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The importance of a radial spoke protein in flagellar motility

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Abstract

The aim of our investigation was to gain insight on the regulation of flagellar movement, at the axonemal level. In our laboratory a panel of monoclonal antibodies (MoAbs) has been produced against the axoneme of the biflagellated algae, *Chlamydomonas reinhardtii*, a well-characterized model for the study of flagellar movement. Of these MoAbs, L2H12 has been selected, because it has a potent inhibitory effect on the motility of demembranated-reactivated flagella of *Chlamydomonas* cells. Using video micrography, we demonstrated that low concentrations of L2H12 cause a progressive decrease in the wave amplitude and beat frequency of the flagella. Results of Western blotting of the axonemal proteins indicates that L2H12 recognizes a 105 kDa protein. Analysis of *Chlamydomonas* radial spoke mutants deficient in one or more radial spoke proteins (RSPs) suggests that this protein is RSP2. Immunoprecipitation of this protein was performed to further characterize it.

Résumé

Notre recherche a porté sur la régulation du mouvement flagellaire au niveau de la structure axonémale. Nous pouvions choisir pour ce faire parmi une série d'anticorps monoclonaux produits dans notre laboratoire à partir d'axonèmes purifiés des algues unicellulaires biflagellées *Chlamydomonas reinhardtii*, un modèle fréquemment utilisé pour ce genre d'étude. Nous avons sélectionné en particulier l'anticorps L2H12 pour cette recherche puisque celui-ci inhibe fortement la motilité des cellules de *Chlamydomonas* démembranées-réactivées. Utilisant des concentrations très faibles de l'anticorps L2H12, nous avons montré, par vidéo micrographie, que ce dernier causait une diminution progressive de l'amplitude et de la fréquence de battement des flagelles de cet organisme. Une protéine d'environ 105 kDa est détectée par immunobuvardage avec cet anticorps. Des analyses similaires faites sur des mutants de *Chlamydomonas* ayant des défiences protéiques au niveau des ponts radiaires suggèrent que cette protéine est la RSP2. Pour permettre une caractérisation plus poussée de celle-ci, nous l'avons immunoprécipitée.

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ATP	:adenosine-5'-triphosphate
ATPγS	:adenosine-5'-O-(3-thiotriphosphate)
BCIP	:5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt
CAM	:calmodulin
CAM-PrPase	:calmodulin-dependent protein phosphatase
cAMP	:cyclic adenosine 3', 5'-monophosphate
cAMP-PK	:cyclic adenosine 3', 5'-monophosphate-dependent protein kinase
cGMP-PK	:cyclic guanosine 3', 5'-monophosphate-dependent protein kinase
Da	:Daltons
DRC	:dynein regulatory complex
DTT	:dithiotreitol
ECL	:enhanced chemiluminescence
EDTA	:ethylenediaminetetraacetic
EGTA	:ethylene glycol-bis β-aminoethyl ether N, N, N', N'-tetraacetic acid
FSH	:follicle stimulating hormone
HBS	:Hepes buffered saline
hr	:hour(s)
Hz	:Hertz
IgG	:immunoglobulin type G subclass
IgG_1	:immunoglobulin type G subclass 1
kDa	:kiloDaltons
l	:litre
LH	:luteinizing hormone
M	:molar
mg	:millligram

ml	:milliliter
mm	:millimeter
mM	:millimolar
min	:minute(s)
MoAb	:monoclonal antibody
NBT	:p-nitro blue tetrazolium chloride
ng	:nanogram
NP40	:nonidet P40
PEG-20 000	:polyethylene glycol-20 000
PMSF	:phenyl methyl sulfonyl fluoride
PPP	:protein phosphatase
RSP	:radial spoke protein
s	:second(s)
SFPF	:serum-free protein-free
TBS	:Tris buffered saline
TTBS	:Tris buffered saline with 0.1 % tween
μg	:microgram
μl	:microliter
μm	:micrometer
μM	:micromolar
°C	:degrees celcius
%	:percent
x g	:times gravity

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1 Introduction

1.1 The decline of male fertilizing potential

1.1.1 Epidemiological evidence

Infertility is a problem that in recent years has evoked a great deal of attention from the public and the scientific community. It has been estimated that approximately 15 % of all couples suffer from infertility (Thonneau et al, 1991, World Health Organization, 1997). In 20 – 25 % of infertility cases, the male alone is responsible for the problem (Gagnon, 1996; World Health Organization, 1997) and in at least 30 % of all cases, the male contributes to the problems of infertility (World Health Organization, 1997). Epidemiological studies have shown that over the past 60 years there appears to be a trend towards a decrease in semen quality. A study performed by Carlsen et al demonstrates that since 1938, the concentration of spermatozoa in semen has dropped from an average of 113 million spermatozoa/ ml of semen in 1938 to 66 million in 1990 (Carlsen et al, 1992). Although this study has been criticized for having numerous confounding factors, other groups have also reported a global trend of increasing defective spermatozoa in semen. Van Waeleghem and his colleagues showed a significant decrease in spermatozoa concentration within semen, in percent motile spermatozoa, and in percent normal morphology, between 1977 and 1995 (Van Waeleghem et al, 1996). Other investigators have reported similar findings (Irvine et al, 1996; Auger et al, 1995; Auger and Jouannet, 1997; Zorn et al 1999).

1.1.2 The importance of studying sperm motility

Sperm motility is an important indicator of the fertilizing potential of males. Several reproductive disorders, related to defective sperm flagellar motility, have been characterized. These include Kartagener syndrome (Kartagener and Zui, 1933) sliding spermatozoa syndrome (Feneux et al, 1985), and epididymal asthenozoospermia (Wilton et al, 1988). Patients suffering from Kartagener syndrome are infertile because spermatozoa lack dynein arms, which are the flagellar motors, involved in motility. The spermatozoa of patients with sliding spermatozoa syndrome are unable to penetrate the membranes surrounding the ovum, because they display flagellar beating with a reduced amplitude (Serres et al, 1986). Men with epididymal asthenozoospermia display a reduction in sperm motility of approximately 80%, which is often accompanied by a high percentage of dead spermatozoa and a high percentage of abnormally shaped sperm. Since testicular biopsies of these men show that the integrity of the spermatozoa are well preserved in the testes, it has been suggested that degeneration occurs in the epididymis (de Kretser and Baker, 1999).

1.2 Spermatogenesis, maturation of spermatozoa and fertilization

1.2.1 Spermatogenesis

Spermatogenesis occurs in the seminiferous tubules of the testes, where spermatozoa are produced under the influence of luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone, estrogens, and growth hormone (Guyton, 1991). Although spermatozoa are produced in the testis, testicular spermatozoa are immobile. Since the focus of this thesis is the motility of spermatozoa, a detailed description of spermatogenesis will not be given.

1.2.2 General morphology of the mammalian spermatozoon

The spermatozoon is composed of the head and the flagellum, which are connected by the neck (fig.1). The head consists of the acrosome, which is responsible for the enzymatic reaction that takes place when the spermatozoon fuses with the oocyte and the nucleus, which consists of 23 single-stranded chromosomes representing the haploid genome. Other components of the head include cytoskeletal structures and a scarce amount of cytoplasm (Eddy and O'Brien, 1994). Morphologically, the flagellum possesses three sections: the middle piece, the principal piece, and the end piece (fig.1). The middle piece contains outer dense fibers wrapped by mitochondria and the principal piece contains a fibrous sheath. In humans, outer dense fibers extend from the head to a variable end point on the principal piece. The axoneme, the internal structure responsible for flagellar movement originates at the base of the neck of the spermatozoon and extends to the endpiece (Eddy and O'Brien, 1994).

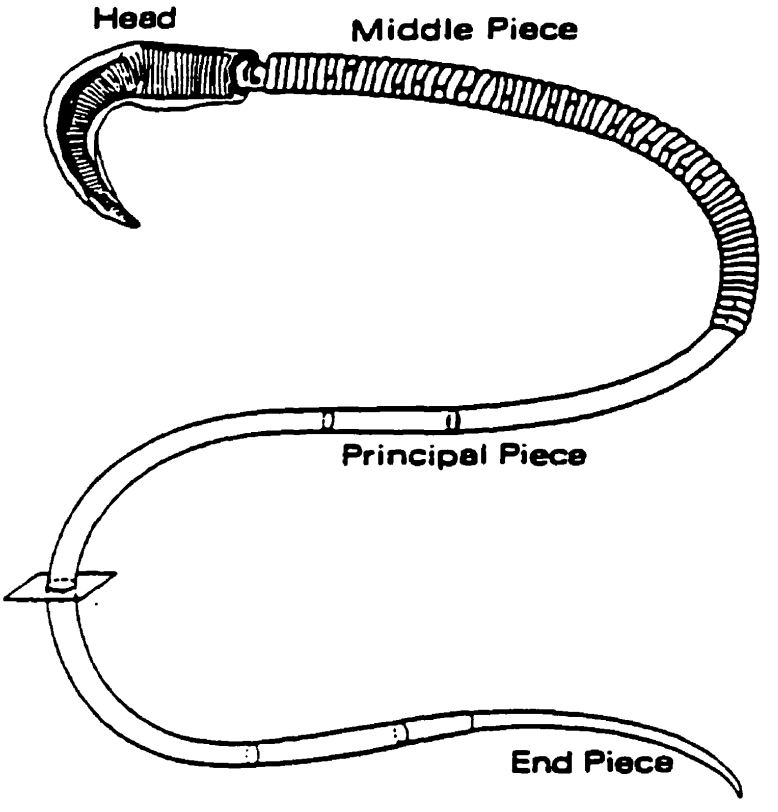


Figure 1: **General features of the mammalian spermatozoon.** The head is attached to a connecting piece of the flagellum. The flagellum is divided into three regions including the middle piece, principal piece, and end piece. Modified from Eddy and O'Brien (1994).

1.2.3 Maturation of spermatozoa in the epididymis

In the epididymis, the spermatozoon undergoes maturation, during which its plasma membrane is modified. Some of the changes that occur include absorption of cholesterol, which stabilizes the membrane (Yanagimachi, 1994), the binding of polypeptides originating from the epididymal epithelium to the sperm surface (Yanagimachi, 1981), changes in lectin binding sites (Yanagimachi, 1994), and glycosylation of membrane proteins (Reynolds et al, 1989). The modifications may be either general or specific to various components of the spermatozoa, such as the acrosomal and postacrosomal region of the head, or the flagellum. These cell surface changes are of particular importance to fertility, because they mediate the ability of spermatozoa to exhibit forward motility, capacitate, bind to the zona pellucida surrounding the oocyte, acrosome react, and fuse with the ovum (Yanagimachi, 1981).

1.2.4 Ejaculation

During ejaculation, spermatozoa from the cauda epididymis are mixed with seminal plasma and are deposited on the female cervix. Approximately 200 – 300 million spermatozoa are initially present in the human ejaculate (Mortimer, 1983). The ejaculate coagulates immediately after it has been released. This is believed to be important for sperm survival and motility. The vagina's acidic environment is detrimental to spermatozoa (Miller and Kurzrok,

1932; Kremer, 1968; Wallace-Haagens et al, 1975). However, coagulated sperm are kept within the seminal plasma. The seminal plasma buffers the vagina's acidic environment, allowing sperm survival. Penetration of the cervix by successful spermatozoa is completed within 15 to 20 minutes of ejaculation (Tredway et al, 1975, 1978). This coincides with liquefaction of the coagulate which takes place within 5 to 20 minutes following ejaculation (Mortimer, 1983).

1.2.5 Migration through the female genital tract

The cervical mucus excludes certain spermatozoa including morphologically and possibly functionally abnormal spermatozoa (Katz et al, 1989). The remaining spermatozoa are retained within the cervical mucus, which keeps spermatozoa viable and stimulates motility (White and Kar, 1973). In the cervix, spermatozoa reach the surface of the female secretory epithelium by traveling along the longitudinal microstructures of mucus glycoproteins. Many spermatozoa remain bound to the epithelium, but others migrate along the longitudinal folds and shallow grooves of the epithelium to reach the uterus. The release of spermatozoa from the cervix may continue for several days (Yanagimachi, 1994).

Once spermatozoa reach the uterine cavity, they become suspended in uterine fluid (White and Kar, 1973). Contractions of the myometrium mix the uterine fluid, distributing spermatozoa uniformly within the uterine lumen (Mortimer, 1983). In some mammalian species, spermatozoa that reach the

junction between the uterine cavity and the Fallopian tubes, or oviduct in non humans, are sequestered in the isthmus of the Fallopian tubes prior to ovulation (Yanagimachi, 1994). Viable spermatozoa remain attached to the epithelial crypts of the isthmus, until they acquire the ability to fertilize (Smith and Yanagimachi, 1991; Demott and Suarez, 1992).

1.2.6 Capacitation and fertilization

Freshly ejaculated spermatozoa are unable to fertilize the oocyte. However, as spermatozoa reside in the female genital tract, they undergo capacitation, which refers to the physiological changes that render the spermatozoa capable of fertilizing the ovum (Yanagimachi, 1994). This is a maturation step (de Lamirande et al, 1997) that is accompanied by a change in the pattern of motility called hyperactivation (Kopf et al, 1999). Capacitation can be induced *in–vitro* by reactive oxygen species and is inhibited when scavengers of reactive oxygen species are introduced in capacitating media (de Lamirande and Gagnon, 1993 a, b; Griveau et al, 1994; Leclerc et al, 1997; de Lamirande et al, 1997). Capacitation is therefore believed to be the result of an oxidative process (Leclerc et al, 1997; de Lamirande et al, 1997).

Capacitated spermatozoa that reach the oocyte, in the Fallopian tube, can initiate fertilization. Although millions of spermatozoa are initially ejaculated, there are very few present near the egg at fertilization (Yanagimachi, 1994). Two extracellular matrices, the zona pellucida and the cumulus oophorus surround the oocyte (Yudin et al, 1988). When the

capacitated spermatozoon comes into contact with the cumulus oophorus, it digests the cumulus matrix to gain access to the zona pellucida (Yanagimachi, 1994). If it is properly capacitated, the spermatozoon acrosome reacts, releasing enzymes that allow it to penetrate through the zona pellucida and fuse with the plasma membrane of the oocyte. Once the spermatozoon passes through the zona pellucida, it can fuse with the cell membrane of the oocyte and fertilization occurs (Yanagimachi, 1994).

1.3 Varying motility patterns and rates displayed by spermatozoa

Motility of the spermatozoon is achieved by flagellar movement, which involves the active production of a sequence of principal and reverse bends (Gibbons and Gibbons, 1974; Woolley, 1977). During migration in the female genital tract, varying degrees of curvature of the bends and different wave propagation rates occur, resulting in different patterns of movement.

1.3.1 Initial patterns of motility

When ejaculated spermatozoa are deposited in the vagina, their viscous environment affects their pattern of motility. The three-dimensional flagellar beat becomes planar (Katz et al, 1989). At the semen-mucus interface, the spermatozoa that are capable, penetrate the mucus by increasing their velocity of motion and amplitude of lateral head displacement (Aitken et al, 1985; Feneux et al, 1985; Mortimer et al, 1986). The spermatozoon now encounters increasingly viscous cervical mucus. It

responds by diminishing the amplitude of curvature of the bends in the proximal flagella, while increasing the amplitude in the distal flagellum (Rikmenspoel, 1984; Ishijima et al, 1986). The result of these changes is that mammalian spermatozoa swim in a pattern referred to as a *figure-eight*, since the distal tip of the flagella traces such a pattern (Katz et al, 1989).

1.3.2 Hyperactivated motility

Hyperactivated motility is a pattern of motion, frequently associated with capacitation, that is essential for successful fertilization (de Lamirande et al, 1997). It is an asymmetrical, irregular pattern of motion involving bends of increased curvature in the midpiece and proximal principal piece (Katz et al, 1989) and is associated with increased velocity (de Lamirande et al, 1997). The two distinct types of hyperactivated motions are a less progressive phase, commonly described as *whiplash* and a more progressive movement, referred to as *darting motion*. During *whiplash*, high curvature flagellar bending bring the proximal midpiece close to the head of the spermatozoon. The bends are not propagated and the head moves in a rapid, hatchet-like manner. The *darting motion* involves extreme reverse bends that bring the head of the spermatozoon close to the flagellum. These bends are propagated along the flagellum and are often followed by bends of lesser curvature. When the extreme reverse bends are followed by bends of lesser curvature, the head of the spermatozoon follows a helical trajectory. Otherwise, it follows a *figure-eight* pattern of motion (Katz et al, 1989). It should be noted that the

description of a *figure-eight* trajectory followed by the head during hyperactivation refers to a pattern of movement that differs from the *figure-eight* movement displayed in the cervical mucus (described in 1.3.1). In the cervical mucus, the distal tip of the flagellum follows the *figure-eight* pattern. A comparison between a low curvature motility and hyperactivated motion, as observed in hamster sperm, is depicted in figure 2.

1.3.3 Pattern of motility when spermatozoa interact with the cumulus oophorus

When the spermatozoon encounters the cumulus oophorus, its pattern of motility is less rapid, but more linear and progressive. The flagellar beat frequency increases and the amplitude of lateral head displacement decreases (Tesarik et al, 1990). This unique pattern of motility was only observed in hyperactivated human spermatozoa that were incubated with human cumulus material when compared with fresh spermatozoa or those incubated in the absence of cumulus material. Therefore, it was suggested that the cumulus exerts a motility-altering effect, specifically on hyperactivated spermatozoa (Tesarik et al, 1990), due to molecular interactions. The specific interaction between hyperactivated spermatozoa and the cumulus oophorus *in-vitro* accurately reflects what occurs *in-vivo*, since uncapacitated spermatozoa that are not hyperactivated do not penetrate the cumulus oophorus (Yanagimachi, 1994). However, Katz et al believe that the altered movement of spermatozoa can be explained in terms of the physical

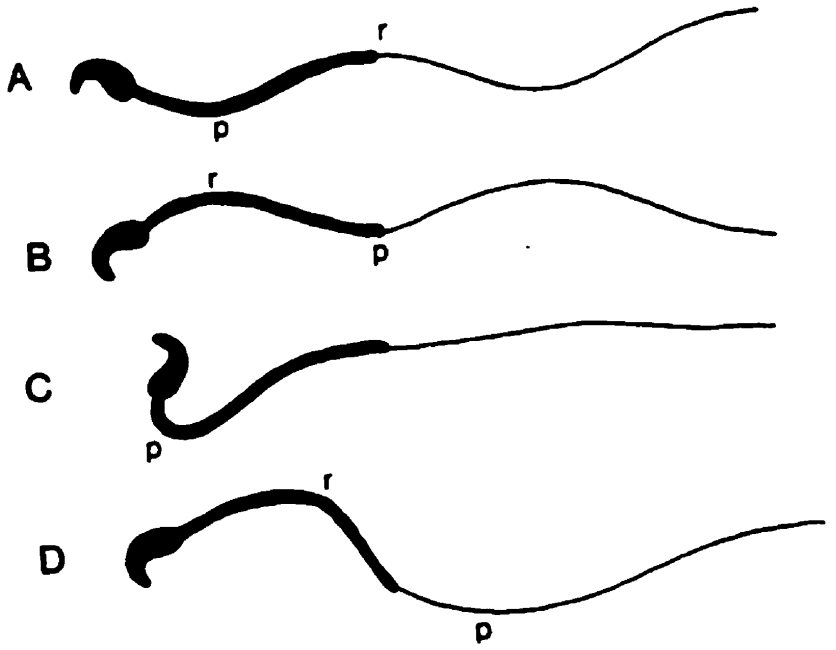


Figure 2: **Illustration of low curvature and high curvature bending for a hamster spermatozoon.** A and B represent low curvature bending, which produces a sinusoidal-like motion. C depicts a high curvature bending of the proximal midpiece, producing a "hatchet-like" motion. D shows an extreme reverse bend , which can produce a "figure-eight" shaped trajectory. Principal bends are labeled as **p**. Reverse bends are labeled as **r**. (Katz et al., 1989)

resistance that the highly viscous cumulus matrix imposes on the spermatozoa (Katz et al, 1986). Consistent with this hypothesis is that once spermatozoa penetrate the cumulus, they resume hyperactivated motility as they encounter the porous zona pellucida (Katz et al, 1986).

1.4 The axoneme

The axoneme is the basic structure found within the flagellum and is mainly responsible for movement (Gagnon, 1995). It is composed of 9 pairs of microtubule doublets that surround a central pair of singlet microtubules. The main components that associate with the microtubules are the outer and inner dynein arms, the radial spokes, the nexin links, and tektins (fig. 3).

1.4.1 Structural composition of the microtubules, nexin links, and tektins

The microtubules are permanently linked together possibly by anchorage to the basal body (Piperno et al, 1990). The microtubules are composed of alpha and beta tubulins. They are modified by acetylation (Piperno and Fuller, 1985), polyglycylation (Redeker et al, 1994), polyglutamylation (Eddé et al, 1990), palmitoylation (Ozols and Caron, 1997), the removal of the C-terminal dipeptide (Paturle-Lafanechère et al, 1991), and detyrosination at the C-terminus (Barra et al, 1988; Raybin and Flavin, 1977). The outer microtubule doublets consist of subfiber A, which is composed of 13 tubulin protofilaments and subfiber B, which is made up of 11 tubulin

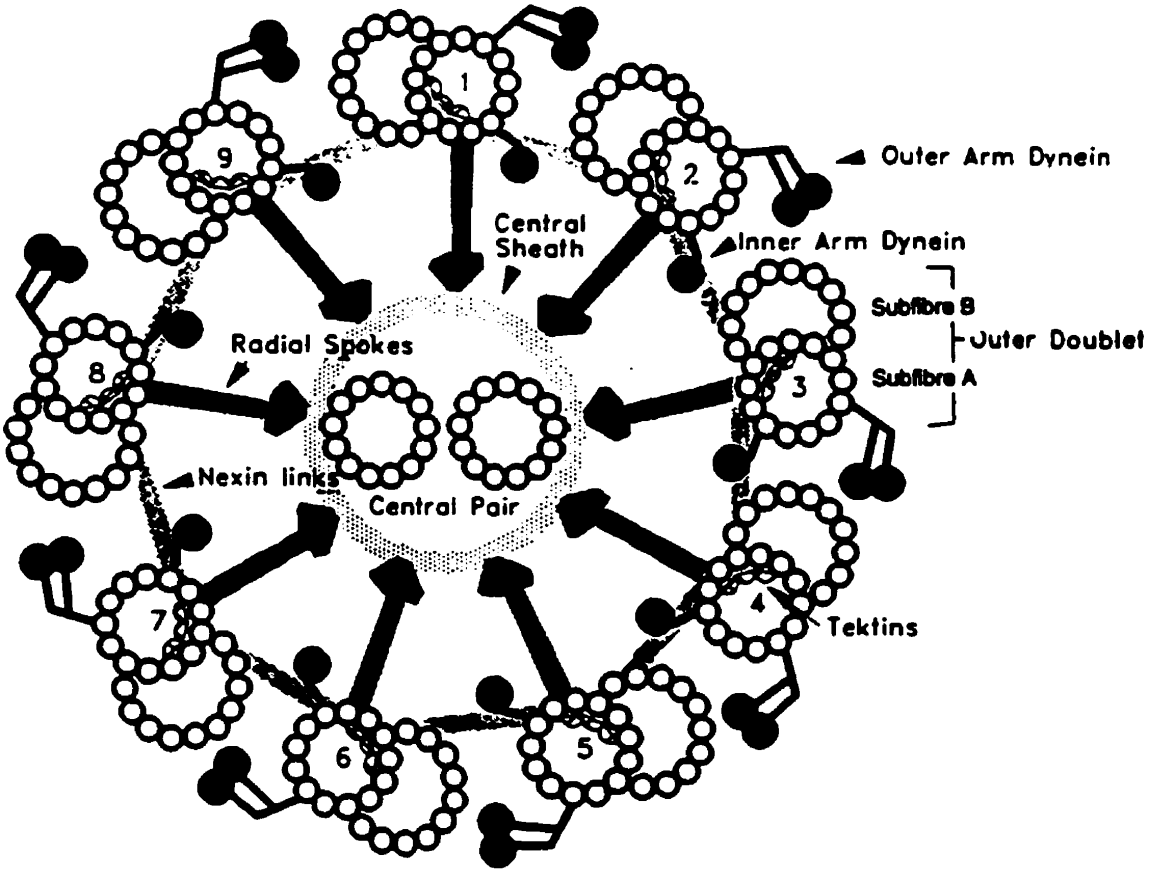


Figure 3: **A cross-section of the axoneme.** The axoneme is made of 9 pairs of outer microtubule doublets that surround a central pair. Dynein arms (outer and inner) and radial spokes attach to microtubule A. Nexin links connect adjacent microtubules. Tektins are found near the junction of microtubule subfibers A and B. Modified from Tash (1990).

protofilaments. The central pair includes 2 microtubules each formed by 13 protofilaments. A central sheath surrounds the pair. Nexin links connect adjacent microtubules and are found every 96nm throughout the structure. Tektins are poorly soluble proteins found near the junction of microtubule subfibers A and B. They are not believed to be important for motility (Gagnon, 1995).

1.4.2 The dynein ATPases

The dynein ATPases are the micromotors of the axoneme. The outer dynein arms can be differentiated from the inner dynein arms by their molecular composition, position within the axoneme, and function. They are composed of 2-3 heavy chains, 2-3 intermediate chains, and 4-10 smaller components. The outer arms assume specific positions on microtubule subfiber A and are spaced every 24nm. This means that 4 outer dynein arms are found between each nexin link, i.e. every 96 nm unit (Gagnon, 1995). Inner dynein arms are diverse. They are composed of one of three distinct subunits termed I1, I2, and I3, each containing 2 heavy chains (Piperno et al, 1990). I1 contains heavy chains 1α, 1β and associates with a bilobed structure to form a triad (Witman, 1992). Three intermediate chains (IC), and three light chains are associated with I1 (Porter, 1996). Only one I1 isoform has been identified. In contrast, several isoforms of I2 and I3 have been identified and have been resolved into at least six distinct heavy chain subspecies (Kagami

and Kamiya, 1992). Inner dynein arms are found every 96 nm on the axoneme. The I1 triad alternates with the I2 and I3 dyads, so that the pattern of dynein inner arms is I1-I2-I3-I1-I2-I3. Depending on their location on the axoneme, the isoform of I2 and I3 will differ. The molecular composition of I1 is constant throughout the axoneme. (Gagnon, 1995).

1.4.3 Radial Spokes

The radial spokes are believed to play a role in the control of movement. They are composed of 17 polypeptides, 12 of which form a stalk that is attached to microtubule subfiber A. The remaining 5 proteins form a globular head that is found at the end of the stalk and projects towards the central pair of microtubules. Pairs of radial spokes separated from each other by 32 nm are found every 96 nm. Several radial spoke proteins (RSPs) are phosphorylated, including RSP2, 3, 5, 13, and 17. These proteins are located on the radial spoke stalks (Huang et al, 1981; Piperno et al, 1981). Phosphorylation may be important for assembly of stalk proteins and/ or for control of motility (Gagnon, 1995). An example of a RSP that is essential for the assembly of the radial spokes is RSP3. It is involved in the attachment of the radial spoke to microtubule subfiber A (Diener et al, 1993).

1.5 Biochemical changes that occur during flagellar movement

The biochemical events that occur during the initiation of movement and as patterns of motility change are poorly understood. However, several

molecules have been implicated as important regulators. Examples of major molecules that influence motility are calcium, bicarbonate, and cyclic adenosine 3', 5'–monophosphate (cAMP).

1.5.1 Calcium

Fluctuations of internal calcium appear within a single flagellar beat cycle and depending on the type of motility displayed by spermatozoa (Suarez et al, 1993). An increase in intracellular calcium has been shown to occur in hyperactivated hamster spermatozoa (Suarez and Dai, 1995). This increase also occurs in acrosome-reacted spermatozoa. However, the levels of intracellular calcium in hyperactivated and acrosome-reacted spermatozoa differ. Furthermore, hyperactivated spermatozoa have elevated levels of calcium in the flagellar midpiece compared to the acrosomal region. In contrast, a higher concentration of calcium was detected in the acrosomal region of acrosome- reacted spermatozoa compared to the flagellum (Suarez and Dai, 1995). *In vitro* demembranation-reactivation assays demonstrated that changes in external calcium influence the pattern of motility displayed by the spermatozoa (Brokaw 1979; Gibbons and Gibbons 1980; Gibbons 1986). Therefore, calcium appears to be responsible for the alteration of waveform and changes in patterns of motion.

1.5.2 Bicarbonate

In-vitro studies with epididymal hamster spermatozoa have demonstrated that bicarbonate stimulates motility (Boatman and Robbins, 1991). In humans, it has been difficult to determine how motility is affected by bicarbonate. A population of infertile men exhibited poor sperm motility associated with lowered levels of bicarbonate in the seminal plasma (Okamura et al, 1986). However, *in-vitro* studies have shown that hyperactivation of ejaculated spermatozoa occurs in the presence or absence of extracellular bicarbonate ions (de Lamirande and Gagnon, 1993 a,b). This may be because these spermatozoa are exposed to a high concentration of bicarbonate ions in the semen during ejaculation (de Lamirande et al, 1997).

1.5.3 cAMP

cAMP has been implicated as the regulator of the initiation of flagellar movement during sperm maturation in the epididymis and in stimulation of motility during the fertilization process (Tash and Means, 1983). The importance of the rise of cAMP for the acquisition of motility was emphasized by an *in-vitro* assay where motility was induced in previously immobile epididymal spermatozoa by artificially raising intracellular cAMP (Vijayaraghavan et al, 1996). Furthermore, in reactivation assays with dog sperm, H-8, a membrane-permeable inhibitor of cAMP and cyclic guanosine 3', 5'-monophosphate-dependent protein kinase (cGMP-PK), inhibited motility (Tash et al, 1986).

1.5.4 Proposed effect of calcium, bicarbonate and cAMP on downstream proteins

Changes in intracellular calcium, bicarbonate and cAMP initiate a cascade of events involving phosphorylation of proteins. Bicarbonate has been demonstrated to increase the intracellular levels of cAMP by stimulating adenylyl cyclase (Kopf et al, 1999). The rise in cAMP during the acquisition of motility in the epididymis was correlated with an increase in activity of cAMP-dependent protein kinase (cAMP-PK) (Hoskins et al, 1974). Furthermore, inhibition of cAMP-PK in reactivated mammalian and sea urchin sperm arrested motility (Ishiguro et al, 1982; Tash et al, 1984). According to Tash this demonstrates that cAMP stimulates the activity of cAMP-PK, which in turn phosphorylates downstream flagellar proteins, leading to the initiation of motility (Tash, 1989). The calcium signal is transduced by calmodulin (CAM). Treatment of live spermatozoa with anti-CAM drugs resulted in modulation of waveform (Tash and Means, 1982; Otter et al, 1984). CAM binding proteins, including CAM-dependent protein phosphatase (CAM-PrPase), have been localized to the flagellum (Klummpp et al, 1983). Tash proposes that calcium modulates movement by stimulating CAM. CAM then activates CAM-PrPase, which selectively dephosphorylates several of the proteins phosphorylated by cAMP-PK. In this way, cAMP is responsible for the generation of motion, while calcium maintains normal waveform (Tash, 1990).

1.6 The mechanics of axonemal motility

1.6.1. The role of the dynein arms in movement

The axoneme is rigid because of the structural arrangement of the microtubules. Therefore, a force must be generated to counteract the inherent inertia of the axoneme to achieve movement. The dynein arms are the micromotors that are responsible for this force production. They are anchored to microtubule A of the axoneme in an adenosine-5'-triphosphate (ATP)-independent manner and form cross-bridges with adjacent B microtubules when associated with ATP. Hydrolysis of ATP causes the dissociation of dynein from the B microtubules, generating a force. This force translates into a sliding movement directed towards the cell body (Satir, 1993). Evidence suggests that the outer dynein arms determine the maximum velocity of motion (Witman, 1992). It has been postulated that due to their more complex molecular composition, the inner arms play a more versatile role. They provide the additional force required for sustaining a high velocity in the presence of a load (Witman, 1992). In addition, they may be involved in the regulation of movement by interacting with the radial spokes (Gagnon, 1995).

1.6.2 The regulation of flagellar bending

In order for flagellar wave formation to occur, microtubule sliding must be converted into bending. Several hypotheses have been proposed to explain how curvature of the axoneme is achieved.

1.6.2.1 Cosson's hypothesis: The formation of transient links

Cosson suggests that the wave propagation in flagella occur due to the formation of transient covalent links (Cosson, 1992). He proposes that rupturing of these links by protease molecules and re-establishment of the connections between microtubules by protein ligases cause bending. With his model, one can envision that bend propagation occurs because a disruption of the links begins locally at the rostral end of the microtubules and as the connection is re-established, the links directly caudal are disrupted. As these links are re-established, links caudal to them are disrupted. This cycle occurs while the dynein arms generate the power stroke. In this synchronous manner, the wave is propagated from the head of the axoneme to the tip. The transient covalent links may involve nexin links, interdoublet links, and/ or radial spokes (Cosson, 1992). In support for Cosson's model, it has been shown that protease inhibitors inhibit wave propagation (Cosson and Gagnon, 1988). In addition, high molecular mass proteasomes and a protein-ligase, called transglutaminase, have been found to be present within the axoneme (Inaba et al, 1992; de Lamirande et al, 1987). Furthermore, a transglutaminase inhibitor interrupted motility (de Lamirande et al, 1987).

1.6.2.2 The "geometric clutch" hypothesis

Satir suggested that bending occurs because relative sliding between groups of microtubules located on opposite sides of the axoneme is controlled by a regulator, which alternatively activates the groups (Satir, 1984). More recently, Lindemann expanded on Satir's hypothesis with his "geometric clutch" hypothesis (Lindemann, 1994). According to Lindemann, bending

occurs when dynein arms on microtubules 2-3 and 3-4 synchronously form interactions in one bend direction, while the dynein arms on microtubules 7-8 and 8-9 cause displacement of adjacent microtubules in an opposite bend direction. At the same time, there is restricted movement between the central pair and doublets 3 and 8, which are in the same plane as the central pair. Lindemann's hypothesis is consistent with previous evidence suggesting that ATP turnover is highest at the junctions between microtubules 2-3, 3-4, 7-8, and 8-9 (Warner 1979). Furthermore, it has been demonstrated that the central pair are physically linked to microtubule doublets 3 and 8 (Tamm and Tamm, 1984; Sale 1986; Lindemann et al, 1992).

1.7 The role of the radial spokes in the control of movement

Evidence suggests that the radial spokes play an essential role in the regulation of flagellar motility. Several mutants of the biflagellate *Chlamydomonas reinhardtii* that are deficient in specific radial spoke proteins are paralyzed or display altered patterns of motion (Huang et al, 1981). Smith and Sale induced dynein driven sliding in these mutants with protease treatment and exposure to ATP. They observed that they could partially restore dynein-arm function in these mutants. However, the sliding velocities were measured at one half to one third the velocity of wild-type *Chlamydomonas* measured under identical conditions. The altered sliding velocities of the mutants were attributed to the absence of an interaction between dynein arms and radial spokes that occurs in wild-type

Chlamydomonas (Smith and Sale, 1992). It was further demonstrated that motility of a mutant lacking all 17 RSPs could be restored when the mutant was reconstituted with inner dynein arms from wild-type *Chlamydomonas*. This suggested that radial spokes activate dynein inner arm activity. Once activated, the dynein inner arms can generate movement in the absence of radial spokes (Smith and Sale, 1992).

1.7.1 Interaction of the radial spokes with the central pair microtubules

The involvement of the radial spokes in the control of motility appears to be associated with the interaction of the central pair microtubules. Observations of the ultrastructure of the axoneme with electron microscopy have revealed that the radial spokes connect transiently with structures that project from the central pair microtubules (Warner and Satir, 1974; Goodenough and Heuser, 1985). When the flagellum bends, radial spoke heads detach from a given set of projections and reattach to a more distal set (Warner and Satir, 1974). Because of their close association, the radial spokes and central pair microtubules are frequently referred to as the radial spoke-central pair system.

1.7.2 The dynein regulatory complex

The radial spoke-central pair system may control motility by transiently inactivating a control system that, in the absence of a signal from the radial spoke-central pair system, inhibits dynein arm activity. This hypothesis was

first suggested by Huang et al (1982), who isolated suppressor mutants using 3 radial spoke mutants and a central pair mutant. Radial spoke mutants pf1, pf24, pf25, and central pair mutant pf6, each exhibiting specific molecular and structural defects, were irradiated with ultraviolet light. This resulted in the isolation of four revertant strains that, when crossed with wild-type *Chlamydomonas*, exhibited restored flagellar function. The suppressor mutants rescued flagellar function in paralyzed radial spoke and central pair mutants without altering their molecular or structural composition. In contrast, motility was not restored in assays where dynein arm mutants were exposed to the suppressors. Tetrad analysis revealed that the new mutant strains, termed "suppressors", contained the original pf mutation and an unlinked suppressor mutation. The suppressors each lacked different components of 6 polypeptides. The absence of these proteins restored motility in the radial spoke and central pair mutants. Therefore, the radial spoke-central pair system was implicated as a regulator of flagellar function. It was hypothesized that this radial spoke-central pair system regulated movement by inactivating "a spoke-specific suppressor system" that in the absence of the radial spokes and central pair inhibits the dynein arms (Huang et al, 1982).

This suppressor system has more recently been referred to as the dynein regulatory complex (DRC). The DRC, which has a total molecular mass of 500 kDa, is composed of 6 peptides with molecular masses between 29 and 192 kDa (Piperno et al, 1992). Characterization of the DRC using DRC mutants suggested that the DRC forms an ATP-independent binding site for

inner dynein arms I2 and I3 on the A microtubule (Piperno et al, 1994). Gardener et al predict that the DRC is positioned between the inner dynein arms and the radial spokes. They suggest that the DRC mediates mechanical and/ or enzymatic signaling between the two structures (Gardener et al, 1994).

1.7.3 Biochemical evidence supporting the importance of the radial spoke-central pair system

The importance of the radial spoke-central pair system in the regulation of motility has been supported by biochemical evidence (Howard et al, 1994; Habermacher and Sale, 1996, 1997). Howard et al (1994) used cAMP-PK inhibitors to restore the sliding velocities of radial spoke and central pair mutants to wild-type levels. Since the velocities of wild-type microtubules with intact radial spokes and central pairs were unaffected by cAMP-PK inhibitors, it was predicted that in wild-type *Chlamydomonas*, the radial spoke-central pair system inhibits endogenous cAMP-PK (Howard et al, 1994). Pretreatment of the central pair and radial spoke mutants with adenosine-5′-O-(3-thiotriphosphate) (ATPγS) blocked the effect of cAMP-PK inhibitors on sliding velocity. This occurred because ATPγS could bypass the effect of cAMP-PK by directly phosphorylating an axonemal protein. Similarly, pretreatment of the mutants with protein phosphatase (PPP) inhibitors also resulted in no change in sliding velocity upon addition of cAMP-PK. This suggests that

dephosphorylation of an axonemal protein is critical for dynein arm activity (Habermacher and Sale, 1996).

Experiments with double mutants have demonstrated that the regulatory protein subunit is associated with inner arm dynein I1 (Habermacher and Sale, 1997). A 138-kDa intermediate chain is the only phosphoprotein in I1 (Habermacher and Sale, 1997) and the phosphorylation state of this protein affects microtubule sliding (Habermacher and Sale, 1997). Therefore, it was concluded that in wild-type *Chlamydomonas*, the phosphorylation state of 138-kDa intermediate chain on I1 affects motility. The radial spoke-central pair system may directly interact with this protein, thereby controlling movement. A model summarizing the conclusions drawn by Howard et al and Habermacher and Sale are presented in figure 4.

1.8 Rationale and outline for the present study

Normal motility is essential for the fertilizing potential of spermatozoa. The ultimate goal of our investigation is to improve our knowledge of male infertility related to abnormal sperm movement. This will be achieved by identifying individual flagellar components involved in the regulation of flagellar motion. Many of the axonemal structures involved in flagellar movement have

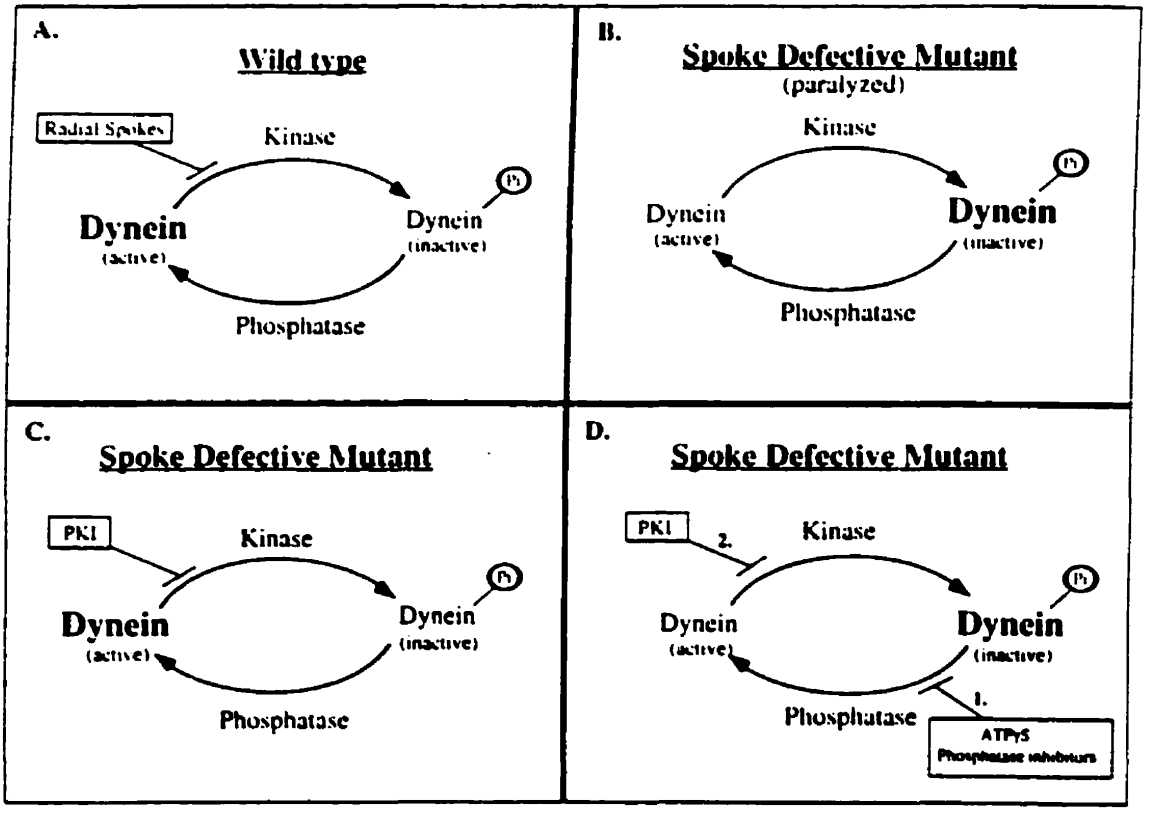


Figure 4: **Predictions for the regulation of dynein activity.** (A) In wild-type *Chlamydomonas*, the radial spokes inhibit cAMP-PK. This leads to the dephosphorylation of the 138-kDa intermediate chain, which activates dynein. (B) In radial spoke and central pair defective mutants, cAMP-PK is not inhibited. The 138-kDa protein remains phosphorylated and motility is impaired. (C) In radial spoke and central pair mutants, cAMP-PK inhibitors can activate dynein to wild-type levels. (D) Pretreatment with PPP inhibitors and ATPγS block the effect of cAMP-PK inhibitors and axonemes remain inactive (Habermacher and Sale, 1995).

been identified. Several groups have hypothesized how these structures interact mechanically and biochemically (Huang et al, 1982; Satir, 1984; Cosson, 1992; Smith and Sale, 1992; Gardener et al, 1994; Howard et al, 1994; Lindemann, 1994; Piperno et al, 1994; Habermacher and Sale 1996, 1997). However, at present, no single mechanism for the control of movement has been proven.

In both cilia and flagella, the 9+2 axoneme forms the basic structure. The basic components of the axoneme have been preserved over evolution among several species of flagella and cilia, as can be viewed by electron microscopy (Gagnon, 1995). Therefore the proteins involved in the regulation of movement in one species may be analogous to those involved in another.

To study the regulation of flagellar movement, we used *Chlamydomonas reinhardtii* as a model. *Chlamydomonas reinhardtii* are unicellular, biflagellate, eukaryotic green algae. The cells have an average diameter of 10 μm. They achieve motility with two 10 to 12 μm flagella located at the anterior of the cell. (Harper, 1999). *Chlamydomonas* normally exhibit ciliary motion. However, when the cells are stimulated by intense light, they reverse their swimming direction by changing their movement to a symmetrical flagellar-type wave, resembling the pattern normally displayed by mammalian spermatozoa (Wakabayashi, 1997). This change is caused by an intracellular increase in calcium. When calcium concentrations greater than 10^{-5} M are added to *in-vitro* assays, *Chlamydomonas* display a flagellar pattern of movement, instead of the ciliary-type that occurs at lower calcium

levels (Hyams and Borisy, 1978; Bessen et al, 1980). Because *Chlamydomonas* have the capacity to display flagellar motion, it can be assumed that they possess axonemal components, analogous to that of human spermatozoa, to regulate motility.

We have chosen to use *Chlamydomonas* as our model, because it is a convenient system for several reasons. Firstly, the axoneme, which is the structure responsible for generating movement in all species, is surrounded by a thin cell wall in *Chlamydomonas* cells (Harper, 1999). This makes it possible to efficiently dissect the axoneme into various components and to use motility assays to identify which components are essential for the regulation of motion. In contrast, spermatozoa of more evolved species are surrounded by dense fibers and a fibrous sheath (Gagnon, 1995) and are more difficult to manipulate. Secondly, *Chlamydomonas* can be easily cultured under laboratory conditions. When constantly exposed to light, the cell life cycle is approximately 20 hours. During this time, the cells will produce between 4-8 "daughter" cells (Harper, 1999). Because *Chlamydomonas* is a haploid genome, it is possible to isolate recessive mutations. In the 1980s, the laboratory of Piperno and Luck extensively analyzed mutant *Chlamydomonas* axonemes (Luck et al, 1982). By comparing mutant axonemes to wild-type axonemes using a two-dimensional gel system, they characterized a variety of *Chlamydomonas* mutants. These mutants and newly characterized mutants are available to establish correlation between loss of function and specific proteins.

In our laboratory, a panel of monoclonal antibodies (MoAbs) against the *Chlamydomonas* axoneme has been generated. These antibodies were selected based on their ability to modify movement in motility assays. The L2H12 MoAb is a potent inhibitor of motility. We therefore believe that it recognizes a protein that is essential for movement.

The objectives of this study were to localize the antigen recognized by L2H12 on the axoneme, isolate, sequence, characterize it, and study the importance of this antigen for flagellar motion.

2 Materials and Methods

2.1 Chemicals, kits and specialty products

Serum-free protein-free (SFPF) medium and mouse immunoglobulins (IgGs) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The following materials were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.): Econo-Pac® Protein A kit including the protein A column, the Econo-Pac 10 DG desalting columns, binding buffer, and elution buffer; protein assay dye. Extracti–Gel D Detergent Removing Gel was bought from PIERCE (Rockford, Illinois, U.S.A.). Protein G Agarose beads were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). An ultrafiltration device was obtained from Millipore Corporation (Bedford, MA, U.S.A). Regenerated cellulose low binding filter with a 100 000 Da molecular weight cut-off was bought from Millipore Corporation. 10 mm width Fisherbrand regenerated cellulose dialysis tubing with a nominal molecular weight cut off range of 12 000-14 000 Da was purchased from Fisher Scientific Company (Pittsburgh, PA, U.S.A.). Supported nitrocellulose (0.22 μm pore size; Micron Separations Inc., Westboro, MA, U.S.A), goat anti-mouse IgG conjugated to horse radish peroxidase (GibcoBRL®, Gaithersburg, MD, U.S.A.), an enhanced chemiluminescence (ECL) kit (Amersham Life Sciences Inc., Oakville, ON, Canada), and x-ray films (Fuji, Minami-Asigara, Japan) were used for immunodetection. Goat anti-mouse IgG conjugated to alkaline phosphatase (Caltag Laboratories, San Francisco, CA, U.S.A), 5-bromo-4-chloro-3-indoyl

phosphate p-toluidine salt (BCIP) / p-nitro blue tetrazolium chloride (NBT) (Bio-Rad Laboratories, Hercules, CA, U.S.A.) were used for immunoblotting with the Miniblotter® (Immunetics, Cambridge, MA, U.S.A)

All other chemicals were of reagent grade.

2.2 Cell Culture

2.2.1 *Chlamydomonas* culture

Chlamydomonas reinhardtii wild type mt+ cc1010; an uniflagellated mutant, uni-1 mt+ cc-1926; radial spoke mutants pf-1 mt+ cc-1024, pf-14 mt+ cc-1032, pf-17 mt+ cc-1035, pf-24 mt+ cc-1384, pf-25 mt+ cc-1385, pf-26 mt+ cc-1386, and pf-27 mt+ cc-1387 were obtained from the *Chlamydomonas* Genetic Center (Department of Botany, Duke University, Durham, NC, U.S.A.).

The cells were cultured using medium I of Sager and Granick (Sager and Granick, 1953) with the following modifications: A total concentration of 2.49 M phosphate was used, instead of a total phosphate concentration of 1.31 M used by Sager and Granick. 1.46 M Na acetate anhydrous was added to provide a carbon source for the *Chlamydomonas* cells. The final pH of the medium was approximately 6.8. The cells were grown in 250 ml Erlenmeyer flasks containing 100 ml of medium, under constant light, at a temperature of 25°C. A cotton plug sealed the flasks and sterile tubing pierced the plug to provide gentle air bubbling. Doubling time was approximately 12 hr.

Stock cultures were maintained by subculturing the cells in petri dishes with hard medium, 1.5% agar, every 3-4 months. 0.4% yeast extract was added to the hard medium to detect bacterial growth. The petri dishes were tightly sealed with parafilm to prevent contamination.

2.2.2 Hybridoma cell culture

L2H12 hybridoma cells, produced in our laboratory (Galfré and Milstein, 1981; Gagnon et al, 1994) and SP2/0 cells were progressively weaned off foetal bovine serum until they could grow in SFPF medium. The cells were incubated at 37°C with 95% air and 5% CO_2. In these conditions, doubling time of the cells was approximately 18-24 hr. As the L2H12 cells were cultured, they released several proteins including the L2H12 monoclonal antibody (MoAb), which is a mouse monoclonal immunoglobulin type G subclass 1 (IgG_1).

2.3 Collection and concentration of proteins released by hybridoma cells

The medium was collected just after the hybridoma cells reached the log phase of growth, where the concentration of antibody is at its peak. The medium was spun at 600 x g for 10 min at room temperature. The supernatant containing the L2H12 MoAb was kept and stored at –20°C with 0.1 % sodium azide. The MoAb was concentrated by ultrafiltration using a stirred cell device with a regenerated cellulose low binding filter that had a 100 000 Da molecular

weight cut-off. Typically, the starting volume of 2 l was concentrated to a volume of 5 ml with a protein concentration between 3-7 mg/ ml. The concentrated medium was spun at 4°C at 19 100 x g for 10 min. It was then dialyzed against Tris Buffered Saline or TBS [20 mM Tris-HCl pH=8, 150 mM NaCl] containing 0.001% sodium azide, using regenerated cellulose dialysis tubing with a nominal molecular weight cut off range of 12 000-14 000 Da, for 24 hr.

2.4 Purification of the L2H12 MoAb

The Econo-Pac® Protein A kit was used for purification of the L2H12 MoAb from the crude L2H12 preparation, obtained after concentration of the culture medium from L2H12 hybridoma cells. The protocol provided by the manufacturer was followed. The sample was prepared with an Econo-Pac 10DG desalting column. Binding buffer (pH = 9.2) was used to equilibrate the column. The sample was introduced to the column and exchanged with binding buffer. The prepared sample was then introduced to the Econo-Pac protein A column, which was previously equilibrated with the binding buffer. The column contained 2 ml of Affi-Gel protein A, which when used with the binding buffer, provided by the manufacturer, had a capacity of 10-14 mg for mouse IgG_1. After the sample was introduced, binding buffer was used to wash the column and the flow through was collected. The antibody was eluted with a pH 4 buffer. The elution buffer was immediately exchanged for TBS

using the 10DG desalting column. A peristaltic pump was used to increase the flow rate of the columns to 1 ml/ min.

2.5 Isolation of *Chlamydomonas* Axonemes

The procedure for isolating *Chlamydomonas* axonemes was a modification of the method described by Witman (Witman et al, 1972, Witman, 1986). *Chlamydomonas* were cultured in 4 l flasks. When a cell density of 4 to 8 million cells/ ml was reached, the cells were harvested at room temperature at 1110 x g for 5 min. The cells were then washed with 10 mM Hepes (pH 7.4) and resuspended in 4% HMS [30 mM Hepes, pH 7.4, 5 mM $MgSO_4$ and 4% sucrose]. The remaining manipulations were performed at 4°C. The flagella were separated from the cell bodies by treatment with dibucaine (5 mM final concentration) for 1 to 2 min. This reaction was neutralized with a HMS-EGTA solution [30 mM Hepes, pH 7.4, 5 mM $MgSO_4$, 4% sucrose, and 0.5 mM ethylene glycol-bis β-aminoethyl ether N, N, N', N'-tetraacetic acid (EGTA)]. A crude sedimentation of the cell bodies was performed by centrifugation at 1600 x g for 8 min. The flagella were sedimented by spinning cells at 14 000 x g. A finer separation of the remaining cell bodies was performed using a one step gradient by layering the flagella resuspended in 30 ml HMS 4% on 10 ml of HMS 25% [30 mM Hepes, pH 7.4, 5 mM $MgSO_4$ and 25% sucrose] in 50 ml conical tubes. The tubes were centrifuged at 1000 x g for 20 min. The flagella were recuperated from the upper layer and interface and were spun at 31 000 x g for 10 min. The pellet was resuspended in HMDEK [30 mM Hepes, pH 7.4,

5 mM $MgSO_4$, 0.5 mM EGTA, 1 mM dithioreitol (DTT), and 25 mM K acetate]. To demembranate the flagella, 1% nonidet P40 (NP40) was added to the medium. The axonemes (demembranated flagella) were spun at 31 000 x g for 10 min. The axonemes were then washed 3 times with HMDEK to remove NP40. The axonemal preparation was then resuspended in HMDEK. A typical yield was between 0.5 and 1 mg of protein/ l of starting volume. The sample was either used immediately, or stored in 25% glycerol with 1 mM benzamidine, 1 μM leupeptin and 1 mM pepstatin.

2.6 Fractionation of the *Chlamydomonas* axoneme

The extracted axoneme (see 2.4) was fractionated following a modification of a protocol introduced by Witman (Witman et al, 1972). All manipulations were performed at 4°C. The original sample was diluted with HMDEK to a concentration of 4-5 mg/ ml and spun at 40 000 x g for 10 min. The axonemal proteins were sequentially extracted with different salt and detergent treatments. Following each extraction, the sample was spun at 40 000 x g for 10 min and the pellet was washed in the appropriate buffer. The first extraction was performed by suspension of the axoneme in HMDEK containing 0.6 M NaCl for 10 min. After centrifugation, the extracted proteins remained in solution, which was saved. The pellet was resuspended in a low salt solution [5 mM Hepes, pH 7.4, 0.2 mM EDTA, 1 mM DTT, and 1 mM phenyl methyl sulfonyl fluorid (PMSF)]. The sample was dialyzed against 1 l of low salt solution using regenerated cellulose dialysis tubing with a nominal

molecular weight cut off range of 12 000-14 000 Da for 16 hr, for maximal extraction of the proteins soluble in this solution. Following the low salt extraction, the remaining proteins were suspended in HDE [Hepes 5 mM, pH 7.4, 1 mM DTT, and 0.2 mM ethylenediaminetetraacetic (EDTA)] containing 0.2% sarkosyl and 2 M urea for 10 min. The proteins soluble in this sarkosyl/ urea solution underwent dialysis against HDE with 0.05% of Extracti-Gel®D Detergent Removing Gel for 72 hr, with 4-5 changes of buffer, to reduce the final concentration of sarkosyl in the sample. The same type of dialysis membrane used for the low salt extraction was used. Note that throughout the experiment 1 mM PMSF was added to each fraction after the pellet was resuspended.

2.7 Demembranation–reactivation assay

Chlamydomonas, in the log phase of growth, were spun at room temperature at 200 x g for 3 min, washed twice with 10 mM Hepes (pH 7.4), then resuspended in 200 μl of Hepes. To allow access of the antibodies to the axoneme, *Chlamydomonas* cells were demembranated by diluting 5-fold with a solution containing 30 mM Hepes (pH=7.4), 1 mM EGTA, 5 mM $MgSO_4 \cdot 7H_2O$, 1 mM DTT, 25 mM K acetate, 0.5% polyethylene glycol-20 000 (PEG-20 000), 1% Ficoll, and 0.1% NP40. Motility was re-initiated by diluting 20-fold in 30 mM Hepes (pH=7.4), 1 mM EGTA, 5 mM $MgSO_4 \cdot 7H_2O$, 1 mM DTT, 25 mM K acetate, 2% PEG-20 000, and 1 mM ATP (Goodenough, 1983; Kamiya and Witman, 1984).

The crude and purified L2H12 MoAb preparations were tested at various concentrations, under an inverted microscope with 100x magnification, to determine their effect on the motility of wild-type *Chlamydomonas*. The total number of *Chlamydomonas* cells in each well was approximately 40 000. The final volume of each well was approximately 100 μl.

The effect of L2H12 on the movement of a uniflagellar mutant was recorded by video micrography using the following materials: An Olympus microscope with a 100 x oil immersion lens and 10 x ocular; Panasonic WWF video camera, S-VHS format; Panasonic recorder in B&W position. The video sequence was recorded at a 50 Hertz (Hz) frequency (European standard), which corresponds to the average beat frequency of the flagellum. Individual successive images were digitalized with a Macintosh computer and the contour of the flagellum was digitally extracted using Videoshop 5.0. Images corresponding to a complete cycle of movement were obtained by taking successive images. The rotation of the cell body that occurs during motility was corrected for by rotating the images so that the images were superimposed on each other. The anchoring point of the flagellum into the cell body was used as a reference point. Flagellar movement was recorded under normal reactivation conditions and after 10 ng of pure L2H12 was introduced to the assay. The images were superimposed to obtain a single illustration of the beating cycle of the flagellum.

The overlapping flagellar images allowed for the visualization of a beating envelope, corresponding to the amplitude of the displacement of the

flagellar trajectory. The width of this beating envelope corresponded to the maximal wave amplitude. The width was measured at the proximal point, located at 1/3 the distance from the flagellar origin at the cell body; the median point, located at 2/3 the distance from the flagellar origin at the cell body; and the distal point, located at the tip of the flagellum. The beat frequencies for individual flagella were measured by counting the number of images necessary to cover one full cycle. For this purpose, cells under phase contrast microscopy were illuminated at 300 Hz, while the microscope stage was manually translated, so that an average of 6 images of the same flagellum were visualized in the same video frame (1/50 s European frequency). Combination of data from one video frame to the following video frame allows the determination of the average beat frequency.

2.8 Immunoprecipitation

2.8.1 Precipitation of the antigen recognized by L2H12

The sarkosyl/urea extract containing an enrichment of the antigen recognized by L2H12 was used for immunoprecipitation. All manipulations were performed at 4°C. To remove aggregates, the extract (1 mg) was spun at 110 000 x g for 30 min. 0.5 ml of immunoprecipitation buffer [50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% TritonX-100 (TX-100) and 0.5% deoxycholate] with the following protease and phosphatase inhibitors:, 4 mM NaF, 100 μM Na_3VO_4, 5 mM $Na_2MoO_4 \cdot 2H_2O$, 2 mM benzamidine, 5 mM iodoacetamide, 5 mM 4-nitrophenylphosphate (PNPP), and 0.5 mM PMSF

was added to the sample with 1 mM $MgCl_2$. The sample was left on ice for 30 min and was spun at 10 000 x g for 5 min. One mg of total protein of the crude L2H12 preparation was added to the supernatant and was incubated for 3 hr. A volume of 100 μl of 20% slurry of protein G agarose beads in Hepes Buffered Saline or HBS [50 mM Hepes, pH 7.5, 150 mM NaCl, and 10% glycerol] was added and incubated for 1 hr. To allow for sedimentation of the antigen-antibody-protein G complex, the sample was centrifuged at 10 000 x g for 30 s. The supernatant was discarded and the antigen-antibody-protein G complex was washed with immunoprecipitation buffer containing the protease/ phosphatase inhibitors. The complex was then washed with HBS, which was subsequently removed by aspiration. To release the antibody-antigen complex from the protein G beads for analysis by SDS-PAGE and Western blotting, a volume of 25 μl of Laemmli's sample buffer (Laemmli, 1970) was added to the complex and heated for 5 min at 100°C.

2.8.2 Precipitation of IgGs with protein G agarose

The 50% Protein G slurry was spun at 500 x g for 5 min and washed twice with HBS. After the washings, the beads were re-suspended in HBS. Crude L2H12 and commercial mouse IgG were incubated with 5 μl of 20% slurry of protein G agarose, in a solution containing TBS and 0.2% Tween. The volumes of the antibody preparations were adjusted so that each sample contained approximately 15 μg or 0.09 μg/ ml of pure antibody. Aprotinin (10 μg/ ml), leupeptin (10 μg/ ml), and pepstatin (35 μg/ ml) were added.

Incubation was performed for 2 hr at 4°C with constant shaking. The sample was centrifuged at 10 000 x g for 30 s. The supernatant was discarded and the antibody-protein G complex was washed 3 times with TBS, which was subsequently removed by aspiration. A volume of 30 µl of Laemmli's sample buffer (Laemmli, 1970) was added to the complex and heated for 5 min at 100°C for analysis by SDS-PAGE and Western blotting.

2.9 Protein Determination

Protein concentrations were determined by the Bradford method (Bradford, 1976) using the Bio-Rad Protein Assay Dye Reagent. Bovine serum albumin (BSA) was used as the standard.

2.10 Western Blot Analysis

Wild-type *Chlamydomonas* axonemes and fractions, radial spoke mutant axonemes, and products of immunoprecipitation were processed for SDS-PAGE. Laemmli's sample buffer (Laemmli, 1970) was added to all samples, which were then heated at 100°C for 5 min. The proteins were separated on a 10% SDS-polyacrylamide gel by electrophoresis. The gel was then electrotransferred onto a supported nitrocellulose membrane (0.22 µm pore size) for immunodetection (Towbin et al, 1979). Forty volts was used for the transfer for a maximal of 16 hr. A cooling system maintained a temperature of 10-12°C. The following manipulations were performed at room temperature with constant shaking: To block non-specific binding sites, the

membranes were treated with TBS containing 0.1% Tween-20 and 10% porcine serum (TTBS + 10% porcine) for 1 hr. They were then incubated with the L2H12 for 1 hr and washed with TTBS 5 times for 5-10 min each. The membranes were incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1:5000) for 30 min. The membrane was washed with TTBS and positive immunoreactive bands were detected with an enhanced chemiluminescence (ECL) kit, following the manufacturer's instructions. Briefly, membranes were incubated with the luminescent substrate for 5 min and exposed with x-ray films for an appropriate period to produce a clear representation of the bands.

The Miniblotter® was used for the simultaneous assessment of the reactivity of different antibody preparations at different concentrations. Western blotting was performed, following a protocol similar to the one described above. However, instead of using a goat anti-mouse IgG conjugated to horseradish peroxidase, the IgG used was conjugated to alkaline phosphatase (1:2500). Immunoreactive bands were detected by incubating the membrane with a 1:1 ratio of BCIP and NBT diluted 1:50 in alkaline phosphate buffer [100 mM Tris-HCl pH=9.8, 1 mM $MgCl_2$]

3 Results

3.1 Purification of L2H12 MoAb, which recognizes a 105 kDa protein

L2H12 hybridoma cells secreted the L2H12 MoAb, along with other proteins of varying molecular weights, in the SFPF medium. After concentrating the medium by ultrafiltration with a 100 000 Da molecular weight cut-off membrane, several high molecular weight proteins including the IgG_1 MoAb were retained. The crude concentrated antibody preparation was purified by affinity chromatography with a protein A column. The purified sample was analyzed by SDS-PAGE, which resolved two protein bands (fig.5). The upper and lower bands corresponded to molecular weights of approximately 54 and 28 kDa, respectively. These values approach the typical molecular weights of 55 and 25 kDa for heavy and light chain IgGs, respectively, given in the literature (Harlow and Lane, 1988).

Western blot analysis of *Chlamydomonas* axonemal proteins, separated by SDS-PAGE on a 10% gel, revealed that L2H12 recognizes a single axonemal protein with a molecular weight estimated at 105 kDa. These results confirmed that the purified L2H12 eluted from the protein A column reacts with the same axonemal protein as crude L2H12 (fig.6).

Figure 5: **Purification of L2H12 analyzed by SDS-PAGE.** Crude L2H12 was obtained after ultrafiltration of the supernatant collected from the L2H12 hybridoma cell culture. Purified L2H12 was obtained after purification of crude L2H12 with protein A affinity chromatography. The flow through represents the first three fractions of proteins that were not bound by protein A, diluted in 10 ml of binding buffer. The samples were processed for SDS-PAGE under denaturing conditions, as described in *Materials and Methods*. Depicted in the center lane are the purified heavy (top) and light (bottom) chains of L2H12 IgG_1, as indicated by the arrows. The bands can be seen among several impurities in the first lane representing L2H12.

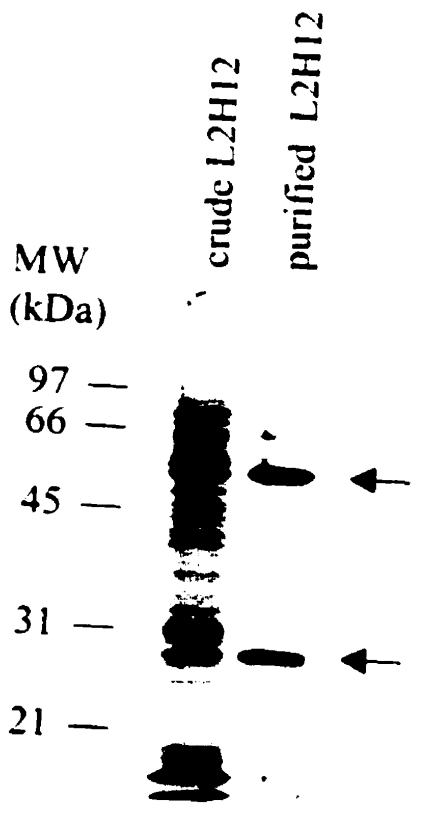
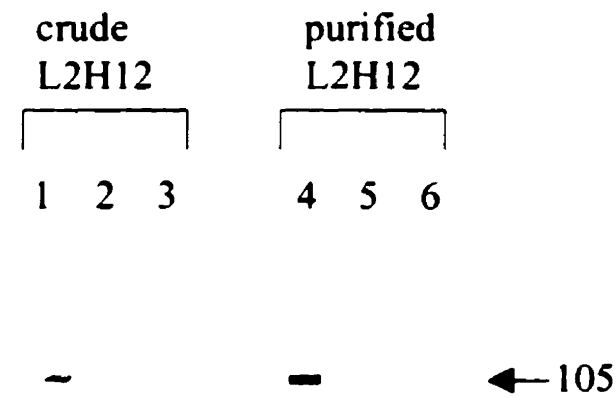

MW
(kDa)
97
66
45
31
21
crude L2H12
purified L2H12

Figure 6: **Recognition of a 105 kDa axonemal protein by crude and pure L2H12.** The affinity L2H12 for its antigen was tested at various concentrations by Western blotting, using the Miniblotter system, as described in *Materials and Methods*. Both the crude and pure antibody preparations were tested. Lanes 1-3 represent crude L2H12 reacting with the protein at diminishing concentrations of 10 µg/ ml, 1 µg/ ml, and 0.1 µg/ ml. The reaction with pure L2H12 at concentrations of 4 µg/ ml, 0.4 µg/ ml, and 0.04 µg/ ml is depicted in lanes 4, 5, and 6 respectively.

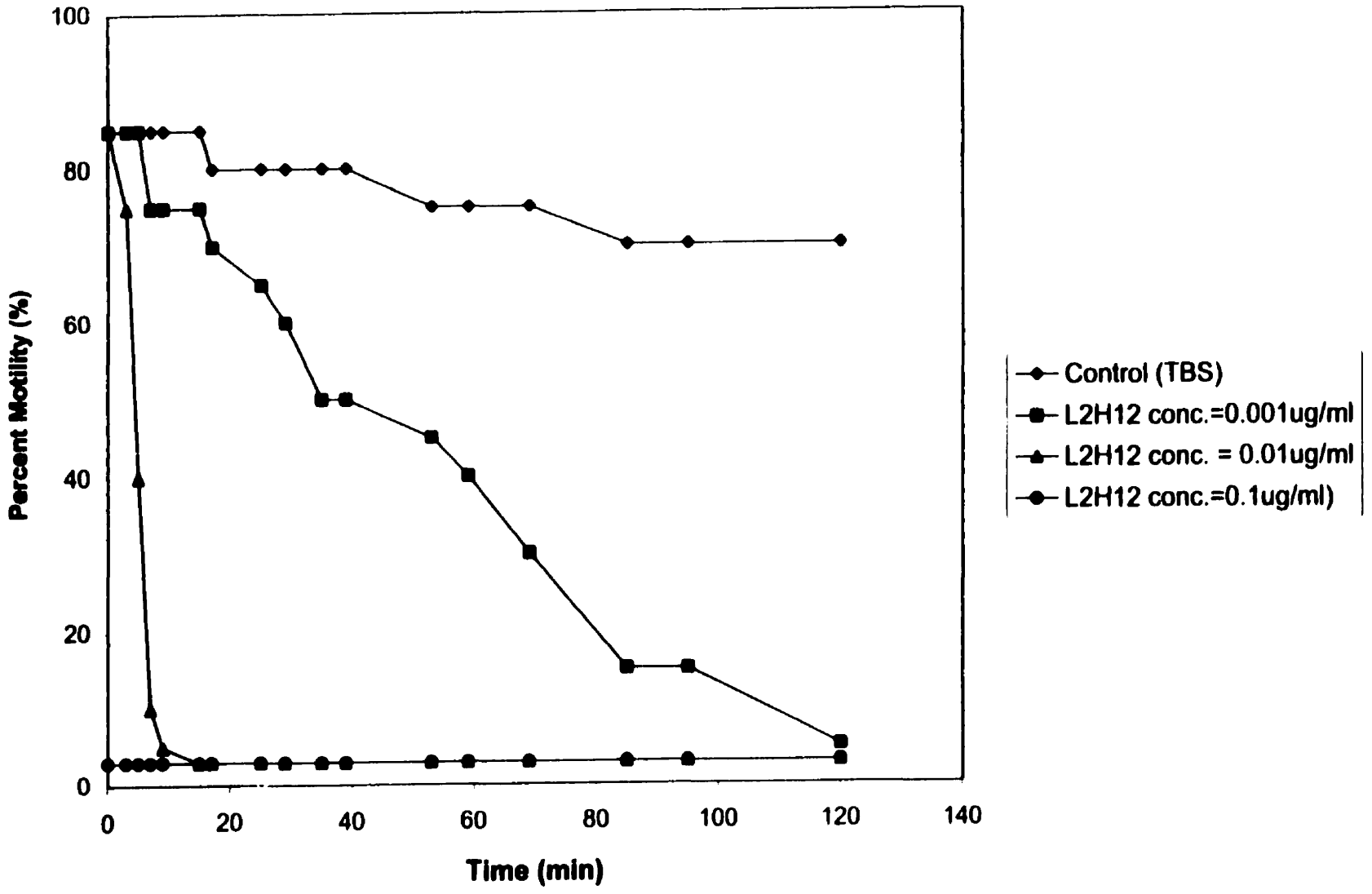

crude
L2H12
purified
L2H12
1 2 3
4 5 6
105

3.2 The effect of the L2H12 antibody on the motility of demembranated-reactivated *Chlamydomonas*

Under normal conditions, *Chlamydomonas* cells, suspended in a pH 7.4 Hepes buffer solution, display a ciliary-type of movement with a helicoidal pattern of forward progression (Hyams and Borisy, 1978), as seen with 100 x magnification under the light microscope (not shown). Using its eyespot as a light sensor, cells can make frequent and rapid changes of direction, responding to the light intensity in their immediate environment (Schmidt and Eckert, 1976). To allow access of anti-axonemal MoAbs to the axoneme and to test their effect on movement, the flagellar membrane must be dissolved. Motility is then reinitiated with the introduction of fresh medium containing ATP. In demembranation-reactivation assays, motility is generally reinitiated in approximately 85% of all *Chlamydomonas*. The motility pattern observed in demembranation-reactivation medium is similar to that of cells suspended in pH 7.4 Hepes buffer. The cells follow the same helical trajectory and are constantly changing directions. The main difference is that the velocity of movement is slightly reduced. Motility of the cells in demembranation-reactivation medium is typically maintained for 1-2 hr. When the effect of L2H12 on motility was observed, the movement of *Chlamydomonas* treated with L2H12 was always compared to a control that was untreated.

We observed that 1 μg/ ml of the crude L2H12 antibody preparation and 0.1 μg/ ml of purified L2H12 instantly inhibited motility in more than 95% of all reactivated *Chlamydomonas* (fig.7). We arbitrarily defined the specific

Figure 7: **Effect of L2H12 on the motility of *Chlamydomonas*.** Demembranation – reactivation assays were performed on *Chlamydomonas* with different concentrations of pure L2H12, as described in *Materials and Methods*. Motility was immediately arrested in more than 95% of the 40 000 cells when L2H12 at a concentration of 0.1 μg/ ml (represented by the circles) was introduced to the demembranation-reactivation medium. At 0.01 μg/ ml (triangles) cell motility decreased for 10 minutes. After 10 minutes, more than 95% of the cells were inhibited. When 0.001 μg/ ml of L2H12 (squares) cell motility decreased for two hours. After this time, 95% inhibition was achieved.


100
80
60
40
20
0
Percent Motility (%)
0
20
40
60
80
100
120
140
Time (min)
Control (TBS)
L2H12 conc.=0.001ug/ml
L2H12 conc. = 0.01ug/ml
L2H12 conc.=0.1ug/ml)

activity of the crude L2H12 as 1. Since purified L2H12 was 10 times more potent than crude L2H12, its specific activity was increased 10-fold for a purification factor of 10.

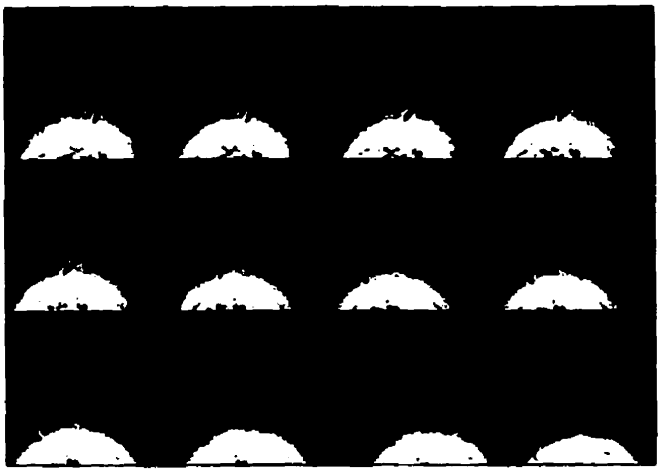
When initially submaximal concentrations of purified L2H12 were added to the demembranation-reactivation medium, motility decreased exponentially (fig 7). At a concentration of 0.01 μg/ ml the percentage of motile cells continuously decreased for 10 min. At this time, more than 95% of cells were inhibited. At a concentration of 0.001 μg/ml, 2 hr were required for the cells to reach 95% inhibition of motility.

Controls with TBS or TBS plus nonspecific proteins secreted by SP2/0 myeloma cells had no significant effect on *Chlamydomonas* cell motility, when added to the demembranation-reactivation medium at the same concentration range as that of L2H12. This suggests that specific binding of L2H12 to its antigen caused the inhibition of motility.

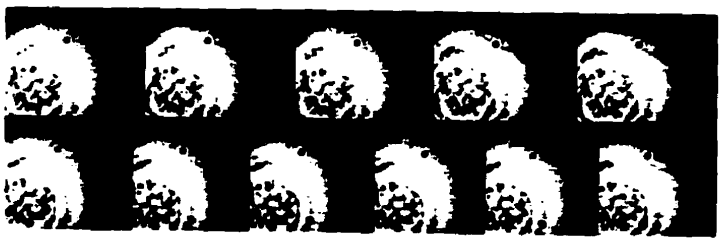
As mentioned above, under normal conditions in demembranation-reactivation assays, *Chlamydomonas* exhibit a ciliary pattern of movement. More specifically, this involves a power-stroke, where the flagella are fully extended allowing for maximal contact and thrust with the surrounding medium and a recovery stroke, where the flagella bend to decrease the forces of friction (Cosson, 1996). During the recovery stroke, the flagella return to their initial position where they then re-initiate the power-stroke. The ciliary movement of a uniflagellar *Chlamydomonas* mutant is shown in figures 8A and 9A.

Figure 8: **Video sequence of flagellar movement with L2H12.** A video sequence recorded with video micrography, as described in *Materials and Methods*, depicts the movement of the flagellum of the uniflagellar mutant under (**A**) normal conditions, when (**B**) 10 ng of L2H12 is added to the demembranation – reactivation medium, and (**C**) after prolonged exposure to L2H12. When the mutant is not exposed to L2H12, it exhibits a ciliary pattern of movement involving a power-stroke, followed by a recovery phase (**A**). L2H12 continually affects the recovery phase (**B**), until the flagellum can no longer re-initiate movement (**C**).

A



B



C

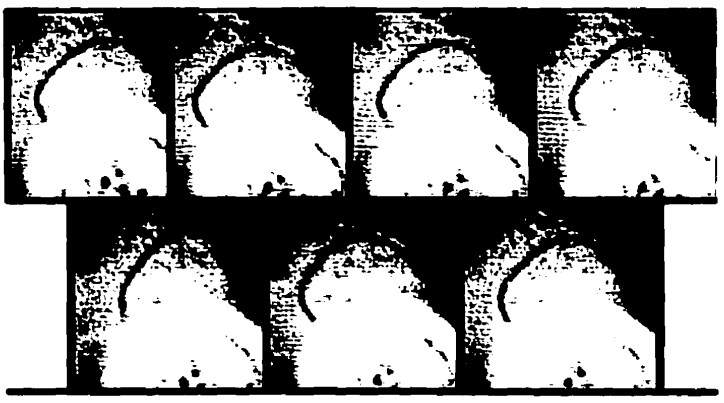
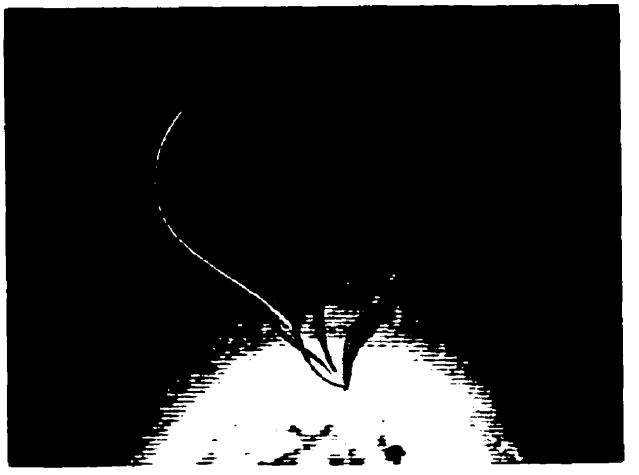


Figure 9: **Digital representation of flagellar movement with L2H12.** A complete flagellar beating cycle was obtained by digitally superimposing the images depicted in figure 8. Seven different positions of the flagella during ciliary beating are depicted in different colors for differentiation purposes. Under normal conditions (**A**), the initiation of the power-stroke occurs at position 1, represented in yellow. At position 2 (pink), the flagellum is extended to allow maximal contact with the surrounding medium. Positions 3 (brown) and 4 (red) depict the final stages of the power-stroke. At position 5 (green), the recovery phase is initiated by the bending of the flagellum. The recovery phase continues at position 6 (black), and 7 (blue), until the flagellum returns to the starting position where it re-initiates the power-stroke. When the axoneme of flagellum is exposed to L2H12 (**B**), the bending that occurs during the recovery phase is decreased (**B**), so that the flagellum cannot return to position 1. This results in a power-stroke with reduced force. After prolonged exposure to L2H12 (**C**), the recovery phase is completely inhibited. The flagellum can no longer produce the power-stroke and motility is arrested.

A



B

C

A

B

C

When 10 ng/ ml of the L2H12 MoAb was added to the demembranation-reactivation medium, the recovery stroke was incomplete. The flagellum did not return to its initial position. Re-initiation of the power stroke occurred from where the recovery stroke ended. Over time, the recovery stroke ended progressively further from the initial position (fig 8B and 9B). The power-stroke that followed traced a progressively shorter trajectory with a weaker force, until motility was inhibited. The flagellum was paralyzed in a bent position (fig. 8C and 9C).

To quantify the effect of L2H12 on cell movement, changes in the maximal wave amplitude of the proximal, median, and distal tip of the flagellum and in the beat frequency of several *Chlamydomonas* cells were measured after treatment with 10ng/ ml of the L2H12 MoAb (table 1). Before treatment, the average maximal amplitude, calculated from the measurements of 6 cells, was 2.2 +/- 0.8 μm, 5.3 +/- 1.4 μm, 7.5 +/- 1.6 μm in the proximal, median, and distal regions, respectively. Between 5-10 min after the addition of 10 ng/ ml of L2H12, the average maximal amplitude, calculated from 9 cells, decreased to 0.9 +/- 0.3 μm, 1.4 +/- 0.4 μm, and 3.0 +/- 0.7 μm, in the proximal, median, and distal regions, respectively. In the late stages of the reaction between L2H12 and its antigen (30-45 min), the average maximal amplitude, for 4 cells, measured in the proximal and median sections of the flagellum remained constant at 1.0 +/- 0.3 μm and 1.2 +/- 0.3 μm, respectively. There was a further decrease in average maximal amplitude in the distal

Table 1: **The effect of L2H12 on the maximal wave amplitude of the proximal, median, and distal flagellum.** The maximal wave amplitude was measured in demembranated-reactivated *Chlamydomonas* cells before treatment with L2H12, between 5-10 min after L2H12 was added (early), and 10-45 min after the addition of L2H12 to the demembranation-reactivation medium. Averages were taken for measurements of amplitude for 6, 9, and 4 cells before treatment, early and late after L2H12 was added, respectively.

	Proximal	Median	Distal
Before L2H12	2.2 +/- 0.8 μm	5.3 +/- 1.4 μm	7.5 +/- 1.6 μm
Early after L2H12 (5-10 min)	0.9 +/- 0.3 μm	1.4 +/- 0.4 μm	3.0 +/- 0.7 μm
Late after L2H12 (30-45 min)	1.0 +/- 0.3 μm	1.2 +/- 0.3 μm	1.1 +/- 0.4 μm

region of the flagellum to 1.1 +/- 0.4 μm. It was observed that shortly after the flagellum was exposed to L2H12, wave propagation only occurred in the proximal region of the flagellum. The median and distal regions appeared to display a passive movement that resulted from proximal "shaking".

The beat frequency for 8 *Chlamydomonas* cells was measured (data not shown). Before treatment with 10ng/ ml of the L2H12 MoAb, the beat frequency averaged 65.6 +/- 2.9 Hz. Upon exposure to the MoAb, the beat frequency continually decreased until it reached a plateau at an average of 22.9 +/- 3.8 Hz. This decrease in beat frequency was frequently followed by periods of full arrest of flagellar beating lasting several seconds, followed by reinitiation of beat frequency equivalent to the value measured when the initial plateau was reached. Ultimately, motility was arrested a few minutes later.

3.5 The antigen recognized by L2H12 is enriched in the sarkosyl/ urea extract

To obtain an enriched preparation of the antigen recognized by L2H12, wild-type *Chlamydomonas* axonemes were sequentially fractionated under different salt and detergent conditions. The axoneme was first treated with 0.6 M NaCl, which extracts mainly dynein arms from the axoneme (Piperno and Luck, 1979). Axonemal proteins that were insoluble in NaCl underwent a low salt treatment, which typically solubilizes mainly inner dynein arms (Smith and Sale, 1991). The remaining proteins were treated with a solution containing 0.2% sarkosyl and 2M Urea, which extracted 45-50% of all axonemal proteins including the radial spokes (Witman et al, 1978).

The antigen recognized by L2H12 was predominately detected in the low salt and sarkosyl/ urea extract (fig.10). However, the intensity of the band representing the antigen in the sarkosyl/ urea extract was greater than the one representing the low salt extract. This suggests that the sarkosyl/ urea extract contained larger quantities of the antigen compared to the low salt extract. The antigen was not detected in the high salt extract or in the unextracted proteins (fig.10).

3.6 Localization of the antigen recognized by L2H12 by analysis of *Chlamydomonas* radial spoke mutants

It was previously demonstrated that the antigen recognized by L2H12 is a RSP (Aghigh, 2000). These results were ascertained by Western blotting analysis of axonemes of wild-type and various *Chlamydomonas* mutants lacking different axonemal components, using the L2H12 MoAb as a probe. The antigen recognized by L2H12 was detected in wild-type *Chlamydomonas* axonemes and in all mutants except for pf-14. Pf-14 is the radial spoke mutant where all 17 radial spoke polypeptides are absent on the axoneme (Huang et al, 1981; Piperno et al, 1981). Since the antigen recognized by L2H12 is missing in pf-14 mutants, the author concludes that it is, most likely, a RSP (Aghigh, 2000). However, which particular RSP is recognized by the MoAb, remained to be elucidated.

To determine which RSP is recognized by L2H12, axonemal proteins of different *Chlamydomonas* radial spoke mutants, along with those of wild-type

Figure 10: **The presence of the 105 kDa protein in fractionated axonemal proteins.** Wild-type *Chlamydomonas* axonemes were sequentially fractionated, processed for SDS-PAGE, and electrotransferred for Western blotting, as described in *Materials and Methods*. The proteins in the resulting fractions were visualized with Coomassie blue staining (**A**). Western blot analysis of the untreated axoneme, the high salt extract, the low salt extract, the sarkosyl/ urea extract, and the unextracted proteins (**B**) demonstrated that the antigen recognized by L2H12 is enriched in the sarkosyl extract. It is also present in the low salt extract, but is absent in the high salt extract. Note that the wells were loaded with 20 μl of volume containing approximately 20, 3, 3, 20, and 1.5 μg of total protein for the unfractionated axoneme, the high salt extract, low salt extract, sarkosyl/ urea extract, and the unextracted proteins, respectively.

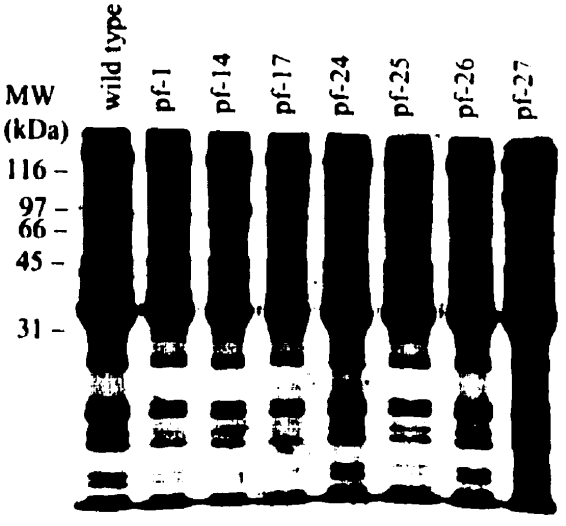
MW
(kDa)
116 –
97 –
66 –
45 –
31 –
full axoneme
high salt
low salt
sarkosyl/0.2 M urea
unextracted proteins
A
full axoneme
high salt
low salt
sarkosyl/0.2 M urea
unextracted proteins
105 kDa
B

Chlamydomonas, were analyzed by Western blotting using L2H12 as a probe (fig.11). The L2H12 MoAb reacted with the axonemes of wild-type *Chlamydomonas* and with those of pf-1, pf-17, pf-25, pf-26, and pf-27 mutants each missing gene products RSP4, RSP9, RSP11, RSP6, and a gene product that inhibits the phosphorylation of 5 polypeptides, respectively (Huang et al, 1981; Piperno et al, 1981). As expected, L2H12 did not react with pf-14. Furthermore, it did not react with pf-24, which is almost completely deficient in RSP2. This suggests that the L2H12 MoAb reacts with RSP2.

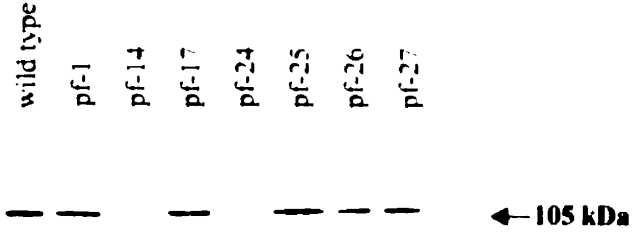
3.5 Immunoprecipitation of the 105 kDa protein

Since the sarkosyl/ urea extract was enriched with the antigen recognized by L2H12, this extract was used for immunoprecipitation with the L2H12 antibody. Under the conditions described in *Materials and Methods*, a single protein with a molecular weight of approximately 105 kDa was immunoprecipitated, as analyzed by immunoblotting (fig.12). Ample protein G beads were added, so that based on the theoretical binding capacity of the beads, maximal amount of antigen was retrieved. However, a small amount of antigen could be detected in the immunoprecipitation buffer after the antigen-antibody-protein G complex was sedimented (fig.12). Similar results were obtained using an IgG_1 anti-tubulin MoAb, as a positive control (data not shown).

Figure 11: **SDS-PAGE and Western blot analysis with radial spoke mutant axonemes using L2H12 as a probe.** Extraction of purified axonemes from wild-type *Chlamydomonas* and radial spoke mutants pf-1, pf-14, pf-17, pf-24, pf-25, pf-26, and pf-27 SDS-PAGE, and Western blot analysis were performed, as described in *Material and Methods*. **B** demonstrates that the antigen recognized by L2H12 is present in wild-type *Chlamydomonas* and in all the radial spoke mutants tested, except pf-14 and pf-24. Pf-14 is a mutant where all the radial spoke polypeptides are absent from the axoneme (Piperno et al., 1981). Pf-24 is deficient in RSP2. In **A**, a Coomassie-blue staining of the axonemal proteins separated by SDS-PAGE is depicted.

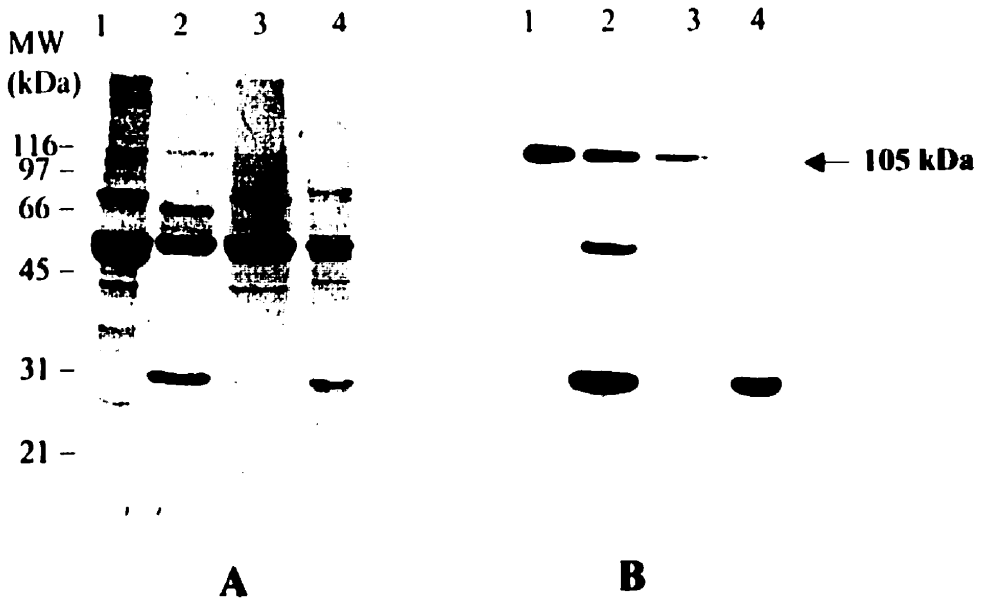

MW
(kDa)
116 –
97 –
66 –
45 –
31 –
wild type
pf-1
pf-14
pf-17
pf-24
pf-25
pf-26
pf-27

A


wild type
pf-1
pf-14
pf-17
pf-24
pf-25
pf-26
pf-27
105 kDa

B

Figure 12: **Immunoprecipitation of the 105 kDa antigen recognized by L2H12**. The antigen recognized by L2H12 was immunoprecipitated, as described in *Materials and Methods*. In **B**, the antigen recognized by L2H12 was detected in the sarkosyl extract (lane 1), the immunoprecipitate (lane 2, upper band), and in the fraction of proteins that were not precipitated (lane 3). The lower 2 bands in lane 2 are the heavy (≅ 50 kDa) and light (≅ 25 kDa) chains of the L2H12 MoAb. The L2H12 MoAb alone was used as a reference (lane 4). Panel A is an amido black stain of the electrotransferred proteins.


MW
(kDa)
1
2
3
4
116–
97 –
66 –
45 –
31 –
21 –
A
1
2
3
4
105 kDa
B

4 Discussion

Since the axoneme is the functional component of nearly all flagella, it must exhibit versatility to accommodate several types of motility patterns (Gagnon, 1995). This requires the contribution of different axonemal structures. It has been previously shown that the outer dynein arms control the velocity of movement (Kamiya et al, 1991), while the inner dynein arms, which exhibit more diversity than the outer dynein arms, may participate in the regulation of movement (Gagnon, 1995). Evidence suggests that the dynein inner arms are involved in the initiation and maintenance of flagellar bending and also contribute to the force required to sustain movement (Witman, 1992; Gagnon, 1995).

The radial spoke-central pair system has been demonstrated to be essential for flagellar bending (Huang et al, 1981, 1982; Smith and Sale; 1992; Piperno et al, 1992, 1994; Gardener et al, 1994; Howard et al 1994; Habermacher and Sale 1996, 1997). Several radial spoke and central pair mutants of the biflagellate *Chlamydomonas reinhardtii* are unable to initiate movement (Huang et al 1981, 1982). It has been suggested that the paralysis of these mutants occurs because in the absence of components of the radial spoke-central pair system, inner dynein arm activity is suppressed by the DRC (Huang et al, 1982; Smith and Sale, 1992; Piperno et al, 1992, 1994; Gardener et al, 1994). Flagellar bending appears to take place when interaction of the radial spoke-central pair complex with the DRC releases the

suppression of inner dynein arm activity (Piperno et al, 1994; Gardener et al, 1994). However it is unclear how the 17 radial spoke polypeptides interact with one another and with other axonemal structures to regulate bending.

In the current study, we identified a 105 kDa radial spoke protein essential for normal flagellar movement in *Chlamydomonas reinhardtii*. We immunoprecipitated this protein using the L2H12 antibody that recognizes it. The protein was immunoprecipitated from the sarkosyl/ urea extract of wild-type *Chlamydomonas* axonemal proteins, which was found to be enriched with the 105 kDa protein. Treatment of the *Chlamydomonas* axoneme with sarkosyl/ urea typically extracts the radial spokes, along with other axonemal components (Witman et al, 1978). Our finding that sarkosyl/ urea yields the largest amount of the 105 kDa protein compared to other treatments is consistent with previous localization of the antigen recognized by L2H12 to the radial spokes (Aghigh, 2000). A small amount of the 105 kDa protein was found in the low salt extract. This may be explained by the fact that inner dynein arms are closely associated with the radial spokes. Other researchers have also found that several RSPs are extracted upon exposure to a low ionic strength solution (Piperno et al, 1981).

In a previous study, Luck's laboratory isolated and characterized several radial spoke and central pair mutants (Piperno et al, 1981; Huang et al, 1981). They found that the deficiency of RSP3 in pf-14 mutants causes the absence of all 17 RSPs on the axoneme of these mutants. Preliminary results suggested that L2H12 may recognize a RSP (Aghigh, 2000).

In the current study, we tested the L2H12 MoAb against purified axonemes isolated from all available radial spoke mutants. We observed that L2H12 reacted with the 105 kDa protein in radial spoke mutants pf-25, pf-26, pf-27, each missing RSP11, RSP6, and a gene product that inhibits the phosphorylation of 5 polypeptides, respectively (Huang et al, 1981; Piperno et al, 1981). L2H12 reacted with pf-1 and pf-17, two mutants deficient in RSP4 and RSP9, respectively. These mutants are missing all the components of the radial spoke heads, but contain all the radial spoke stalk proteins. This suggests that the antigen it recognizes is not localized to the radial spoke heads and is instead found on the radial spoke stalks. There was no significant reaction between L2H12 and pf-24, a radial spoke mutant almost completely deficient in RSP2 (Huang et al, 1981). This suggests that RSP2, which has been localized to the radial spoke stalk (Piperno et al, 1981) is the antigen recognized by L2H12. Piperno et al (1981) estimated that the molecular weight of RSP2 is 118 kDa. This approaches the molecular weight value that we assigned to the antigen recognized by L2H12. The differences in molecular weight estimation of RSP2 by Piperno et al and our estimation of the antigen recognized by L2H12 can be explained by the differences in the conditions used to resolve the proteins. Piperno et al (1981) used 4-11% gradient gels that have a surface area of 168 cm^2 to resolve RSP2. Furthermore, they solubilized their sample in 5 M Urea. In our study, uniform 10 % gels with a surface area of 70 cm^2 were used and the samples were solubilized in a buffer that did not contain Urea. Since pf-24 mutants are

paralyzed and since L2H12 inhibits motility, the data strongly suggest that the antigen recognized by L2H12 is, most likely, RSP2 and plays a key role in flagellar motion.

The importance of the 105 kDa protein for normal flagellar function was demonstrated when the L2H12 antibody caused inhibition of motility by impairing the function of the 105 kDa protein in demembranated-reactivated *Chlamydomonas* cells. We have ruled out the possibility that exposure to TBS, the medium used to keep the L2H12 antibody in solution, caused the arrest of motility, because the addition of TBS alone to the demembranation–reactivation medium did not affect the movement of the cells. When SP2/0 myeloma cells, used for the production of L2H12 hybridoma cells, are cultured alone, they secrete neither heavy nor light antibody chains, although other proteins are released in the culture medium. We cultured SP2/O myeloma cells in parallel to L2H12 hybridoma cells under similar conditions and concentrated the medium, containing nonspecific proteins released by the cells, by ultrafiltration. When testing the concentrated medium against the demembranated-reactivated *Chlamydomonas* at concentrations comparable to that of L2H12, motility was not significantly affected. This suggests that the nonspecific proteins released both from L2H12 hybridoma cells and SP2/O cells were not contributing to the inhibition of movement.

The L2H12 cell line secretes several high molecular weight proteins with L2H12 IgG_1 that are not released by SP2/O cells alone. To demonstrate that these proteins were not interrupting motility instead of L2H12, we purified

the crude antibody preparation with affinity chromatography. We confirmed that purification was successful by visualizing the eluted fractions with SDS-PAGE using Coomassie blue staining. The purified L2H12 obtained in the first eluted fraction from the protein A column contained two bands with molecular weights that were typical of the heavy and light chains of IgGs. Bands of equivalent molecular weight to those of purified L2H12 were present within the several proteins of L2H12. Immediate inhibition of motility in greater than 95% of demembranated-reactivated *Chlamydomonas* cells could be achieved when the cells were exposed to purified L2H12 at a 10-fold lower total protein concentration than that of the crude L2H12 solution. This suggests that the proteins present in the crude L2H12 preparation were not major contributors to the inhibitory activity of the L2H12 solution. The fact that TBS and the medium from SP/20 cells do not significantly affect cell motility and that higher concentrations of crude L2H12 are required to inhibit movement strongly suggests that the inhibition of motility occurs because of specific interaction between the L2H12 MoAb and the 105 kDa protein.

L2H12 is a potent inhibitor of the motility of demembranated-reactivated *Chlamydomonas* cells. It arrests movement at significantly lower concentrations than other monoclonal IgGs recognizing different *Chlamydomonas* axonemal proteins. L1F1, L3G4, L6G5 MoAbs were produced in our laboratory at the same time as L2H12 (Aghigh et al, 2000). These antibodies were selected based on their ability to recognize a single protein on the *Chlamydomonas* axoneme, as tested by Western blot analysis.

L1F1, which recognizes a 55 kDa protein had no effect on motility in demembranation-reactivation assays. L6G5 recognizes a 34 kDa protein, which was enriched in the sarkosyl extract of the fractionated axoneme, but is not localized to a particular axonemal structure according to mutant analysis. It affected the motility of *Chlamydomonas* by slightly decreasing the velocity of movement, but did not completely arrest movement. L3G4 recognizes a 110 kDa protein localized to the radial spoke heads of the axoneme. It inhibits motility, but at a slower rate than L2H12. When equivalent concentrations of L2H12 and L3G4 were used on demembranated-reactivated *Chlamydomonas* cells, it took 6-fold more time to completely arrest motility by the antibody L3G4 than for L2H12 (Aghigh, 2000).

The flagella of wild-type *Chlamydomonas reinhardtii* generally display a ciliary pattern of motion that allows the cells to progress forward. This involves the power-stroke, where the flagella extend to provide maximal contact with their surrounding medium and the recovery stroke, where the flagella bend as they sweep through the medium to return to a starting position where they can reinitiate the power-stroke (Cosson, 1996). Previously, Huang et al (1982) demonstrated that the radial spoke heads are involved in the modification of microtubule sliding to generate the asymmetrical pattern of motility observed during ciliary motion. Using a central pair mutant, pf-6 and radial spoke mutants pf-1, pf-24, and pf-25, they successfully restored wild-type flagellar function in assays where these mutants were exposed to suppressor mutants, as was described in detail in the *Introduction (section 1.7.2)*. However, radial

spoke head-deficient mutant, pf-17 did not regain wild-type flagellar motion and instead displayed an increased curvature during the recovery stroke, which was retained during the diminished power-stroke. This observation suggested that the presence of radial spoke heads on the axoneme was essential for normal flagellar function and that the radial spoke heads may be particularly important for the recovery phase.

In the current study, we used video micrography to provide detailed analysis of the inhibition pattern by the MoAb, L2H12 on the flagellar movement of demembranated-reactivated *Chlamydomonas* cells. We demonstrated that when L2H12 MoAb interrupts the activity of the radial spoke stalk protein it recognizes, the recovery phase of ciliary motion is affected. Exposure to submaximal concentrations of L2H12 prevents the return of the flagellum to the position required for the re-initiation of a normal power-stroke. Instead, the flagellum recovers incompletely. The power-stroke that follows involves a shorter extension of the flagellum. Therefore, the flagellum cannot produce an effective force upon contact with the surrounding medium. As L2H12 continues to interact with its antigen, the recovery stroke finishes progressively further from the starting position. This results in a progressively weaker power-stroke, until motility is arrested.

To quantify the effect of the interruption of the function of the 105 kDa protein recognized by L2H12 on flagellar activity, motility parameters including maximum beat amplitude and beat frequency were measured when *Chlamydomonas* cells were exposed to submaximal levels of L2H12 in

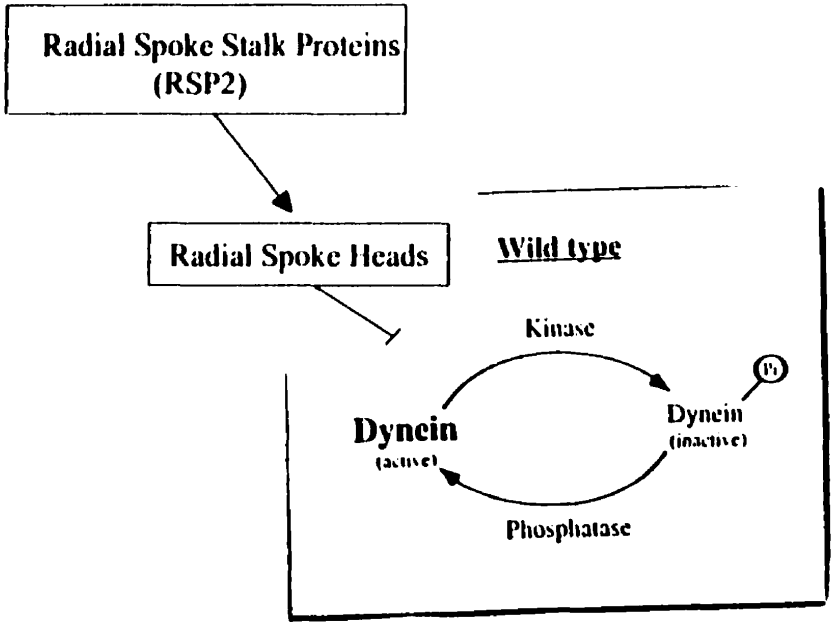
demembranation-reactivation medium. It has been previously demonstrated that *Chlamydomonas* mutants lacking dynein inner arms show a reduction in the amplitude of flagellar beating compared to wild-type *Chlamydomonas*, whereas the beat frequency is not significantly different from that of the wild-type cells (Brokaw and Kamiya, 1987). In mutants where the outer dynein arms are absent on the axoneme, beat frequency is reduced and no significant effect on beat amplitude is observed (Gibbons and Gibbons, 1973). This suggests that the outer dynein arms determine the velocity of movement, measured as beat frequency and the inner dynein arms are responsible for force production, which affects the amplitude of the beating wave (Witman, 1992). The absence of components of the radial spokes and central pair microtubules results in the arrest of movement (Huang, 1986), indicating that these structures are essential for the initiation of movement. In addition, as has been mentioned above, the radial spoke-central pair apparatus may play a regulatory role during motility. Our finding that both the maximum wave amplitude and beat frequency are significantly reduced when L2H12 is added to demembranated-reactivated *Chlamydomonas*, may implicate that the 105 kDa protein plays a role in the regulation of both outer and inner dynein arm activity.

Since both the 105 kDa radial spoke stalk protein and the radial spoke heads have been implicated as essential regulators of the recovery stroke (Huang et al, 1982), we speculate that the 105 kDa RSP interacts with radial spoke heads during flagellar bending. The RSP may participate in the

activation of radial spoke heads, which remove the constitutive suppression of inner dynein arm activity by interacting with the DRC. Luck's laboratory has demonstrated that RSP2, 3, 5, 13, and 17 are phosphorylated (Piperno et al, 1981). It is of interest to note that these RSPs have been localized to the radial spoke stalks. Perhaps it is the phosphorylation states of these stalk proteins that controls how they interact with the radial spoke heads during the regulation of flagellar movement. It was previously predicted that in wild-type *Chlamydomonas*, the radial spokes inhibit cAMP-PK, leading to the dephosphorylation of the 138-kDa intermediate chain, which activates dynein (Habermacher and Sale, 1997). More specifically, we speculate that the radial spoke stalk proteins, including RSP2, activate the radial spoke heads, which then inhibit cAMP-PK, leading to the activation of dynein (fig.13). At present the radial spoke-central pair system has not been shown to interact with the dynein outer arms. However since impairing the activity of the RSP recognized by L2H12, significantly reduces the beat frequency of *Chlamydomonas* by more than 50%, it is plausible that the radial spoke-central pair system interacts with the dynein outer arms, while controlling inner dynein arm activity.

During our experimental work, we encountered a major problem that slowed down our progress. We noticed that the L2H12 hybridoma cell line stopped synthesizing intact L2H12 MoAb. This conclusion was evidenced by the fact that purification of crude L2H12 with affinity chromatography and immunoprecipitation using protein G beads were successful only when

Figure 13: **Involvement of the radial spokes in the regulation of dynein actvity.** In wild-type Chlamydomonas, the radial spoke stalk proteins, including RSP2, activate the radial spoke heads, which inhibit cAMP-PK. This leads to the dephosphorylation of the 138-kDa intermediate chain, which activates dynein.

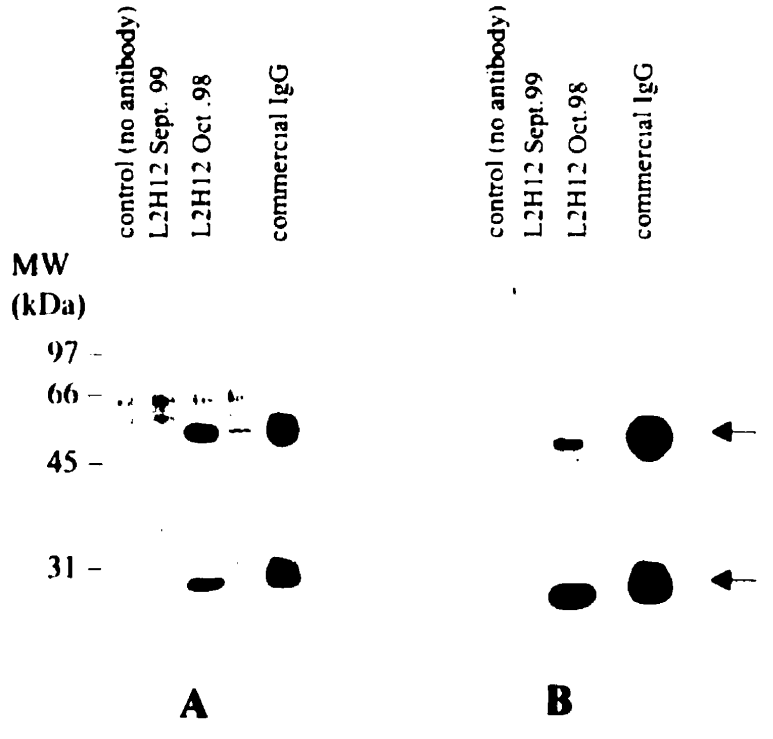

Radial Spoke Stalk Proteins
(RSP2)
Radial Spoke Heads
Wild type
Kinase
Dynein
(active)
Dynein
(inactive)
P
Phosphatase

batches of crude MoAb collected prior to and including Oct. 1998 were used (fig.14).

To test whether there were discrepancies in the recognition of different batches of antibody by protein G, we incubated several batches of L2H12 antibody with protein G agarose beads, along with mouse IgG as a positive control. Protein G beads precipitated the L2H12 MoAb from the batch of antibody collected Oct. 1998. Similarly control mouse IgG was precipitated. However, we could not detect precipitation of L2H12 from the batches of antibody collected after Oct.1998 (fig.14). Based on the results, it appears as though there was a modification in the binding affinity of the Fc portion of the L2H12 MoAb. A change in binding affinity may be caused by partial deletions or variations in IgG synthesis, which occurred during prolonged culturing of L2H12 hybridoma cells. This is plausible, particularly since it has been previously shown that the loss of heavy chain production by hybridoma cells is common (Cotton et al, 1973; Scharff 1974; Köhler, 1980). Further experimentation beyond the scope of the thesis would be required to elucidate the mechanism by which the binding affinity of the MoAb has decreased.

In summary, our findings support the importance of the radial spokes in the regulation of flagellar function. We have demonstrated that the inactivation of a single radial spoke protein, with the binding of the specific MoAb, L2H12, is sufficient to arrest motility in *Chlamydomonas* cells. Furthermore, we have shown that L2H12 causes a decrease in the flagellar wave amplitude and beat

Figure 14: **Affinity of protein G agarose beads for L2H12.** The affinity of protein G for different batches of antibody was tested as described in *Materials and Methods*. **A** depicts a silver stain of the membrane used for Western blotting with goat anti-mouse IgG conjugated to horse radish peroxidase as a probe. In **B**, Western blot analysis revealed that commercial IgG and the batch of L2H12 prepared Oct. 1998 were precipitated by the protein G beads. The batch of L2H12 prepared Sept. 1999 and other batches of antibody prepared after Oct. 1998 (data not shown) were not detected as precipitation products. The first lane represents a negative control with protein G beads incubated with TBS and 0.2% Tween only.


control (no antibody)
L2H12 Sept. 99
L2H12 Oct .98
commercial IgG
MW
(kDa)
97 -
66 -
45 -
31 -
A
control (no antibody)
L2H12 Sept.99
L2H12 Oct.98
commercial IgG
B

frequency. After observing video sequences of flagellar beating in the presence of L2H12, we suggest that the protein recognized by this MoAb participates in the control of the flagellar bending that occurs during the recovery phase of ciliary movement. Analysis of several radial spoke mutants, revealed that the antigen recognized by L2H12 is, most likely, RSP2.

Future goals include stabilizing the L2H12 hybridoma cell line, reculturing the MoAb, and purifying it. The 105 kDa protein will then be immunoprecipitated and separated with 2D-SDS-PAGE. Peptides from this protein will be sequenced. These sequences will be used to determine whether the 105 kDa protein has similarities to proteins in available data banks. The sequences will also be used to isolate and sequence the cDNA.

References

Aghigh S. Characterization of novel axonemal proteins that modulate the motility of *Chlamydomonas reinhardtii* flagella. MSc thesis, McGill University, Montreal; 2000.

Aitken RJ, Sutton M, Warner P, Richardson DW. Relationship between the movement characteristics of human spermatozoa and their ability to penetrate cervical mucus and zona-free hamster oocytes. J. Reprod. Fertil. 1985; 73: 441-449.

Auger J, Krustmann JM, Gzyglik F, Jouannet P. Decline in semen quality among fertile men in Paris during the past 20 years. N. Engl. J. Med. 1995; 332: 281-285.

Auger J, Jouannet P. Evidence for regional differences of semen quality among fertile French men: Federation Française des Centres d'Etude et de Conservation des Oeufs et du Sperme humains. Hum. Reprod. 1997; 12: 740.

Barra HS, Arce CA, Argarana CE. Posttranslational tyrosination-detyrosination of tubulin. Mol. Neurobiol. 1988; 2: 133-153.

Bessen M, Fay RB, Witman GB. Calcium control of waveform in isolated flagellar axonemes of *Chlamydomonas*. J. Cell Biol. 1980; 86: 446-455.

Boatman DE and Robbins RS. Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. Biol. Reprod.1991; 44: 806-813.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. Anal. Biochem. 1976; 72: 248

Brokaw CJ. Calcium-induced asymmetrical beating of Triton-demembranated sea urchin sperm flagella. J. Cell Biol. 1979; 82: 401-411.

Brokaw CJ and Kamiya R. Bending patterns of *Chlamydomonas* flagella. IV. Mutants with defects in inner and outer dynein arms indicate difference in dynein arm function. Cell Motil. Cytoskel. 1987; 8: 68-75.

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. BMJ 1992; 305: 609-613.

Cosson J. The covalent oscillator: a paradigm accounting for the sliding/ bending mechanism and wave propagation in cilia and flagella. Biol. Cell 1992; 76: 319-327.

Cosson J. A moving image of flagella: news and views on the mechanisms involved in axonemal beating. Cell Biol. Int. 1996; 2: 83-94.

Cosson MP and Gagnon. Protease inhibitor and substrates block motility and microtubule sliding of sea urchin and carp spermatozoa Cell Motil. Cytoskel. 1988; 10: 518-527.

Cotton RGH, Secher DS, and Miltein C. Somatic mutation and origin of antibody diversity. Clonal variability of the immunoglobulin produced by MOPC-21 cells in culture. Eur. J. Immunol. 1973; 3: 135-140.

de Krester DM, Baker HWG. Infertility in men: recent advances and continuing controversies. J. Clin. Endocrinol. Metab. 1999; 84: 3443-3450.

de Lamirande E, Cosson MP, and Gagnon C. Is transglutaminase involved in sperm motility? Ann. NY Acad. Sci. 1987; 513: 592-594.

de Lamirande E, Gagnon C. A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. Int. J. Androl.
1993a; 16: 21-25.

de Lamirande E, Gagnon C. Human sperm hyperactivation and capacitation as parts of an oxidative process. Free Rad. Biol. Med. 1993b; 14: 157-166.

de Lamirande E, Leclerc P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol. Hum. Reprod. 1997; 3: 175-194.

Demott RP, Suarez SS. Hyperactivated sperm progress in the mouse oviduct. Biol. Reprod. 1992; 46: 779-785.

Diener DR, Ang LH, and Rosenbaum JL. Assembly of flagellar radial spoke proteins in *Chlamydomonas*: identification of the axoneme binding domain of radial spoke protein 3. J. Cell Biol. 1993; 123: 183-190.

Eddé B, Rossier J, Le Caer JP, Desbruyères E, Gros F, Denoulet P. Posttranslational glutamylation of α-tubulin. Science 1990; 47: 83-85.

Eddy EM, O'Brien DA. The Spermatozoon. In: Knobil E and Neill JD, eds. The Physiology of Reproduction, Second Edition. New York: Raven Press Ltd.; 1994: 29-77

Feneux D, Serres C, Jouannet P. Sliding spermatozoa: a dyskinesia responsible for human infertility. Fertil. Steril. 1985; 44: 508-511.

Gagnon C. Regulation of sperm motility at the axonemal level. Reprod. Fertil. Dev. 1995; 7: 847-855.

Gagnon C. The study of male infertility : a fertile ground for research. ACSI/IAAC 1996; 3: 10-14.

Gagnon C, White D, Huitorel P, Cosson J. A monoclonal antibody against the dynein IC1 peptide of sea urchin spermatozoa inhibits the motility of sea urchin, dinoflagellate, and human flagellar axonemes. Mol. Biol. Cell 1994; 5: 1051-1063.

Galfré G, Milstein C. Preparation of monoclonal antibodies: strategies and procedures. Meth. Enzymol. 1981; 73: 3-46.

Gardner CL, O'Toole E, Perrone CA, Giddings T, and Porter ME. Components of a "dynein regulatory complex" are located at the junction between the radial spokes and the dynein arms in *Chlamydomonas* flagella. J. Cell Biol. 1994; 127: 1311-1325.

Gibbons IR. Transient flagellar wave forms in reactivated sea urchin sperm. J. Muscle Res. Cell Motil. 1986; 7: 245-250.

Gibbons BH, Gibbons IR. The effect of partial extraction of dynein arms on the movement of reactivated sea urchin sperm. J. Cell Sci. 1973; 13: 337-357.

Gibbons BH, Gibbons IR. Properties of flagellar "rigor waves" formed by abrupt removal of adenosine triphosphate from actively swimming sea urchin sperm. J. Cell. Biol. 1974; 63: 970-985

Gibbons BH and Gibbons IR. Calcium-induced quiescence in reactivated sea urchin sperm. J. Cell. Biol. 1980; 84: 13-27.

Goodenough UW, Heuser JE. Substructure of inner dynein arms, radial spokes, and the central pair/ projection complex of cilia and flagella. J. Cell Biol. 1985; 100: 2008-2018.

Griveau JF, Renard P, Lelannou D. An *in-vitro* promoting role for hydrogen peroxide in human sperm capacitation. Int. J. Androl. 1994; 17: 300-307.

Guyton AC. Reproductive and harmonal functions of the male. In: Wonsiewicz MJ, ed. Textbook of Medical Physiology Eighth Edition, Philadelphia: W.B Saunders Company; 1991: 885-898.

Habermacher G and Sale WS. Regulation of flagellar dynein by an axonemal type-1 phosphatase in *Chlmaydomonas*. J. Cell Sci. 1996; 109:1899-1907.

Habermacher G and Sale WS. Regulation of flagellar dynein by phosphorylation of a 138-kDa inner arm dynein intermediate chain. J. Cell Biol. 1997; 136: 167-176.

Harlow and Lane, eds. Antibodies – A Laboratory Manual, New York: Cold Spring Harbor; 1988: 616-623.

Harper JDI. *Chlamydomonas* cell cycle mutants. International Review of Cytology 1999. Vol 189

Hoskins DD, Stephens DT, Hall ML. Cyclic adenosine 3':5'-monophosphate and protein kinase levels in developing bovine spermatozoa. J. Reprod. Fertil.1974; 37: 131-133.

Howard DR, Habermacher G, Glass DB, Smith EF, and Sale WS. Regulation of *Chlamydomonas* flagellar dynein by an axonemal protein kinase. J. Cell Biol. 1994; 127: 1683-1692.

Huang B. *Chlamydomonas reinhardtii*: a model system for the genetic analysis of flagellar structure and motility. Int. Rev. Cytol. 1986; 99: 181-215.

Huang B, Piperno G, Ramanis Z, Luck DJL. Radial spokes of *Chlamydomonas* flagella: Genetic analysis of assembly and function. J. Cell Biol.
1981; 88: 80-88.

Huang B, Ramanis Z, Luck DJL. Suppressor mutations in *Chlamydomonas* reveal a regulatory mechanism for flagellar function. Cell 1982; 28: 115-124.

Hyams JS, Borisy GG. Isolated flagellar apparatus of *Chlamydomonas*: characterization of forward swimming and alteration of waveform and reversal of motion by calcium ions *in vitro*. J. Cell Sci. 1978; 33: 235-253.

Inaba K, Akazome Y, Morisawa M. Two high molecular mass proteases from sea urchin sperm. Biochem. Biophys. Res. Commun. 1992; 182: 667-674.

Irvine S, Cawood E, Richardson D, MacDonald E, Aitken J. Evidence of deteriorating semen quality in the United Kingdom: Birth cohort study in 577 men in Scotland over 11 years. B.M.J. 1996; 312: 467.

Ishiguro K, Murofushi H, Sakai H. Evidence that cAMP-dependent protein kinase and a protein factor are involved in reactivation of triton X-100 models of sea urchin and starfish spermatozoa. J. Cell Biol. 1982; 92: 777-782.

Ishijima S, Oshio S, Mohri H. Flagellar movement of human spermatozoa. Gamete Res. 1986; 13: 185-197.

Kagami O, Kamiya R. Translocation and rotation of microtubules caused by multiple species of *Chlamydomonas* inner-arm dynein. J. Cell Sci. 1992; 103: 653-664.

Kamiya R, Kurimoto E, Muto E. Two different types of *Chlamydomonas* flagellar mutants missing different components of inner arm dynein. J. Cell Biol. 1991; 112: 441-447.

Kamiya R., Witman GB. Submicromolar levels of calcium control the balance of beating between two flagella in demembranated models of *Chlamydomonas*. J. Cell Biol. 1984; 98: 97-107.

Kartagener M, Zui P. Pathogenesis der bronchiectasen. Beitr. Klin. Tuberk Spezifschen Tuberk Forsch 1933; 83: 489-501.

Katz DF, Cherr GN, Lambert H. The evolution of hamster sperm motility during capacitation and interaction with the ovum vestments *in vitro*. Gamete Res. 1986; 14: 333-346.

Katz DF, Drobnis EZ, Overstreet JW. Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. Gamete Res. 1989; 22: 443-469.

Klumpp S, Steiner AL, Schultz JE. Immunocytochemical localization of cyclic GMP, cGMP-dependent protein kinase, calmodulin and calcineurin in Paramecium tetaurelia. Eur.J. Cell Biol. 1983; 32: 164 –170.

Köhler G. Immunoglobulin chain loss in hybridoma lines. Proc. Natn. Acad. Sci. U.S.A. 1980; 77: 2197-2199.

Kopf GS, Ping Ning X, Visconti PE, Purdon M, Galantino–Homer H, and Fornés M. Signaling mechanisms controlling mammalian sperm fertilization competence and activation. In: Gagnon C, ed. The Male Gamete: From Basic Science to Clinical Applications. Vienna: Cache River Press; 1999: 105-118.

Kremer J. The *in-vitro* spermatozoal penetration test in fertility investigations. PhD dissertation, University of Groningen, the Netherlands 1968.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.

Leclerc P, de Lamirande E, Gagnon C. Regulation of protein–tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Rad. Biol. Med. 1997; 4: 643-656.

Lindemann CB. A "Geometric Clutch" hypothesis to explain oscilllations of the axoneme of cilia and flagella. J. theor. Biol. 1994; 168: 175-189.

Lindemann CB, Orlando A, Kanous KS. The flagellar beat of rat sperm is organized by the interaction of two functionally distinct populations of dynein bridges with a stable central axonemal partition. J. Cell Sci. 1992; 102: 249-260.

Luck DJL, Huang B, Piperno G. Genetic and biochemical analysis of the eukaryotic flagellum. Synp. Soc. Exp. Biol 1982; 35: 399-419.

Miller EG, Kuzrok R. Biochemical studies of human spemen. III. Factors affecting migration of sperm through the cervix. Am. J. Obstet. Gynec. 1932; 24: 19-26.

Mortimer D. Sperm transport in the human female reproductive tract. In: Fin CA, ed. Oxford Review of Reproductive Biology vol.5. 1983: 30-61.

Mortimer D, Pandya IJ, Sawers RS. Relationship between human sperm motility characteristics and sperm penetration into human cervical mucus *in vitro*. J. Reprod. Fertil. 1986; 78: 93-102.

Okamura N, Tajima Y Ishikawa H, Yoshii S, Koiso K, Sugita Y. Lowered levels of bicarbonate in seminal plasma cause poor sperm motility in human infertile patients. Fertil. Steril. 1986; 45: 265 – 272.

Otter T, Satir BH, Satir P. Trifluoperazine-induced changes in swimming behavior of paramecium: evidence for two sites of drug action. Cell Motil. 1984; 4: 249-267.

Ozols J, Caron JM. Posttranslational modification of tubulin by palmitoylation: II. Identification of sites of palmitoylation. Mol. Biol. Cell 1997; 8: 637-645.

Paturle-Lafanechère L, Eddé B, Denoulet P, Van Dorseelaer A, Mazarguil H, Le Caer JP, Wehland J, Job D. Characterization of a major brain tubulin variant which is tyrosinated. Biochemistry 1991; 30: 10523-10528.

Piperno G, Fuller MT. Monoclonal antibodies specific for an acetylated form of α-tubulin recognize the antigen in cilia and flagella from a variety of organisms. J. Cell Biol. 1985; 101: 2085-2094

Piperno G, Huang B, Ramanis Z, Luck DJL. Radial spokes of *Chlamydomonas* flagella: polypeptide composition and phosphorylation of stalk components. J. Cell Biol. 1981; 88: 73-79.

Piperno G, Luck DJL. Axonemal adenosine triphosphatases from flagella of *Chlamydomonas reinhardtii*. Purification of two dyneins. J. Biol. Chem. 1979; 254: 3091-3099.

Piperno G, Mead K, LeDizet M, Moscatelli A. Mutation in the "dynein regulatory complex" alter the ATP-insensitive binding sites for inner arm dyneins in *Chlamydomonas* axonemes. J. Cell Biol. 1994; 125 (5): 1109-1117.

Piperno G, Mead K, Shestak W. The inner dynein arms I2 interact with a "dynein regulatory complex" in *Chlamydomonas* flagella. J. Cell Biol. 1992; 118: 1455-1463.

Piperno G, Ramanis Z, Smith EF, and Sale WS. Three distinct inner dynein arms in *Chlamydomonas* flagella: molecular composition and location in the axoneme. J. Cell Biol. 1990; 110: 370-389.

Porter ME. Axonemal dyneins: assembly, organization, and regulation. Curr. Opin. Cell Biol. 1996; 8: 10-17.

Raybin D, Flavin M. Modification of tubulin by tyrosylation in cells and extracts and its effect on assembly *in vitro*. J. Cell Biol. 1977; 73: 492-504.

Redeker V, Levilliers N, Schmitter JM, Le Caer JP, Rossier J, Adoutte A, and Bré MH. Polyglycylation of tubulin: a posttranslational modification in axonemal microtubules. Science 1994; 266: 1688-1691.

Reynolds AB, Thomas TS, Wilson WL, Oliphant G. Concentration of acrosome stabilizing factor (ASF) in rabbit epididymal fluid and species specificity of anti-ASF antibodies. Biol. Reprod.1989; 40: 673-680.

Rikmenspoel R. Movements and active moments of bull sperm flagella as a function of temperature and viscosity. J. Exp. Biol. 1984; 108: 205-230.

Sager R, Granick S. Nutritional studies with *Chlamydomonas reinhardtii*. Ann. N.Y. Acad. Sci. 1953; 56: 831-838.

Sale WS. The axonemal axis and the Ca^{2+}-induced asymmetry of active microtubule sliding in sea urchin sperm tails. J. Cell Biol. 1986; 102: 2042-2052.

Satir P. The generation of ciliary motion. J. Protozool. 1984; 3: 8-12.

Satir P, Barkalow K, Hamasaki T. The control of ciliary beat frequency. Trends Cell Biol.1993; 3: 409-412

Scharff MD. The synthesis, assembly and secretion of immunoglobulin: A biochemical and genetic approach. Harvey Lect. 1974; 69: 125-142.

Schmidt JA, Eckert R. Calcium couples flagellar reversal to photostimulation in *Chlamydomonas reinhardtii*. Nature (Lond.) 1976; 262: 713-715.

Serres C, Feneux D, Jouannet P. Abnormal distribution of peri axonemal structures in human sperm flagellar dyskinesia. Cell Motil. Cytoskel. 1986; 6: 68-76.

Smith EF, Sale WS. Regulation of dynein-driven microtubule sliding by the radial spokes in flagella. Science 1992; 257: 1557-1559.

Smith TT, Yanagimachi R. Attachment and release of spermatozoa from the caudal isthmus of the hamster oviduct. J. Reprod. Fertil. 1991; 91: 567-573.

Suarez SS, Dai X. Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome-reacted hamster sperm. Mol. Reprod. Dev. 1995; 42: 325-333.

Suarez SS, Varosi SM, Dai X. Intracellular calcium increases with hyperactivation in intact, moving hamster spermatozoa and oscillates with the flagellar beat cycle. Proc. Natl. Acad. Sci. USA 1993; 90: 4660-4664.

Tamm SL, Tamm S. Alternate patterns of doublet microtubule sliding in ATP-disintegrated macrocilia of ctenophore. Boroë. J. Cell Biol. 1984; 99: 1364-1371.

Tash JS. Protein phosphorylation: the second messenger signal transducer of flagellar motility. Cell Motil. Cytoskel. 1989; 14: 332-339.

Tash JS. Role of cAMP, calcium and protein phoshorylation in sperm motility. In Gagnon C, ed. Controls of Sperm Motility: Biological and Clinical Aspects. Boca Raton FL: CRC Press; 1990: 229-241.

Tash JS, Hidaka H, Means AR. Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for activation of sperm flagellar motility. J. Cell Biol. 1986; 103: 649-655.

Tash JS, Kakar SS, Means AR. Flagellar motility requires the cAMP-dependent phosphorylation of a heat-stable NP-40 soluble 56 kd protein, akokinin. Cell 1984; 38: 551-559.

Tash JS, Means AR. Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate. Biol. Reprod. 1982; 26: 745-763.

Tash JS, Means AR. Cyclic adenosine 3', 5' monophosphate, calcium, and protein phosphorylation in flagellar motility. Biol. Reprod. 1983; 28: 75-104.

Tesarik J, Oltras CM, Testart J. Effect of the human cumulus oophorus on movement characteristics of human capacitated spermatozoa. J. Reprod. Fert. 1990; 88: 665-675.

Thonneau P, Marchand S, Tallec A, Ferial ML, Ducot B, Lansac J, Lopes P, Tabaste JM, Spira A. Incidence and main causes of infertility in a resident population (1,850,000) of three french regions (1988-1989). Hum. Reprod. 1991; 6: 811-816.

Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 1979; 76: 4350-4354.

Tredway DR, Buchanan GC, Drake TS. Comparison of the fractional postcoital test and semen analysis. Am. J. Obstet. Gynec. 1978; 130: 647-52.

Tredway DR, Settlage DS, Nakamura RM, Motoshima M, Umezaki, CU, Mishell DR. Significance of timing for the postcoital evaluation of cervical mucus. . Am. J. Obstet. Gynec. 1975; 121: 387-393.

Van Waeleghem K, De Clercq N, Vermeulen L, Schoonjans F, Comhaire F. Deterioration of sperm quality in young healthy Belgian men. Hum. Reprod. 1996; 11: 325-329.

Vijayaraghavan S, Syephens DT, Trautman K, Smith GD, Khatra B, da Cruz e Silva EF, Greengard P. Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase –3 and protein phosphatase 1 activity. Biol. Reprod. 1996; 54: 709 – 718.

Wakabayashi K, Yagi T, Kamiya R. Ca^{2+}-dependent waveform conversion in the flagellar axoneme of *Chlamydomonas* mutants lacking the central-pair/radial spoke system. Cell Motil. Cytoskel. 1997; 38: 22-28.

Wallace-Haagens MJ, Duffy BJ, Holtrop HR. Recovery of spermatozoa from human vaginal washings. Fertil. Steril. 1975; 26: 175-179.

Warner FD. Cilia and flagella: microtubule sliding and regulated motion. In: Roberts K and Hyams JS, eds. Microtubules. New York: Academic Press 1979, 359-380

Warner FD, Satir P. The structural basis of ciliary bend formation. Radial spoke positional changes accompanying microtubule sliding. J. Cell Biol. 1974; 63: 35-63.

White IG, Kar A. Aspects of the physiology of sperm in the female genital tract. Contraception 1973; 3: 183-194.

Wilton LJ, Temple-Smith PD, Gordon Baker, HW, de Krester DM. Human male infertility caused by degeneration and death of sperm in the epididymis. Fertil. Steril. 1988; 49: 1052-1058

Witman GB. Isolation of *Chlamydomonas* flagella and flagellar axonemes. Meth. Enzymol. 1986; 134: 280-290.

Witman GB, Carlson K, Berliner J, Rosenbaum JL. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. J. Cell Biol. 1972; 54: 507

Wilton LJ, Temple-Smith PD, Gordon Baker HW, de Kretser DM. Human male infertility caused by degeneration and death of sperm in the epididymis. Fertil. Steril. 1988; 49: 1052-1058.

Witman GB, Plummer J, Sander G. *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules. J. Cell Biol. 1978; 76: 729-747.

Witman GB. Axonemal dyneins. Curr. Opin. Cell Biol. 1992; 4: 74-79.

Woolley DM. Evidence for "twisted plane" undulations in golden hamster sperm tails. Journal of Cell Biology 1977; 75: 851-865.

World Health Organization. Towards more objectivity in diagnosis and management of male fertility. Int. J. Androl. 1997; 7(Suppl):1-53.

Yanagimachi R. Mechanisms of fertilization in mammals. In: Mastroianni L, Biggers JD, eds. Fertilization and Embryonic Development *In Vitro*. New York: Plenum Press; 1981: 81-187

Yanagimachi R. Mammalian Fertilization. In: Knobil E and Neill JD eds. The Physiology of Reproduction, Second Edition. New York: Raven Press; 1994: 189-317.

Yudin AI, Cherr GN, Katz DF. Structure of the cumulus matrix and zona pellucida in the golden hamster: a new view of sperm interaction with oocyte-associated extracellular matrices. Cell Tissue Res. 1988; 251: 555-564.

Zorn B, Virant-Klun I, Verendik I, and Meden-Vrtovec H. Semen quality changes amoung 2343 healthy Slovenian men included in an IVF-ET programme from 1983 to 1996. Int. J. Androl. 1999; 22: 178-183.