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HISTORICAL SKETCH

First isolation of actomyosin from a non-muscle cell: first isolated platelet protein

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How it all started

I (M.B-G.) was born in 1928 in Beauvais (France) where my family lived until 1929, but because of the economic depression, my parents moved to Geneva where we were citizens. Both my parents were interested in the scientific aspects of biology, which was certainly important for my education. Living in Geneva was optimal, as we could easily go to high school and the University by tram, although I preferred my bike. As the 1939–45 war affected everybody, I worked during the holidays, first on farms, but I soon noticed that working in a factory was better paid. After the war during school time I gave French lessons to United Nations personnel. At the University, as I had good marks in biochemistry, I was asked to be a teaching assistant at the Botanical Institute.

Nevertheless, I was the 'ugly duckling' of the family because since birth, every beat of my heart produced a murmur, which could not be diagnosed then, so that the physicians warned my parents that I would die very young. Nevertheless, when I was 20 years old, the correct diagnosis was made: I had an open 'ductus Botalli', which could then be operated on. Prof. Max Grob, from the University of Zürich Children's Hospital, performed the operation. Being 22 years old, I was an exception in a children's clinic. A young assistant in surgery, Marcel Bettex, was working there then. This was how we met, and after a time we decided to unite our lives. I nevertheless stubbornly finished my studies in biochemistry, which postponed our marriage for 2 years (1952).

In Zürich I found a job as an assistant at the Physiological Institute, the head of which was Prof. F. Leuthardt. The salary was poor but the topic was interesting. After a time Prof. Leuthardt wanted me to prepare the demonstrations for the students. As his lessons began at 08.00 h, I often had to work at night. Once I had to prepare actomyosin from rabbit muscle, so I learned to handle this protein using the classical method of Szent Gyorgyi [1]. I had to demonstrate the 'superprecipitation', that is the contraction of the normal precipitate under the influence of ATP.

I also made some investigations on the role of biotin in the fixation of carbon dioxide by animal tissues and used these as the basis of my PhD; however, first I had to submit to an examination board. The chairman was Paul Karrer, who had been awarded the Nobel Prize in chemistry and had written a thick book on organic chemistry, which I had to learn. So I memorized it, sitting for 3 months at my desk and in the examination was asked to describe the synthesis of morphine: I got the maximum mark (1958).

At that time, my husband, who was a very gifted pediatric surgeon, was appointed as head of the Paediatric Surgery Clinic in Berne. As a consequence I wanted to find a job in Berne. Among two opportunities I chose to work with Ernst Lüscher, because he offered me a laboratory, a technical assistant (Madeleine Schneider), and a good salary.

Platelets and thrombosthenin

E. F. Lüscher had come back to Switzerland after almost 3 years in Pasadena, California, performing research on immunochemistry with Dan Campbell in Linus Pauling's department. He found a place to work with Prof. A. von Muralt in the Physiological Institute, who gave him a book to read written by Dr A. Fonio (Head surgeon in the hospital of Langnau, near Berne) and Dr J. Swendener: *Der Thrombozyt des menschlichen Blutes* (published by Huber Verlag). The authors were fascinated by blood platelets, the anuclear fragments of megakaryocytes, capable of projecting long pseudopods and responsible for the retraction of the blood clot. Lüscher was interested in this as platelets also play a major role in blood coagulation and hemostasis. The role of platelets in the production of factor III (the exposure of negatively charged phospholipids, involved in procoagulant activity) was one of his main preoccupations. On the other hand Ernst Lüscher did not work in the laboratory because, as he said himself, 'he was not gifted with his hands'. However, he read the literature thoroughly and had many contacts with other researchers in the field of

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Fig. 1. Madeleine Schneider and I examining thrombosthenin retraction at the Theodor Kocher Institute in 1959.

Fig. 2. At a joint Congress of French and Swiss physiologists in 1965, Ernst Lüscher spoke about coagulation and hemostasis, after which I had to report my work on platelet actomyosin. The photograph shows Lüscher and I discussing with Prof. Ephraim Katchalski-Katzir (President of Israel 1973–78), member of the Institute of France and the Academy of Sciences, who established the Department of Biophysics at the Weizmann Institute of Sciences and who came to congratulate us warmly for our work on platelet actomyosin.

coagulation and hemostasis. He gave me the task of studying the contraction of the blood clot.

We were fortunate that the Swiss Red Cross in its Central Laboratory centrifuged large amounts of blood from healthy donors to prepare freeze-dried plasma for the army, as well as for the isolation of various blood factors. What remained after removing the plasma over the red blood cells was a thin whitish sheet containing the leukocytes and the blood platelets called 'buffy coat'. The procedure was not easy, the centrifuges were in the cellar of the arsenal situated on the other side of the city. I was happy to have a car to transport the buckets with ice to refrigerate the cells.

Madeleine Schneider (Fig. 1) was skilful at the multiple centrifugation steps needed to obtain pure platelets in suspension. These were already in a more or less stellate form (a shape change caused by the cooling), the state before 'viscous metamorphosis', which leads to blood clot retraction. Nevertheless, they still contained a fair amount of ATP.

We first examined their glucose consumption and respiratory capacity, as well as their ability to retract a blood clot or pure fibrinogen suspensions. We also checked the ATP content of the platelets and found that the ATP level decreases during clot retraction [2]. Gustav Born, from London who could measure ATP in minute amounts with firefly extracts, also came to the same conclusion [3].

We concluded that the retraction of a blood clot was directly dependent on the ATP-level, indicating mechanical work. Therefore, I proposed to Ernst Lüscher (Fig. 2) to try to extract an actomyosin-like protein from platelets. Lüscher was quite sceptical about this idea!

We prepared a washed platelet suspension containing 10^7 platelets μL^{-1} and less than one leukocyte per 10^5 platelets. The suspension was homogenized in buffered 0.6 mol L^{-1} KCl in a small refrigerated mechanical blender at 17 000 r.p.m. If the ionic strength was lowered by diluting with water to 0.1 mol L^{-1}, a precipitate forms, which in the presence of ATP and Mg^{2+} contracts or 'superprecipitates' like muscle actomyosin (Fig. 3). We published these observations first in *Nature*, which is known to publish rapidly [4].

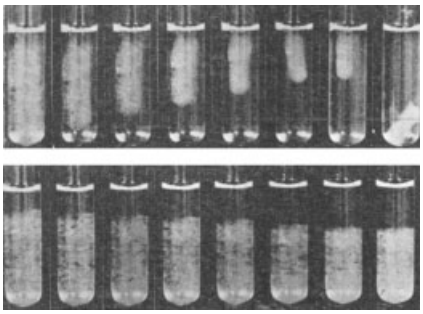

Fig. 3. Contraction of thrombosthenin in response to ATP. The upper series of pictures shows the contraction of the actomyosin precipitate in response to ATP, the lower series the sedimentation in the absence of ATP. The platelet extract was reprecipitated once. Protein concentration 0.4%, ionic strength 0.08 mol L^{-1}, pH 6.3, $MgSO_4$ 6 mmol L^{-1}, total volume 1.5 mL.

Later, we measured the nitrogen content of a given number of platelets compared with the nitrogen content of contractile material extracted from the same number of platelets using the Kjeldahl method. The contractile material represented 15% of the total platelet protein, a figure close to that of present day estimates.

Our laboratories were in the Theodor Kocher Institute, built with a gift of the Nobel Prize for Medicine from the surgeon of that name. Prof. Alexandre von Muralt (who was behind the organization of the Swiss National Science Foundation and chairman of the commission administrating the Theodor Kocher Institute) had carried out some experiments on muscle actomyosin with J. T. Edsall at Harvard when he was in the USA. He was very interested in our work, and requested that we give a name to this new protein (what Prof. von Muralt

wanted, had to be done). My husband, who had studied Greek at school, proposed 'thrombosthenin' meaning the strength of the blood clot, with the result that French hematologists called me 'Miss Thrombosthenin' [5].

Glory and renown

Very soon after having discovered the fact that not only muscle cells contain an actomyosin-like protein, E. Lüscher and I received the Otto Naegeli prize (1965), amounting to 100 000 Swiss francs, then the highest Swiss scientific award. As at that time I had some problems with my children, I was at home when the phone call came, announcing the news. My husband and Madeleine Lüscher organized a celebration of this award, to which all the members of the Theodor Kocher Institute and of the neighboring laboratories were invited. It ended in a country restaurant where various speeches were made. Prof. Wildbrandt, head of the Pharmacology Institute, remarked: 'Generally, when a scientist receives such a prize, he stops working immediately or goes on working just a few more months. But nobody has ever seen somebody like Micheline, who stopped working 6 months before and then received such an award!' Various newspapers published articles about the prize, and as I became known from this publicity, I was elected to the executive council of seven members of our local community of some 4000 people and there took charge of the department of construction, buildings and roads. In Switzerland it was the first year (1971) that women could vote and be eligible for election!

It was really a breakthrough and a great success! When I applied to the European Society of Haematology, 8th Congress in Vienna in 1961, I was invited as a keynote speaker [6]. I had 20 minutes to speak in the main hall instead of 10 minutes like the other participants. The isolation of platelet actomyosin was the first in a long series of discoveries establishing the Theodor Kocher Institute as a major center for platelet research.

Follow-up research

The next step in our research was trying to dissociate an actin protein from a myosin protein to test if 'thrombosthenin' was identical to muscle actomyosin [7]. Indeed, actomyosin from muscle seemed a likely candidate based on ATPase activity. Like actomyosin from muscle, the viscosity of 'thrombosthenin' also decreased drastically after treatment with ATP [8].

Having carried out some electron microscopy experiments in Geneva, I wanted to try this method on platelets. The first pictures of platelets were made by Parmeggiani and showed degranulation of the platelets after treatment with thrombin. The quality of the pictures was not very good, but they were the first to show the platelet in a state of viscous metamorphosis. Prof. E. R. Weibel, head of the Institute of Anatomy in Berne, was also interested, he invited me to use his electron microscope and provided a technical assistant. The α-granules from the platelets are equivalent to the Weibel–Palade bodies in endothelial cells which Prof. Weibel had discovered during a

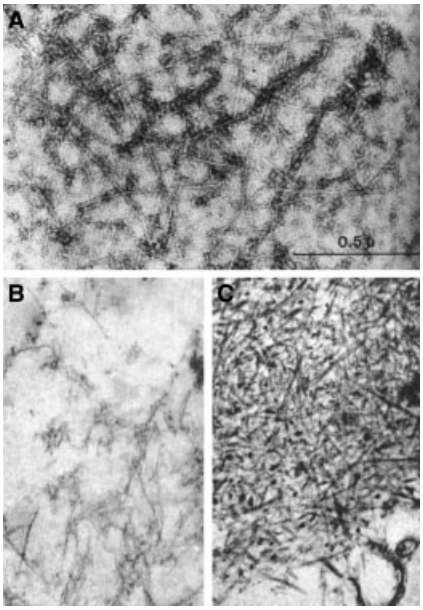

Fig. 4. (A) Electron micrograph of highly purified thrombosthenin-A (actin) on thin carbon film, negative contrast with uranyl acetate, magnification ×144 000. Sections of precipitates of platelet thrombosthenin at 0.1 mol L^{-1} KCl: (B) without ATP; (C) with addition of ATP. Dark spindles of thrombosthenin-M (myosin) are clearly visible in the precipitate in (C). The precipitates were fixed with buffered glutaraldehyde, postfixed in OsO_4, and contrasted with uranyl acetate and slide-contrast made with lead citrate. Magnification ×125 000.

stay in the USA [9]. To distinguish the two proteins derived from the contractile material of the platelets we called the actin-like part thrombosthenin-A and the myosin-like part thrombosthenin-M.

Acetone-dried powder from human platelets was extracted with KI and dialyzed against buffered 0.1 mol L^{-1} KCl, then centrifuged at 100 000 × *g*. When the pellet was resuspended in the same solution, electron microscopy on thin carbon film after negative contrast with 2% uranyl acetate clearly showed the fine filamentous structure of actin (Fig. 4A) [10]. Thrombosthenin-A in its globular state could be compared with actin from striated rabbit muscle by polyacrylamide gel electrophoresis: both proteins migrate at almost the same speed.

Ultrathin sections were made from fixed thrombosthenin at 0.1 mol L^{-1} in precipitates and in superprecipitates with the addition of ATP, as well as from platelets treated after extraction with glycerine under the same conditions. In the non-contracted state the thrombosthenin is diffusely dispersed (Fig. 4B), but under superprecipitation conditions, with the addition of ATP, the massive spindle structure of thrombosthenin-M is clearly distinguished, the structure of thrombosthenin-A is less clear (Fig. 4C). In whole platelets the identical state can be observed: the membrane of the platelet is visible, although not quite intact, and in the absence of ATP the contractile protein is diffuse in the cell, but in the presence of ATP it becomes concentrated as 'superprecipitate' in the center of the platelets (Fig. 5) [10]. Normal platelets were also prepared by allowing the blood to flow directly from the

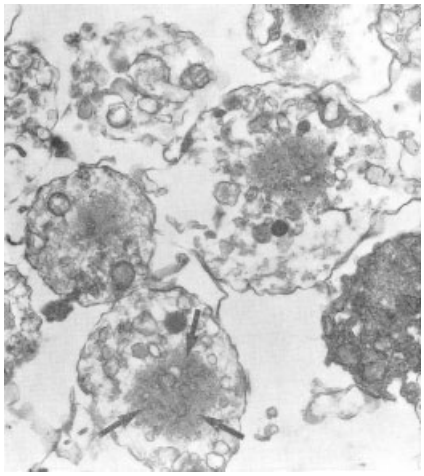

Fig. 5. Section of glycerol-extracted human platelets fixed under conditions of superprecipitation (i.e. ATP treatment): the platelets are spherical and contain various organelles as well as a distinct, heavily contrasted precipitate containing actomyosin contracted in a central mass (indicated by arrows). Magnification ×18 000.

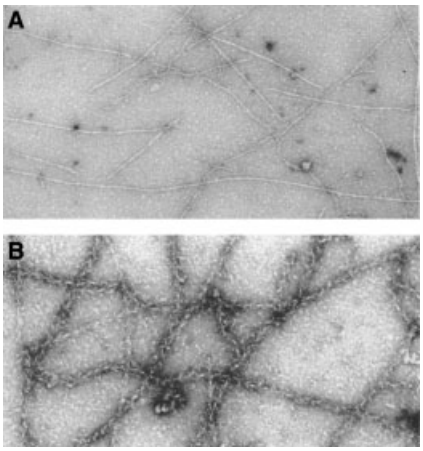

Fig. 6. Upper figure: polymer of a human platelet actin extract. Lower figure: actin reaction with skeletal muscle heavy meromyosin showing continuous arrowhead formation. Magnification ×92 000.

drawing needle into a physiological solution with EDTA as anticoagulant. A short, low-speed centrifugation was used to remove the red and the white cells. In thin section the normal circulating state of the platelet consists of a membrane under which microtubuli can be observed. Along these, fibrillar material could be formed by contractile proteins. The platelets also contain occasionally a certain amount of glycogen associated with a Golgi apparatus. The cytoplasm shows a dense felt-like matrix and dark granules. Some mitochondria are also present [11].

Once we had a visit from G. M. Hughes, from the Research Unit for Comparative Animal Respiration in Bristol. He suggested that in the secondary lamella of fish gills, certain cells might contain a retractile system to regulate the blood flow. We went to a hatchery to buy a trout and prepared the gills in the same way as our platelets. We then examined the pillar cells in ultrathin slices with the electron microscope and observed filaments in a contracted state, the first sign that contractile material could be present in non-muscle cells other than platelets [12].

At that time Swiss scientists had to organize the International Congress of Biochemistry, as an emergency, in Lucerne and Montreux, two towns with enough hotel rooms to accommodate everybody, because in Rome, where it was meant to take place, the Italian workers were threatening to go on strike. I was in charge of organizing the congress in Montreux. At the final dinner I met a scientist named O. Behnke from Copenhagen. He interrogated me about our results with thrombosthenin-A. He proposed to make a test to combine it with H-meromyosin from muscle tissue. So I travelled once to Copenhagen with a refrigerated thermos flask containing thrombosthenin-A in monomeric form. In his laboratory, equipped with an electron-microscope, I polymerized the protein: alone on a grid it formed thin filaments. When H-meromyosin was added arrowheads 'decorated' the thrombosthenin-A, showing that the two proteins could combine (Fig. 6) [13].

On another occasion Colonel-Médecin Bernard Maupin from the French army, wanted to collaborate with me to make a list of all the enzymes detectable in platelets. The list was very long [14], it seemed that circulating platelets have a large set of enzymes, and that they metabolize actively.

Many editors invited us to write reviews: we had the honor to publish a chapter in *Advances in Protein Chemistry* [8], in *Der Thrombozyt* by Rudolf Marx [15], in *Metabolism and Membrane Permeability* by E. Deutsch, E. Gerlach and K. Moser [16] and in *Thrombosis and Bleeding Disorders* by N. U. Bang, F. K. Keller, E. Deutsch and E. F. Mammen [17].

We were cited so often that *Current Contents* published a retrospective as 'This Week's Citation Classic' for our article in *Biochimica Biophysica Acta* that had been cited in 220 publications since 1961 [18].

Thrombosthenin = actomyosin; yes, but not just any actomyosin

Of course, since I worked on 'thrombosthenin' much progress has been made. At that stage we thought that 'thrombosthenin' was possibly identical to muscle actomyosin although we found some distinct differences. It is now known that the myosin component of platelet actomyosin contains non-muscle myosin heavy-chain type IIA. This has a critical role in many non-muscle cells and defects in its gene, *MHY9*, lead to the autosomal dominant, giant platelet disorders May–Hegglin anomaly, Fechtner, Sebastian, Epstein and Alport-like syndromes [19]. While nearly all patients have giant platelets, thrombocytopenia and leukocyte inclusions, some also suffer from high-tone deafness, cataracts and nephritis. Any linkage between the mutation site and the symptoms in a given individual remain obscure, implying that other factors also play a role. Abnormal folding of the defective protein leading to interference with the activity of the normal protein may be the

reason for this. Recently, mice with the non-muscle myosin heavy-chain type IIA gene 'knocked out' have been prepared and showed an embryonic development defect that was fatal by the day 7.5 [20]. Mice with the gene for non-muscle myosin heavy-chain type IIB ablated also show many defects but this change is somewhat less catastrophic. This knock out is fatal for about 65% of embryos, the rest suffering from congenital heart failure [21]. The effects of ablation of the type IIA gene on platelets and their function are awaited with interest and will require a tissue-specific knock out. The studies on mouse embryos suggest that non-muscle myosin heavy-chain type IIA has a unique role in early embryonal development in maintaining adhesion junctions and cellular organization.

Unexpected consequences

The story of my association with the Theodor Kocher Institute did not stop with thrombosthenin and, by a curious coincidence, changed one of the directions of research there. We had the unique opportunity to obtain so many buffy coats, containing platelets and leukocytes, from the Central Laboratory of the Swiss Red Cross in Berne, a fact that was noted by my sister Mathilde Krim PhD (Fig. 7), when she joined the Sloan-Kettering Institute for Cancer Research as a research scientist. She wanted to test the effect of interferon on Kaposi sarcoma cells. At that time (1970s) interferon was a rare commodity. So she came once to Berne, and contacted Dr A. Hässig, the then director of the Central Laboratory of the Red Cross, who was delighted to invite her to perform cancer cell experiments in his laboratory, but as the danger of contamination had to be considered, it was evidently impossible to carry out such experiments in the Central Laboratory, which prepared blood for transfusion and immunoglobulin G for infusion. She was depressed, having obtained a grant sufficient for this project. I had the idea that maybe Ernst Lüscher could find a solution. He could indeed, knowing that a large laboratory in the Theodor Kocher Institute, where he was now director, was empty. Interferon-α could be prepared there, starting with the buffy coats from the Central Laboratory of the Swiss Red Cross. This laboratory was equipped accordingly and under a succession of scientists (J. M. Hoskins, Victor Edy and Alfred Walz) went on to produce and supply interferon to the Sloan-Kettering Institute where its effects on tumor growth were tested. Unfortunately, interferon-α, as is now known, was not very effective against cancer and finally, after several years, the project was dropped. However, it was then thought that supernatants from the activated, cultured leukocytes, used to produce interferon, might contain other biologically active proteins and a search for such molecules was instigated. It was this work that led to the discovery of interleukin-8 in 1987 [22] at the Theodor Kocher Institute, now with Marco Baggiolini as director, the first of the major chemotactic chemokines and the start of the explosion of interest in chemokines and their receptors which still continues. I also worked there for a time on this subject [23]. Later studies in this area involving the Theodor Kocher Institute led to the recognition that chemokine receptors are involved in the transmission of human immunodeficiency virus (HIV) [24]. These discoveries have allowed the development of targeted pharmacological approaches to the prevention of HIV transmission [25]. During this time my sister's interests had also moved from treatment of cancer to prevention and treatment of acquired immune deficiency syndrome (AIDS), a major factor in Kaposi syndrome. Thus, her earlier efforts in organizing interferon production had finally not been unrewarded, by one of those curious pathways that basic research often takes. This is something not always clear to some non-scientists, especially certain politicians, who think that it should be possible to rationalize research to reach a desired goal more efficiently.

Fig. 7. My sister, Mathilde Krim, together with President Kennedy at a gala performance for charity in New York in 1963. She had started work at the Sloan-Kettering Institute for Cancer Research in 1962 and from 1981 to 1985 was director of its Interferon Laboratory. She founded the AIDS Medical Foundation in 1983 afterwards becoming chairman (1985) of the American Foundation for AIDS Research.

Final thoughts

My husband (Professor for Children's Surgery, who was awarded the medal of the European Society for Paediatric Urology) and myself, both worked hard, were successful in our respective fields, compensating long working days with wonderful holidays, sometimes combining congresses with trips, and were fanatics about scuba-diving, which allies sport with observation of nature.

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