiative and surely became the single most-significant experience during my membership in the *Heidelberg Life-Science Lab* (LSL) – probably one of the most important group and learning experiences so far in my life.
In the LSL, I was a member of the Neurobiology Group and Biochemistry Group and participated in an EMBL project*.
My main hobby is reading – almost anything – it is a great way to broaden your horizon.
The San Francisco Academy was a great experience. I learned a lot in various ways – not only concerning my scientific project in Berkeley, but also in terms of organization, communication, and group leadership. Discussions with other academy participants were inspiring and the international scientific community on campus was stimulating and impressive – quite thought-provoking the differences in the people we encountered daily at the bustling YMCA.
After a great time with the *Heidelberg Life-Science Lab*, I am now at a point to move on – I have clearly learned "to think for myself."

* reference: Andrew Moore *EMBO Reports* **1**(5)384–386 (2000)

Sarah Claus

was born on the 7th of February, 1983. My high-school years at the Elisabeth von Thadden School in Heidelberg ended with graduation (Abitur) in 2003. Now, I have enrolled in pharmaceutical studies at the Ruprecht-Karls-University of Heidelberg. Since the summer of 2002 I have been a member of the *Heidelberg Life-Science Lab*. Over the years I have attended two LSL workgroups, biochemistry and pharmacology, and completed an intermship at the European Molecular Biology Laboratory (EMBL).

My hobby is music – playing the clarinet – as a member of the Mannheim Wind Philharmonic orchestra.

For myself, the San Francisco Academy 2003 was a very interesting and challenging experience. I appreciated the lab work and found it very exciting to participate in the ongoing research of a leading institute.

Christoph Fischer

was born on July 25, 1984 in Heidelberg. In 1993 my family moved to Antwerp, Belgium where I lived for 7 years. In 2001, directly after my return to Germany, I joined the *Heidelberg Life-Science Lab* where I am member of the "Physics group". I graduated from high school (Abitur) in Heidelberg at the St. Raphael Gymnasium in 2003.

Currently I am performing my civil service at the Hörsprachzentrum Heidelberg, a school for disabled children with speech disorders. After that I am thinking about studying law or physics. I have enjoyed the San Francisco Academy 2003 and will do my best to foster its continuation in the future.

Monika Gessat

Mentor of the San Francisco Academy 2003 and teacher of biology, chemistry, and physics at the Edith-Stein-Gymnasium in Bretten, Germany. There my main focus is to awaken and to encourage student interest in the natural sciences. My hobbies are travelling, hiking, dancing, listening to classical music, and reading contemporary literature.

The San Francisco Academy 2003 remains as a most impressive event in my mind. For my occupational activity as a teacher, I took advantage of the opportunity of updating my knowledge of current techniques in biochemistry. It was interesting to get to know the American way of teaching science. The engagement of the American scientists in supporting us during our projects in the labs has been very impressive.

I want to thank all members of the San Francisco Academy. It was a pleasure to accompany them. Their staying power made the realization of the academy possible and their thorough preparation allowed them to successfully complete their projects. A good basis for future academies in San Francisco was provided by the new-established contacts to the professors and departments at Berkeley and Stanford.

Felix Gut

was born on the 30th of August 1983. I graduated from the Dietrich-Bonhoeffer-Gymnasium of Eppelheim in 2003. Prensently I am enrolled in pharmacy at the Ruprecht-Karls-University of Heidelberg. I joined the *Heidelberg Life-Science Lab* in 2000 attending workgroups in molecular biology, biochemistry, chemistry, and pharmacy serving as tutor since July 2003. During this time I have completed an internship at the European Molecular Biology Laboratory (EMBL) and was a participant of the International Science Academy in Portugal 2002. As a member of the LSL office staff I have helped arrange the Science Academy Baden-Württemberg 2003.

My hobby is rowing and serving as a youth representative and instructor at the rowing association of Heidelberg (RGH).

Philipp Gut

graduated from high school in Eppelheim/Heidelberg in 1999 with profile courses in biology and mathematics.

As a medical student at the University of Heidelberg I am now working as a doctoral fellow at the Institute of Anatomy and Cell Biology under Prof. Klaus Unsicker. Before, I completed my civil service "Zivildienst" at the German Cancer Research Center (DKFZ), Department of Immunology with Prof. Peter H. Krammer doing various jobs including lab work, administration, and research materials management.

Hobbies: My passion is running and triathlon - also as an instructor.

The San Francisco Academy 2003 has left me deeply impressed. The students were not going to wait for the future to come, but rather create their own future by setting up and arranging the San Francisco Academy all by themselves. The meaningful experiences we made in the labs, the positive atmosphere and mutual responsibility among the group members, and the "easy-going" Californian way of life were unique for this kind of program – and I hope it will be carried on in coming years.

Laura Michel

graduated from high school (St. Raphael Gymnasium, Heidelberg) in 2003. Presently I am enrolled in medical studies at the University of Munich.

As an early member of the San Francisco Academy, I am now looking back in pride to a great experience. It seemed to be the ideal time for an internship abroad. After school graduation when decisions need to be made for the future, it was extremely helpful to look at my previous life from a distance – a taste of independence.

At first I was a bit concerned about visiting a lab, working on a project all on my own, with limited practical experience, in a completely unfamiliar working environment. But, I got well integrated in the lab and it was great to work with Alenka, my supervisor in the Schekman lab. After the first week, I was able to work more and more independently and this made me more self-confident.

I hope that the San Francisco Academy will be carried on, because it is a great way of starting an academic career, and I think further participants of the LSL should grab this opportunity.

Markus Schindler

born on the first of May 1983. After my elementary education I attended the Elisabeth von Thadden Gymnasium in Heidelberg. At the beginning of my school career I was interested in archaeology particularly the ancient history of the different Egyptian dynasties. But in the course of time my interest shifted more and more towards the sciences. When I joined the *Heidelberg Life-Science Lab* in 2000, I realized the unprecedented opportunity to gain insight into different research areas and to share my passion about the discovery of new aspects and horizons. After a scholarship at the Canadian Eastview Secondary School and two Portugal Academies, I realized how crucial abroad experiences are and along with others initiated the San Francisco Academy in 2001. Right from the start many interested and talented people worked hard to accomplish our project and in retrospect it was worth every single minute, because "Life is like a book, and those who do not travel, read but a page" (St. Augustine).

Gunther Schmitt

was born on December 6, 1983. I attended the Heinrich-Böll-Gymnasium in Ludwigshafen and graduated (Abitur) in 2003. Currently, I am enrolled in psychology at the University of Mannheim.

Since 2001, I have been a member of the *Heidelberg Life-Science Lab*, as one of the first students from the Rhine Palatinate (Rheinland-Pfalz). In the neurobiology workgroup we focused on the nervous system and the influence of the mind to the human body, whereby I discovered a lively interest in psychology. Furthermore, I completed an internship at the Max Planck Institute for Medical Research (MPI) in Heidelberg. In my free time I enjoy biking, reading, and going to the movies.

I would like to thank all the people who made the SFA 2003 possible by planning the trip, organizing the internships with the professors, and supporting and preparing us in Germany. For me this academy was a great experience, as for all of us, because we could visit the USA, get to understand and appreciate the "American way of life", and have an opportunity to do exciting own research in top-ranking biology labs. I hope that this will encourage other students to organize further academies in the U.S. in coming years.

Martin Schorb

graduated from high school in Eppelheim in 2003 with profile courses in mathematics and physics.
After returning from the San Francisco Academy, I enrolled in physics at the University of Heidelberg.

Within the *Heidelberg Life-Science Lab* I attended workshops in molecular biology and biochemistry, and participated in internships at the DKFZ (German Cancer Research Center) and EMBL.

As a member of the International Science Academy in Portugal 2002, I explored bioenergetics in thermophilic bacteria.
As tutor and student coordinator I will be involved in carrying on the exciting project of the San Francisco Academy, trying to make the following academies as successful as this one.

Other than pursuing my interests in the sciences, I play saxophone and percussions.

Jonas Tesarz

born on June 22, 1979. As a third-year medical student, I have also been engaged in philosophical studies at the Ruprecht-Karls-University of Heidelberg. My doctoral thesis, which I have recently started, is on neural processing of muscle pain in the spinal cord at the Department of Anatomy and Cell Biology with Prof. Siegfried Mense.

As a member of the *Heidelberg Life Science Lab* I have been an enthusiastic participant and tutor of the San Francisco Academy 2003. The academy was an impressive scientific and group experience.

Next to science, I am interested in almost anything.

Sebastian Urban

was born in 1984 in Ludwigshafen, close to Heidelberg, and finished secondary school in 2003; since 2001, a member of the Heidelberg Life-Science Lab; there, responsible for communication systems, web pages, and administrating the Lab-owned laptop iBooks. My focus within the *Heidelberg Life-Science Lab* is on neurobiology, especially on techniques for measuring activity in the nervous system. Inspired by an internship at the Neurological University Clinic in Heidelberg in 2002, together with two other participants I built an electro-encephalograph device.

Besides my scientific activities I am a rescue swimmer and -teacher, I play the piano and enjoy dancing. Upon returning from San Francisco I started my civil service at the German Cancer Research Center (DKFZ) in Heidelberg; my intention is to study mathematics at the University of Karlsruhe.

Florestan Colin Ziem

born on February 7, 1986. I am a Dornier Scholar at Birklehof Boarding School and will graduate in summer 2004. My intension is to study physics or mathematics. In my free time I enjoy reading, playing basketball, playing the drums, and taking photographs. I have been a *Heidelberg Life-Science Lab* member since summer 2003 and have joined in the San Francisco Academy 2003, so far.

My time spent at UC Berkeley has confirmed my wish to study physics and probably to run for professorship. During my time at UC Berkeley I have obtained insight into different fields of work and the corresponding preparation that researchers handle.

I would like to thank all those, who have worked hard to make the Academy possible.

Epilogue

Going to San Francisco – Reasons Beyond the Californian Sun

Why should freshly graduated German high school students spend time on a Science Academy rather than taking a laid-back vacation on the beach? Why should they work long hours in a lab doing highly specialized research rather than waiting for things to come their way? ... because learning has a different quality after high school. For the first time they can actually decide on their own on what and how much they want to learn and take responsibility for. The decision to attend a university and what field to choose, means a far-reaching commitment, affords good judgment, and needs to be well planned.
The special value of the San Francisco Science Academy 2003 is manifold and any one aspect does not reflect the overall learning experience of this Science Academy.

Why did they choose San Francisco as the site of their International Science Academy – while their hometown Heidelberg actually provides world-class research facilities and offers nearly every field of science and medicine.

The Bay Area is the region with the World‘s highest density of top-class research facilities and start-up companies. California has one of the most efficiently communicating networks between education, research and industry, and for many serves as a role model. The opportunity of gaining insight into both the American and German systems before setting out with their university studies is of great value for the novice, especially for their self-confidence in future decisions.
But there are more reasons for such a journey to a foreign country. For the late teens it was a challenge to contact researchers at top-ranking American universities, arranging housing, flights, and insurance, reading scientific papers, frequent group meetings – all at the same time as preparing for their final graduation exams – they had to be perfectly organized.

Once there, being reliable team players throughout the weeks and adapting to all the „little differences and details“ of American life afforded constant devotion. Group integration in the hosting lab, overcoming language barriers, cultural differences needed to be mastered at the same time as trying to obtain useful and reliable results in their experiments.

They asked for the challenge, they challenged themselves, and they were challenged. The reward of those thrilling “gee-whiz” experiences that make science so much fun – were all based on their own initiative.

Instead of sunbathing on the beach, they decided to “climb a mountain”.
They drafted a plan, made all necessary provisions, secured the ropes and made it to the top – now enjoying the fresh breeze and a taste of their future ahead.

Regarding the success of our San Francisco Academy 2003, I would like to express my admiration for every participant and I am proud to have had the opportunity to work and learn with this group. I wish them all the best for their future projects.

This documentation is meant to reflect our enthusiasm and is hopefully contagious to those who may want to follow along.

Philipp Gut
Tutor and Participant ISA San Francisco 2003

*Special thanks to Theodor C.H. Cole (Ted) for all his support, sharing his enthusiasm, and his energy with us during the last 9 months.

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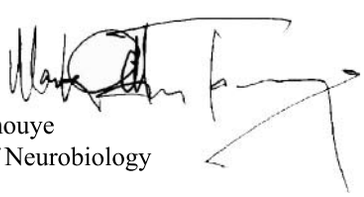
August 8, 2003

To Whom It May Concern:

I am writing to give my opinion of the Heidelberger Life Science Lab. During July 2003, I had the opportunity to have two students from this program work in my laboratory here at UC Berkeley. The students were Felix Gut and Gunther Schmitt. I was very impressed with the quality of these two students. They were very smart, learned quickly and effectively the methodologies that we use in the lab, and produced high-quality and reliable results during the short time they spent here. From this experience, I was pleased that the program placed these students with us; it was a very enjoyable and productive time. I have also spoken to one of my colleagues, Professor Kristin Scott, who had a similarly good experience with students of this program. I recommend that you continue to support and possibly expand this program: it has made a very strong impression on me and my colleagues.

Sincerely,



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