

Kinesin and Kinesin Related Proteins: Common Sites of Acrylamide Toxicity?

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Introduction:

The primary goal of the research is to evaluate the effects of acrylamide and its key metabolites, glycidamide and propionamide, on the function of kinesin related proteins. Previously observed abnormalities in cell division leading to delays or blocks in mitosis, generation of micronuclei and/or aneuploidy, and potential carcinogenicity may be caused by general or specific inhibition of kinesin related proteins (KRPs). This mechanism is being tested as a potential alternative or simultaneous cause of carcinogenicity to toxicant adduction and/or clastogenic activity on DNA. This is a feasible site of action due to the involvement of kinesin motor proteins in cell division, the association of abnormal distribution of genetic material with cancer, and previous observations that kinesin motors purified from brain are inhibited by acrylamide.

Hypothesis:

Acrylamide inhibits the function of kinesin related proteins involved with mitosis

Background: This research program includes the use of molecular biology technology to identify and express DNA sequences that code for kinesin related motor proteins involved in mitosis and to determine the effect of the toxicant acrylamide (ACR) upon their activity. These studies have relevance to determination of kinesin as a common critical site of action of the variety of toxicities produced by acrylamide. It represents the natural continuation of our research that has identified kinesin as a major, and possibly the critical, molecular site of action of acrylamide in neurotoxicity. We will identify and clone kinesin related proteins (KRPs) from rat testis, and use recombinant DNA technology to express these proteins. We will quantitate the effects of ACR upon KRP function to determine if these proteins are molecular sites of toxicant action. Linking these data to the current and emerging cellular biological understanding of their respective functions will provide substantial and timely insight into the molecular mechanisms of ACR-induced alteration of chromosomal segregation in mitosis and consequently to carcinogenicity. Other parameters to be considered will be the production of micronuclei and aneuploidy and distorted or duplicated mitotic spindles.

Acrylamide Toxicity. Acrylamide monomer produces a variety of toxicological responses including neurotoxicity, genotoxicity, developmental toxicity, male reproductive toxicity, and carcinogenicity. The mechanistic action is not completely understood; in particular, the rationale for diverse, yet selective, organ system specificity is unknown. The high reactivity of acrylamide acting on a variety of proteins is one potential explanation for toxicity in diverse systems. Another plausible mechanism is action upon a protein common to all systems, which can account for a diverse spectrum of actions. Acrylamide is known to bind a wide variety of proteins and DNA. However, we hypothesize that action upon the kinesin protein superfamily represents a common action causing, or at least making a major contribution to, selective organ specificity. With kinesin as the site of action, the nervous system is highly susceptible due to the dependence of long nerves upon delivery of newly synthesized materials to distant nerve fibers by the kinesin-driven transport system. Carcinogenicity, mitotic defects and male reproductive events may also be due to the critical involvement of kinesin related proteins into proper segregation of genetic material to the daughter cells.

Motor Proteins and Chromosome Movements: There are several hundred members of the superfamily of kinesin proteins. These are distributed across numerous phylogenies; obviously most are of little interest to mammalian toxicity. However, comparisons of the kinesin-related proteins have identified subfamilies, which have similar sequences and functions (although insufficient information is available to categorize some of the KRPs). Kinesin proteins have a similar common motor domain. However, slight variations within the motor domain correlate with directionality of microtubule interactions, rates of movement and in certain cases, the functions of the protein (e.g. disassembly of microtubules rather than motility). Variations within the tail regions and/or associated proteins endow them with different and specific functions. Variations in the tail domain and/ or associated proteins dictate the specific cargo attaches, e.g. vesicles or chromosomes, moved by each type of motor and hence dictate what each protein moves. Our previous data with bovine brain kinesin suggest an ACR effect on the motor domain. Our observations of selectivity of effect of ACR on chromosome movement during anaphase suggest that specific families of proteins are more sensitive to toxic agents than others. The following figures summarize the mechanics of mitosis, identification of the families responsible for each movement, as well as some of the properties and proposed functions of individual kinesin related protein families. The reviewer is referred to the accompanying review article identifying the motors associated with mitotic spindle functions and potential consequences of kinesin protein inhibitions (Anat Rec 261:14-24,2000). This proposal will include expression and testing of KRP 1 (member of kar3 family), and KRP 2 (member of KIF2 family).

DNA vs Spindle Effects: Acrylamide effects include those that suggest action directly on DNA as well as those that suggest action on an element of the spindle (see list below). Suggestive that ACR acts on both DNA and proteins are observations of micronuclei (pieces of chromosomes) that possess kinetochores. These kinetochore-containing micronuclei and the aneuploidy are most likely the result of maldistribution of whole, or parts of, chromosomes. The spindle effects can be accounted for by inhibition of MT-associated motor proteins. In addition, benign tumors and heritable genetic damage can arise from maldistribution of regulatory genes into daughter cells.

DNA action

1. binds and alkylates DNA, low reactivity
2. genotoxic
3. mutagen
4. delays cell cycle, slows or blocks mitosis
5. micronuclei
6. carcinogenic

Spindle action

1. C mitotic effect (colchicine like)
2. delays cell cycle, slows or blocks mitosis
3. multipolar spindles
4. aneuploid and polyploidy
5. micronuclei
6. carcinogenic
7. dominant lethal

8. spindle disturbances
9. chromosomal aberrations (sister chromatid exchange, recombinogenic, synaptonemal complex irregularities)

ACR Effect on Mitosis (see publication provided for details): We have previously investigated the effects of ACR on the division of cells and the movement of chromosomes. Cultured cells (human fibrosarcoma HT 1080) were used as the model due to high rate of division and availability. The generic nature of mitosis would predict comparable results in other mammalian, as well as human, cells. Addition of colchicine, a known MT poison used as a positive control in the study, blocked mitosis; the condensed chromosomes were randomly dispersed throughout the cytoplasm and immunofluorescence demonstrated the lack of a mitotic spindle, consistent with the known MT disassembly action of this agent. Inclusion of ACR in the incubation media for 4 hours increased the number of cells that did not progress through mitosis (see accompanying paper). However, in contrast to the dispersed chromosomes seen with colchicine, the condensed chromosomes congressed to the metaphase plate but did not separate and progress normally through anaphase. The block in mitosis was dose-dependent within the 1-10mM dose range, significant differences were observed beginning with 5mM ACR. Immunofluorescence demonstrated normal-appearing spindles, except at the highest dosage where the spindles were distorted in shape. Rather than appearing as a symmetric spindle, the spindles were twisted in the long axis and unsymmetrical. The meaning of this was unclear at the time, but will be discussed later in light of new observations of motor protein involvement in spindle stabilization. Morphometry of the spindles did not reveal any significant difference in distances between the poles of the spindle.

Other reports support our finding by observing delays in the cell cycle and slowing of mitosis. Some have reported multipolar spindles and spindle disturbances. The aneuploidy and polyploidy can arise from improper segregation or failure to separate chromosomes during mitosis. The presence of micronuclei, especially those that also possess the kinetochore can arise from improper progression through mitosis. We consider these findings critical to understanding mechanisms of carcinogenicity of ACR; however, the cell biology of the motor proteins is still an emerging field.

The phenotypes associated with the mitotic block caused by acrylamide suggest possible motor targets of action. The inability of acrylamide treated cells to move their chromosomes to the poles imply that an anaphase A, minus-end directed motor is being inhibited. The lack of effect of acrylamide on the lengthening of the spindle does not support the involvement of anaphase B motors. However, the spindle malformations observed at high acrylamide doses indicate some involvement of these motor types as well. The differential effects may be the result of different dose responses for kinesin motor family members and/or differential redundancies or quantities of each family member. In addition, the occurrence of multiple spindle poles in ACR treated cells suggests an effect on spindle stabilizing proteins. Previous work has identified members of each of these potential motor targets for ACR (Sperry and Zhao, 1996)

Materials and Methods:

(Brief description -technical details of experimental protocols have not been included for brevity) These experiments are based upon well-established protocols for cloning (Ausubel et al., 1998), gene expression of motor proteins (Saxton, 1994), and motor activity assays (Wagner et al., 1991) (Chandra et al., 1993)). Dr. Ann Sperry of East Carolina University isolated and characterized the full-length gene for the motor proteins by hybridization to find identical sequence in a DNA library. She sequenced individual DNA isolates to confirm gene identity and length. Selected sequences were then incorporated into the appropriate vector for subsequent expression of the motor protein in bacterial cells. Dr. Sickles optimized the conditions for expression and developed and optimized functional assays for each KRP and then utilized these functional assays to measure the effect of acrylamide upon each function. Details of the functional assays are given in the results section.

Results:

A. KRP 1 Cloning & Sequencing

Twelve potential positives were identified in the initial screen using KRP1 head domain probe (Sperry and Zhao, 1996). The insert size for each clone was determined by PCR using oligonucleotide primers to either the vector sequences flanking the cloning site or the vector and head domain sequences. One clone was identified with an insert size of about 2 kb, sufficient to encode KIFC1, a related motor from mouse, and was further purified by two rounds of infection, plating, and hybridization to the KRP1 head probe.

The KIFC5A cDNA was sequenced, in both directions, with overlapping oligonucleotide primers either directly from the phage DNA or after subcloning into Bluescript plasmid, using the fmol sequencing kit from Promega. Alignments were also generated against sequences in the Genbank databases using the National Center for Biotechnology Information (NCBI) Blast server. This sequence is reported in Navolanic and Sperry (2000) and is in the database.

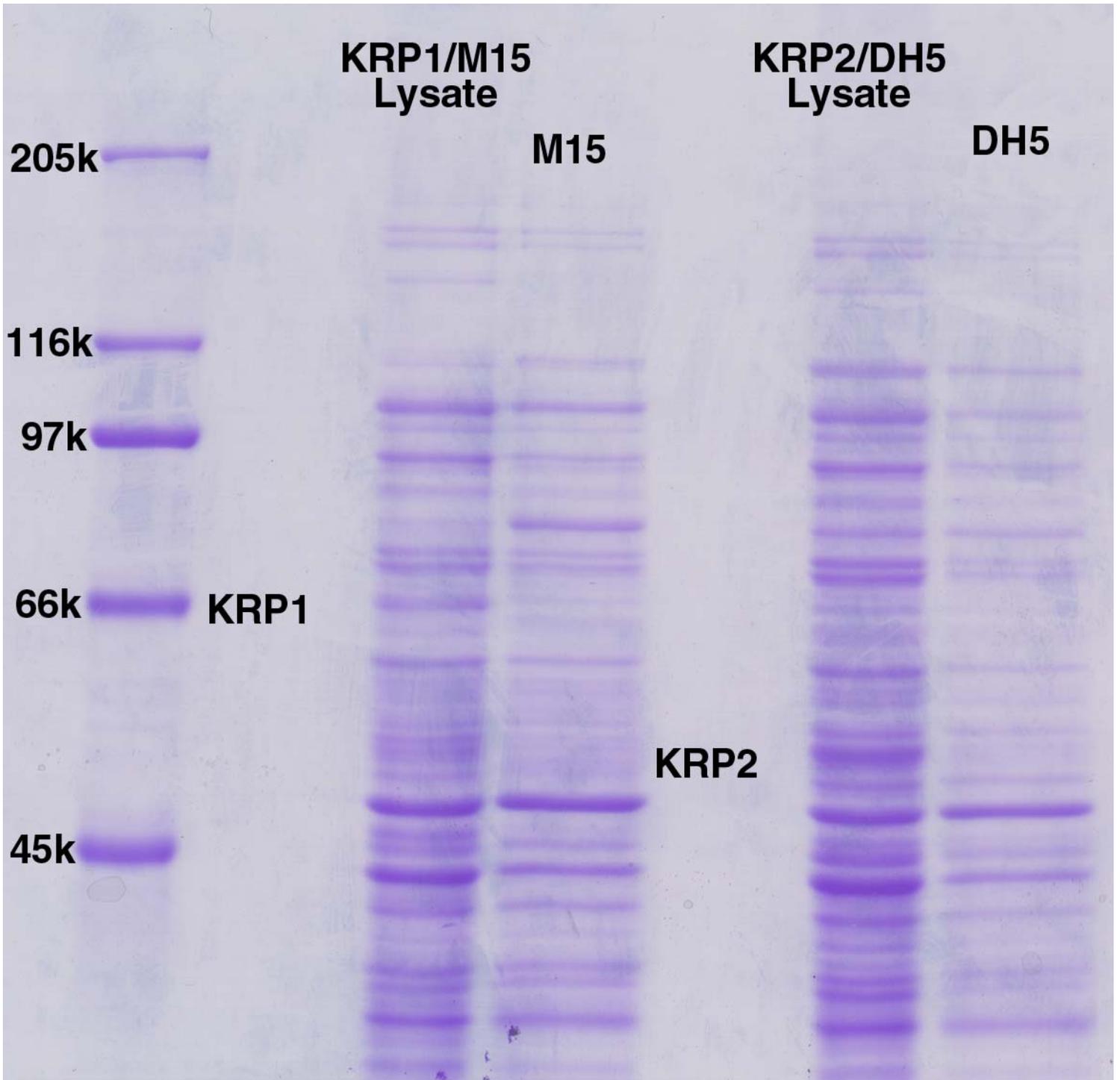
Analysis of the sequence showed that KRP1 is related to motors involved in stabilizing the spindle pole including xenopus **XCTK2**, drosophila **ncd**, and hamster **CHO2**. All are C-terminal motors (therefore may move toward MT minus ends). Comparison to KIFC1 (at first thought to be identical) showed that KRP1 was a closely related isoform (hence the name change). KRP1 is identical to KIFC1 except for the insertion of two sequence blocks (120 nt and 60nt) in the tail domain. This is interesting of course because the tail domain probably attaches to cargo (other MTs).

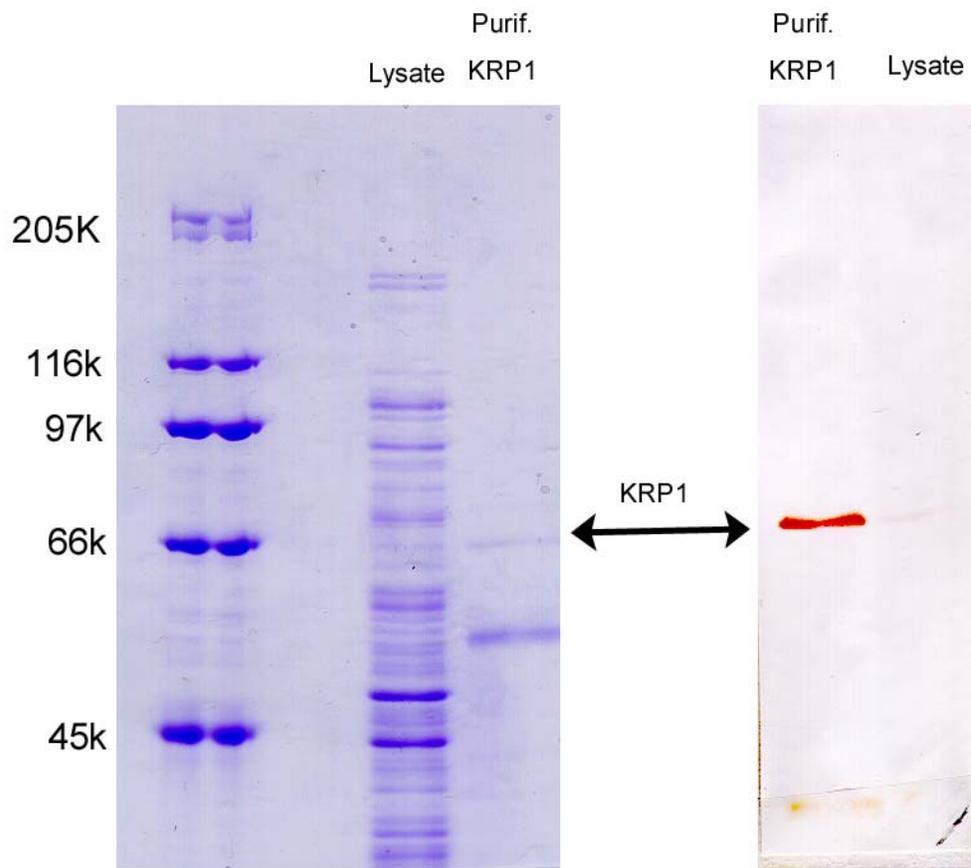
The sequence analysis of KRP1 and comparison to KIFC1 lead us to do RT-PCR experiments using primers to the divergent tail domain to see if there were other isoforms. We found two more, B and C isoforms, which confirms the 4 found by genetic mapping. Fluorescent antibody staining with an antibody (provided by Dr. Ryoko Kuriyama) that recognizes CHO2, KRP1 and the other isoforms to localize these proteins in male germ cells found these motors in multiple microtubule complexes in spermatogenesis (spindle,

manchette, flagella). This is consistent with a common microtubule bundling activity in these structures.

B. Expression and Optimization of KRP1

KRP1 was cloned into the bacterial expression vector QE31 and expressed as a fusion protein to 6HIS tag. The transfected bacteria capable of expressing KRP1 (M15) were cultured numerous times to identify the optimal conditions for protein expression. Modifications in concentration of IPTG, temperature of induction, time of induction, time of initial growth were all modified. The optimal conditions appear to be growth overnight in large culture flasks at 37C with induction of kinesin expression with IPTG (0.2mg/ml) for 4 hours at room temperature. We repeatedly expressed the KRP1 at 37C and were able to obtain a protein that had the proper molecular weight and reacted with the His 6 antibodies (reactive to the engineered 6 histidine residues on the cloned protein). In addition, this protein was reactive with the sea urchin kinesin (SUK4) antibody that recognizes the common motor domain of kinesin proteins. We were encouraged that we had an active protein and repeatedly attempted microtubule motility assays attempting to develop a functional assay by which the inhibition by acrylamide could be measured. Numerous modifications of the motility assay were attempted including variation of KRP1 concentration, multiple adsorptions, addition of His6 antibodies to enhance adhesion, addition of various binding agents and buffer systems to enhance activity. Unfortunately, none of these worked. We finally began expression at room temperature that enhances the quality of protein processing, folding and obtaining the proper quaternary structure. We were able to express the same protein as identified previously. The following gel illustrates our expression of KRP1 in M15 transfected bacteria (as well as KRP2 in DH5 α transfected bacteria – this will be addressed later). The additional protein bands in the bacterial lysates in those cultures transfected with the KRP1-possessing vectors and at the expected molecular weights are identified.





The above gel and Western blot demonstrates the expression of KRP1. The first lane of the gel are Sigma high molecular weight markers, the second lane was left open. The third lane is the protein profile expressed by the M15 bacteria transfected with the KRP1 clone. The fourth lane demonstrates the protein profile in the supernatant following our purification process. The latter was obtained by exposing the lysate to taxol-stabilized microtubules in the presence of AMP-PNP, the non-hydrolyzable analogue of ATP. AMP-PNP prevents the release of the KRP from the MT and they are pelleted with the microtubules at 100,000g. The pellet is resuspended and exposed to ATP and high salt concentration that results in release of the motor protein from the microtubules, subsequent centrifugation results in fairly pure KRP1. The band at 55kD is residual unpolymerized tubulin, the amount of this depends on the age of the tubulin. The Western blot (right) illustrates the KRP1 particularly in the lane loaded with purified KRP1. The protein was identified with His6 antibody, in other preparations we were able to identify this protein with SUK4 (reactive to the motor domain) as well. A slight band is observed in the lysate. This is weak due to low expression

and termination of the coloration reaction prematurely due to the high concentration in the lane with purified KRP1.

C. KRP 1 Functional Assay

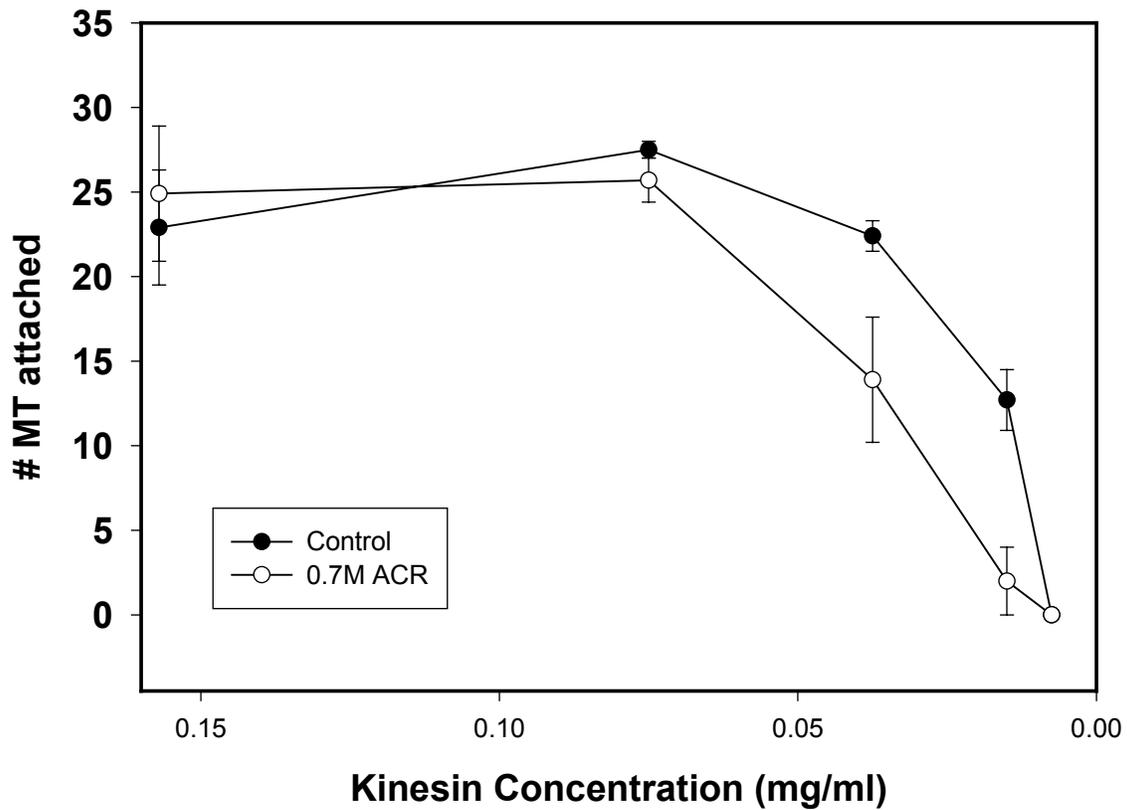
Our initial attempts in developing a functional assay for KRP1 activity were use of a standard microtubule motility assay. We had no success with this approach. As stated above, numerous attempts with protein expressed at 37C never identified KRP1-based MT motility. We repeated all of the above conditions with KRP1 expressed at room temperature. However, a constant observation was the attachment of MT to the glass coverslip as well as formation of bundled MT in the solution above the coverslip, suggesting the ability to crosslink and bundle MT. This activity is consistent with the function of a homologous family of kinesin known to bundle MT. To determine whether this was KRP1 related, we made serial dilutions prior to absorption of the KRP onto the coverslips and then quantitated the number of bound microtubules. At full concentrations of KRP1, the predominant feature was the presence of bundled MT in solution above the coverslip. With dilution, the number of MT attached to the coverslip increased and then fell off to zero at concentrations 1/10 to 1/20 of the purified preparations (original protein concentrations of 0.098-0.199 mg/ml). See control curve in following figure for actual data. This was similar to our previous observations of decreased microtubule motility following dilution of the bovine brain kinesin. We used this “attachment activity” to determine whether acrylamide has any effect on this kinesin related protein.

D. Quantitation of Acrylamide Effect on KRP1

Fresh purified KRP1 was incubated with 0.7M ACR (the concentration used to produce neurotoxicity following multiple exposures) at 37C for 20 minutes prior to adsorption of the KRP1, at various dilutions onto the glass coverslips. MT in solution and attached to the coverslip were viewed with video-enhanced differential interference microscopy using a Zeiss inverted scope equipped with Newvicon camera, Hamamatsu Argus 20 microcomputer and Hamamatsu high resolution monitor. Pictures of five microscopic fields obtained at random were printed for each dilution, with and without acrylamide preincubation. An observer blind to the experimental condition counted the average number of microtubules attached to the coverslips in the five fields of view. The assay was repeated with fresh KRP1 on three different occasions.

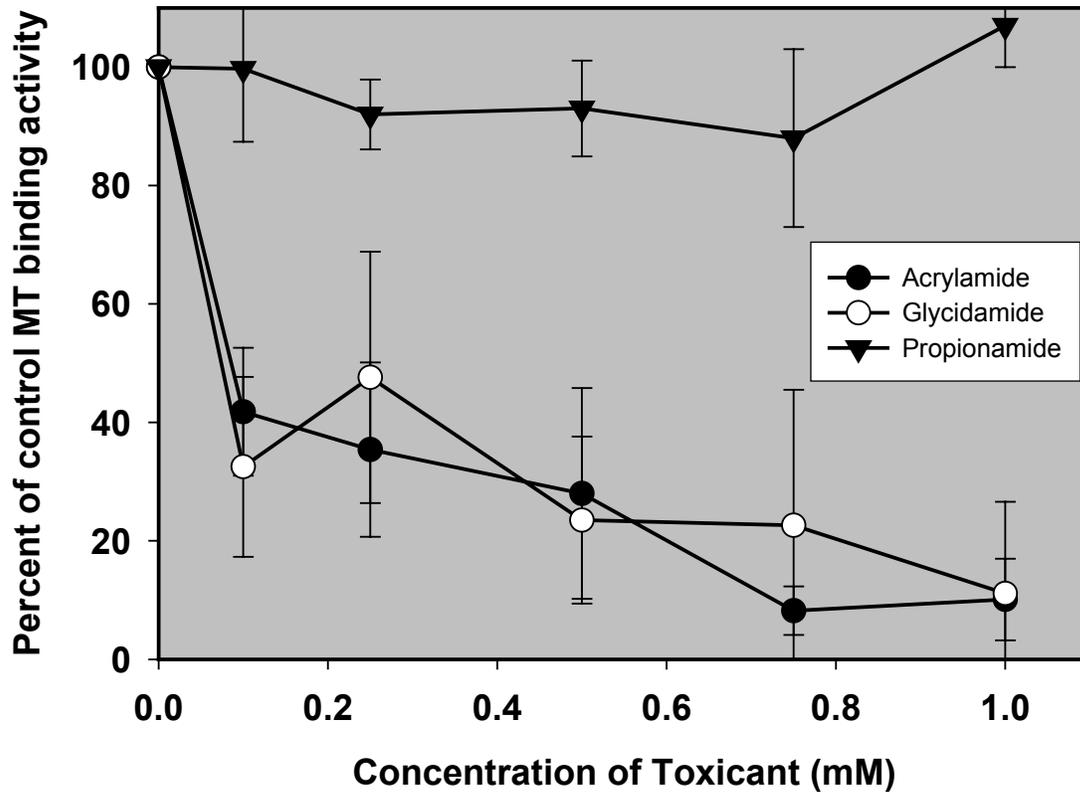
The following figure demonstrates the number of microtubules observed to be bound to the coverslip at various concentrations of KRP1 with and without exposure to ACR. Note that as the KRP concentration decreases that the number of bound MT declines. This activity was inhibited rather dramatically by acrylamide. For example, at absorption concentrations of 0.0375 mg/ml acrylamide inhibited the binding function of KRP1 by 38% and at half this concentration by 84%. The lack of effect at high concentrations of KRP1 and the increasing magnitude of effect at low concentrations is the result in differential sensitivity of the assay at varying KRP1 concentrations. This was previously observed in the microtubule motility assays. Since a minor quantity of KRP1 is necessary to bind the MT to the coverslip (e.g. single molecules); one must titer the kinesin concentration to obtain an assay that is sufficiently sensitive to detect toxicant inhibition. Otherwise almost complete inhibition of kinesin activity would be necessary to identify functional inhibition. This

criteria was also observed in our previous studies using bovine brain kinesin. The dose-response of inhibition of KRP1 was almost identical to that of bovine brain kinesin. Note that these proteins are inhibited at the lowest concentration of acrylamide than any other reported protein inhibitions.



The following figure demonstrates the dose response of effect of acrylamide, glycidamide or non-neurotoxic propionamide on MT attachment by KRP1. In these experiments, KRP1 was incubated with 0.1 to 1.0M of each compound for 20 minutes at 37C. KRP1 was diluted to 0.4 mg/ml for absorption for all cases. There is a dose-dependent inhibition of KRP1 binding activity from 0.1 to 1M with both acrylamide and glycidamide. Although not shown on the figure, all doses of each compound are significantly different from control. There is no difference between acrylamide and glycidamide. Propionamide had no effect at any dose.

Effects of Acrylamide, Glycidamide or Propionamide on KRP1A



E. Interpretation

KRP1 is significantly and equally inhibited by acrylamide and glycidamide. The dose-response is extremely similar to the dose response of inhibition of bovine brain kinesin. KRP1 is a carboxyterminal motor. Some relatives of the protein have been shown to move toward the minus ends of MT but others have had difficulty showing motility with their protein. The function of this group of motors seems to be stabilization of the poles of the mitotic spindle. Interruption of function of this family of kinesin motors results in decreased number of spindles formed and/or malformed spindles (twisted). We have observed considerable bundling of MT in the solutions containing KRP1; attachment of MT to the coverslip is also observed. While bundling might be assayable using diffraction of light in a spectrophotometer, we elected to assay the protein function by the latter "attachment" function. This activity is directly related to the quantity of KRP1 in solution. The reduced number of bound microtubules at the highest concentration of KRP1 is presumed due to a reduction in available MT due to bundling that was visually observed in the solutions above the coverslip (by adjusting the focal plane) The attachment function of KRP was inhibited by acrylamide and glycidamide. This is specific for the parent acrylamide and the toxic metabolite glycidamide as propionamide at doses of 1.0M (ten times the lowest effective dose of acrylamide and glycidamide) has no effect. Therefore, one would predict that ACR would alter the appearance of the mitotic spindle at relatively high doses. In our previous

work, 10mM ACR resulted in mitotic spindles that were distorted in appearance. We attribute the inhibition of proteins such as KRP1 to this alteration in spindle morphology. Therefore, just as nervous system kinesin is inhibited by acrylamide, so is the kinesin related protein 1; and at similar doses. Therefore, inhibition of KRP1 is similar in sensitivity leading one to predict similar dose-response effects in dividing cells as the neurotoxicity. Factors that may affect this correlation include redundancy in motor proteins, the relative safety factors for each KRP (i.e. the excess in quantity versus the amount required for normal function), and the sensitivity of the assays.

KRP2

A. Cloning & Sequencing

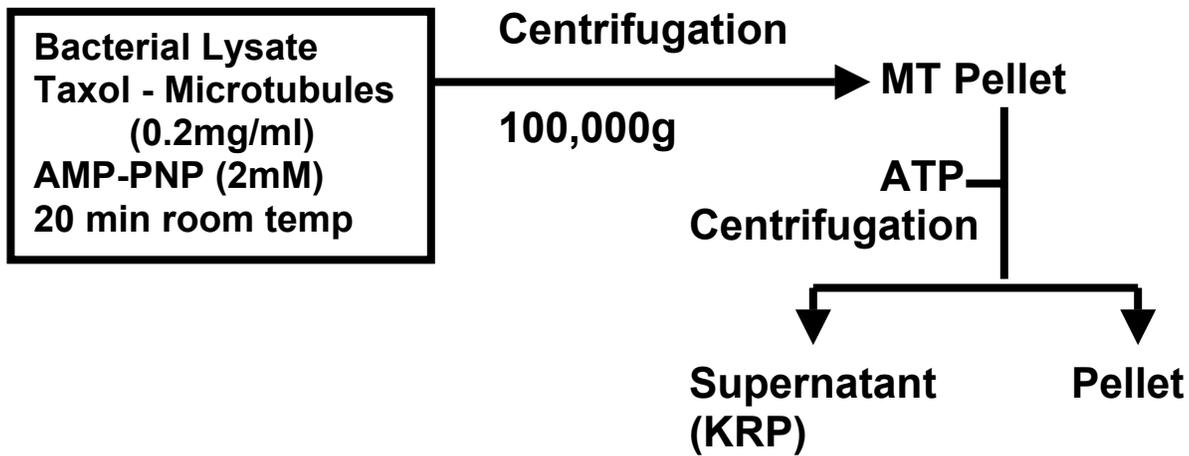
KRP2 was cloned in 1992-93. The sequence may be found in Sperry and Zhao, '96 Mol. Biol. Cell. The full length KRP2 was cloned into aQE vector as was the head domain clone. The head domain clone is actually extends outside what is considered the motor domain and is 443 aa long. It consists of the 1.3kb SacI fragment from the middle of the cDNA. It encodes an about 50 kD protein.

KRP2 is homologous to hamster **MCAK** and xenopus **XKCM1**. These proteins have been shown to be microtubule-destabilizing proteins and are not thought to translocate. Electron microscopy has demonstrated **XKCM1** bound to the ends of microtubules where it makes the protofilaments peel back from the microtubule. MCAK is found at the centromere during mitosis.

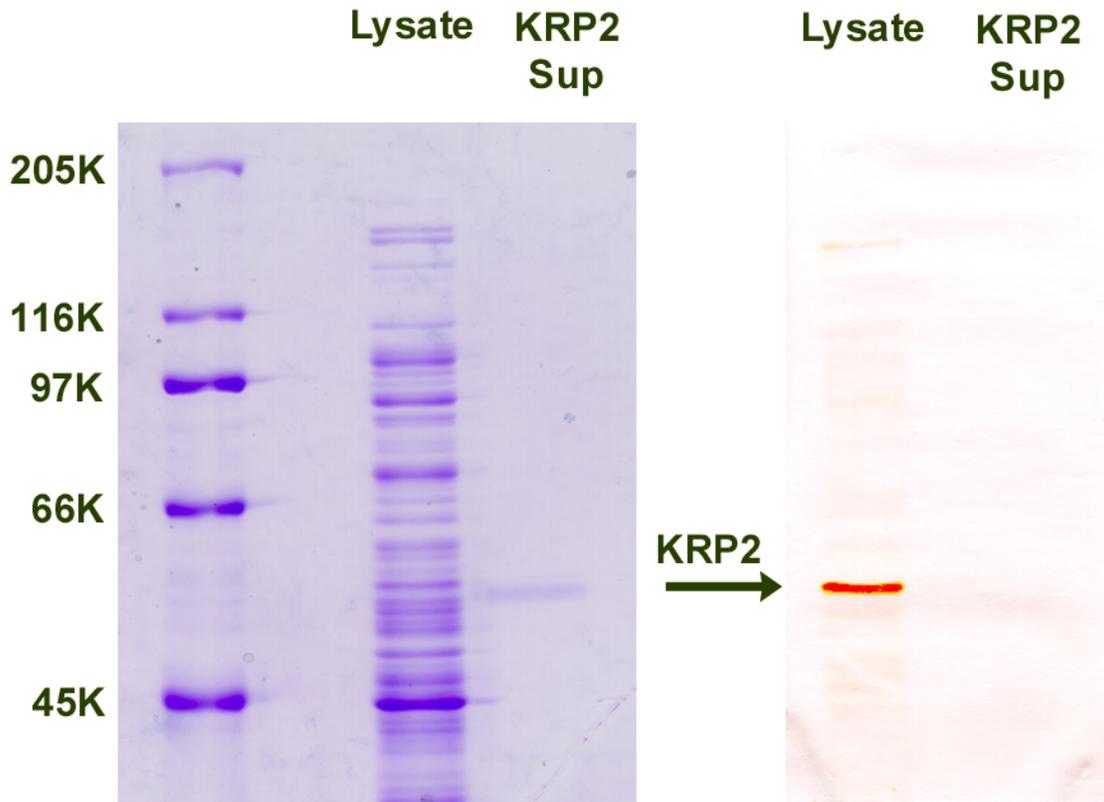
Dr. Sperry believes this is a testis isoform. They conducted microtubule pelleting assays with expressed protein, taxol, and AMPPNP and found that KRP2 does not pellet with MT and when KRP2 is present, tubulin does not pellet, it is soluble. Discussion with Claire Walczak, who studies XKCM1, indicated that they made the same observation when they initially studied XKCM1. As demonstrated below in the purification section, we have made the same observation. Dr. Sperry has done some FISH with KRP2 in the testes and their results support that reported in Sperry and Zhao: KRP2 is found in later stage cells and not only in dividing cells. This may indicate that KRP2 has a role in destabilizing microtubule complexes in spermiogenesis as well as meiosis. They made some polyclonal antibodies against the tail of KRP2 but did not find any staining in the seminiferous tubules.

B. Optimization of Expression

We experience considerable difficulty with what we thought was expression of this protein. As with KRP1, we modified the inducer concentration, the growth of the bacteria, the length of bacterial growth and protein expression. Most of the comments related to KRP1 expression are applicable with KRP2. We were able to produce KRP2 as demonstrated below. This sample was produced by expression at 37C for 4 hours with 0.4 mM IDPG. However, as explained below, we were not successful in purifying the protein using the following MT binding procedure.



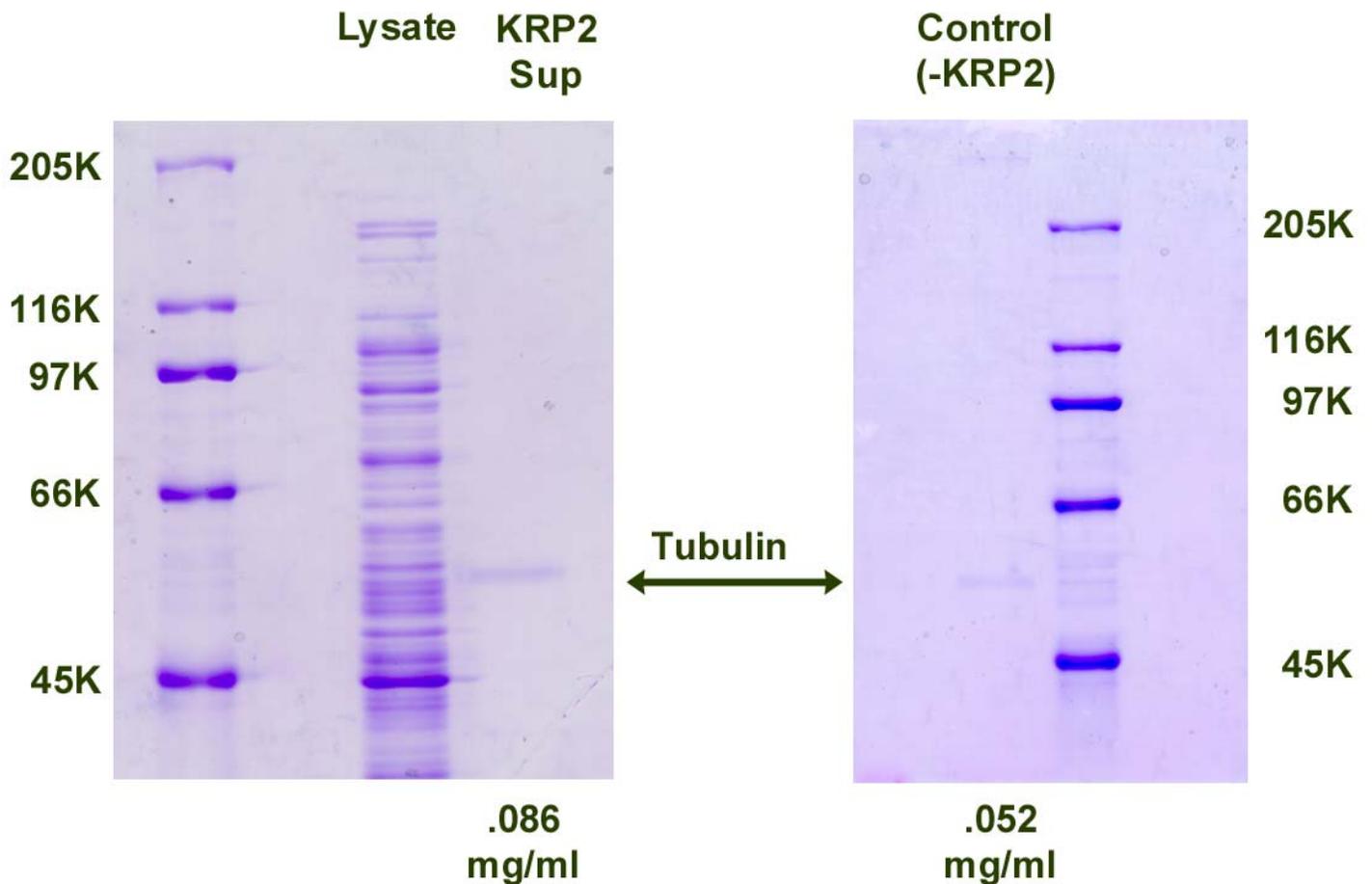
With further experimentation it became obvious that the failure in purification of the protein was due to the destabilizing function of the protein.



The gel and Western demonstrate the expression and purification of the protein. The first lane are molecular weight markers, the second is empty, the third is the protein profile of the bacterial lysate after KRP2 expression, the 4th lane is the supernatant following the MT affinity purification. The band observed here is most likely tubulin and not KRP2, which has a similar molecular weight as tubulin (see following details). The Western blot shows the presence of KRP2 in high concentration in the lysate but not in the supernatant after the MT affinity purification. This indicates that KRP2 was not purified.

C. Development of Functional Assay

Since we could not purify the protein with the microtubule affinity assay and there was good expression of KRP2, we attempted motility assays with the bacterial lysates. All of the various permutations in concentrations of each component of the motility system (identified above in the KRP1 section) were attempted without success. Microtubules were never observed to even attach or locomote. We attempted expression of KRP2 at room temperature, necessary in some laboratories to obtain motility with kinesin related proteins, with success. We made the following observations that allowed for development of a functional assay.

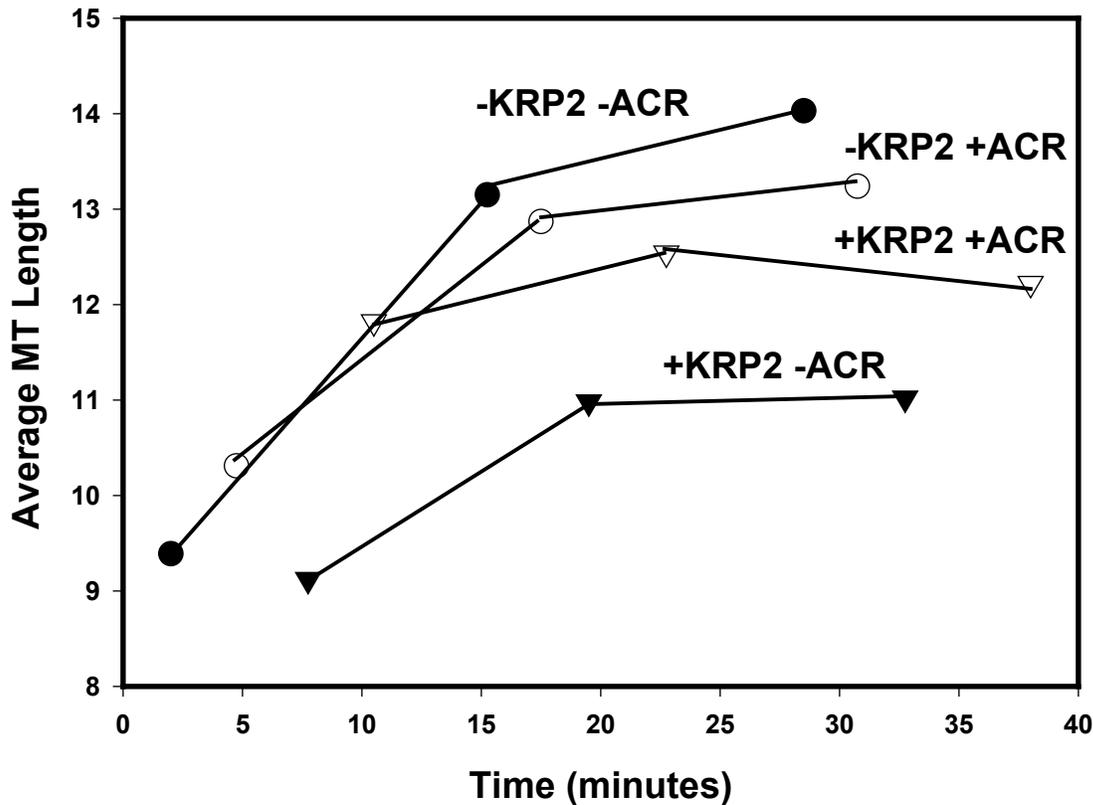


The above figure demonstrates the effect of KRP2 on tubulin during the attempted purification of the motor protein. In the left gel, the first lane are markers, the second lane was empty, the third demonstrates the proteins of the bacterial lysate following KRP2 expression and the last lane is the supernatant following the MT affinity purification step. Note that only one band is present, which has the molecular weight of tubulin. We have noticed that this band is darker than usual whenever purifying KRP2 and suspected this was a result of depolymerization of some microtubules. To confirm this observation the purification assay was run in the absence of KRP2, the results are shown in the gel on the

right (above). The right gel has two lanes; the far right are the markers, the left lane is the supernatant obtained from the MT affinity purification in the absence of KRP2. The supernatant without KRP2 has less tubulin. Quantitation of the protein in the supernatant for each assay is shown below each lane and clearly identifies a higher amount of soluble tubulin whenever KRP2 is present. We have developed several functional assays based on these observations.

Our first attempt to develop a functional assay, using the observations above, was a DIC-microscopy based assay measuring the average length of MT over time with and without the addition of KRP2 and with or without the addition of 0.7M acrylamide. The following figure illustrates the results of a single experiment. In the control (-KRP2 -ACR) the length of MT grew over a time period of 30 minutes, which is consistent with normal assembly of tubulin in solution. With the addition of KRP2 only (+KRP2 -ACR) the average length of MT was less than control, consistent with inhibited growth and/or disassembly of some MT. In the absence of KRP2 but with ACR added (-KRP2 +ACR; additional control to determine the effect of ACR alone) the growth curve was almost identical to control indicating that ACR had no effect on MT dynamics. In the presence of KRP2 and ACR (+KRP2 +ACR) the curve fell between control and the curve resulting from the presence of KRP2 but without ACR. This is consistent with an ACR-induced inhibition of KRP2 disassembly. Altering the concentrations of tubulin and KRP2 to optimize the sensitivity of the assay could be done. However, this approach was technically challenging due to constantly changing oil-immersion coverslips on the microscope, adjusting focus and lighting, resetting the computer algorithms. It was therefore difficult to assure comparable sampling of multiple experimental conditions on the same preparation. This produced variability in sampling times (note different temporal points below), loss of sample points or variations in data due to utilization of different preparations. Therefore, we elected to pursue a different assay.

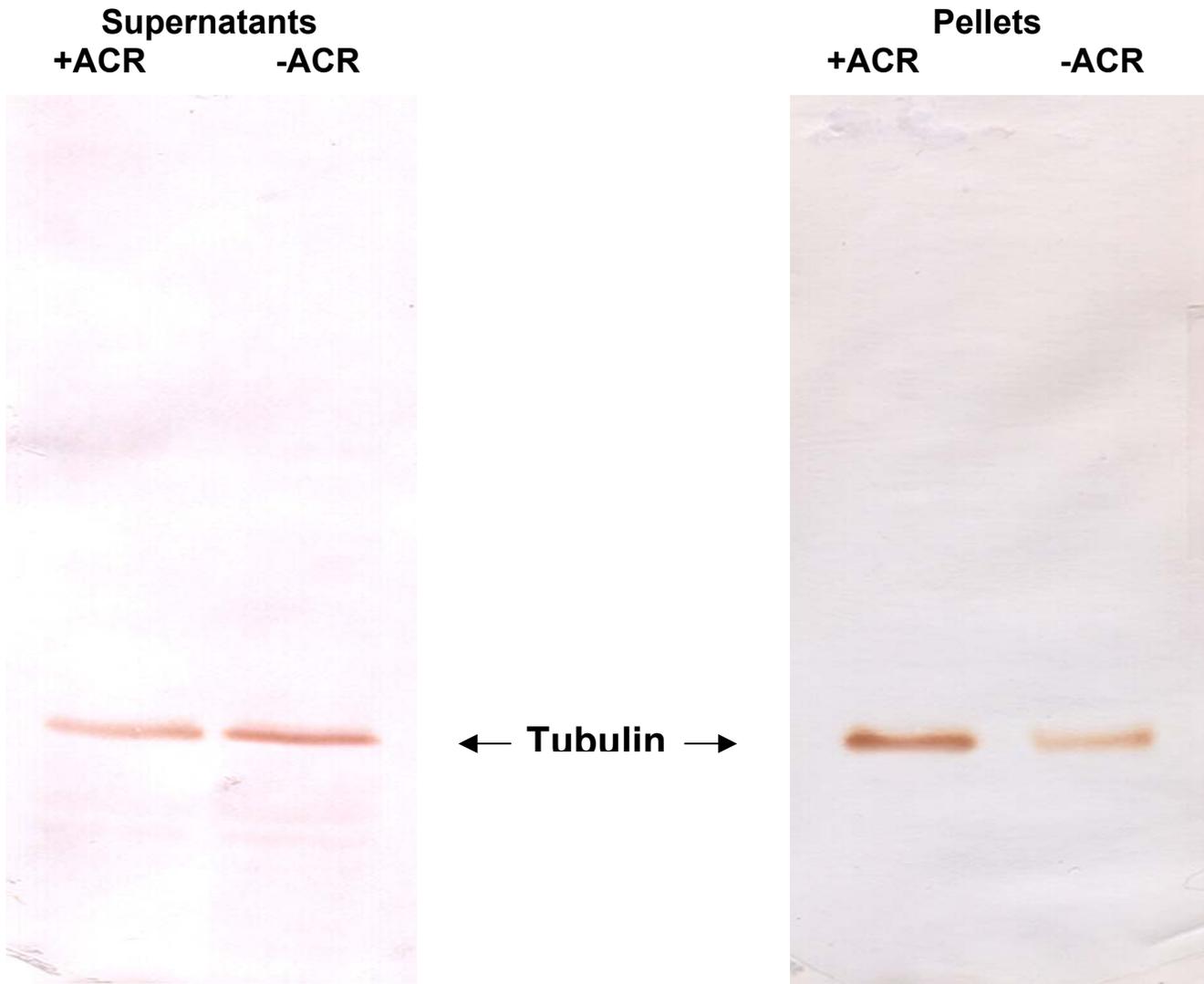
Acrylamide Inhibition of KRP2-induced MT disassembly



Subsequently we determined the effects of KRP2 on microtubules biochemically by analyzing the changes in soluble and polymerized tubulin caused by KRP via Western blots. KRP2 lysate was preincubated with and without 0.7mM ACR for 20 minutes at 37°C. After incubation we added 3 mM MgATP, taxol-stabilized MT @ 0.05 mg/ml final concentration and incubated for 10 min at RT. The solution was spun at 24K rpm for 20 min in an SW40Ti rotor. The pellets and supernatants were run on a 7.5% SDS-PAGE gel and stained with Coomassie blue or transferred for Western blot analysis of tubulin. The pellets were resuspended into the original volume; the supernatants required concentration to 1/3 original volume using a Centricon 10 for 2 hr at RT. The nitrocellulose was incubated with monoclonal anti- α tubulin (Sigma #T5168, clone B-5-1-2, mouse ascites fluid at 1:2000 dilution for 2 hr at 37°C. The secondary antibody was a Bio-Rad goat anti-mouse IgG HRP conjugated at 1:2000 dilution for 1 hr at RT. The blots were developed with DAB (50 mg/100ml; half this concentration for the supernatants) using 10ul of 30% H₂O₂. The following figure shows the amount of tubulin in the final supernatants and pellets with and without ACR. ACR preincubation with KRP2 reduced the quantity of tubulin in the supernatant and increased the quantity of tubulin in the pellet. Therefore, exposure to

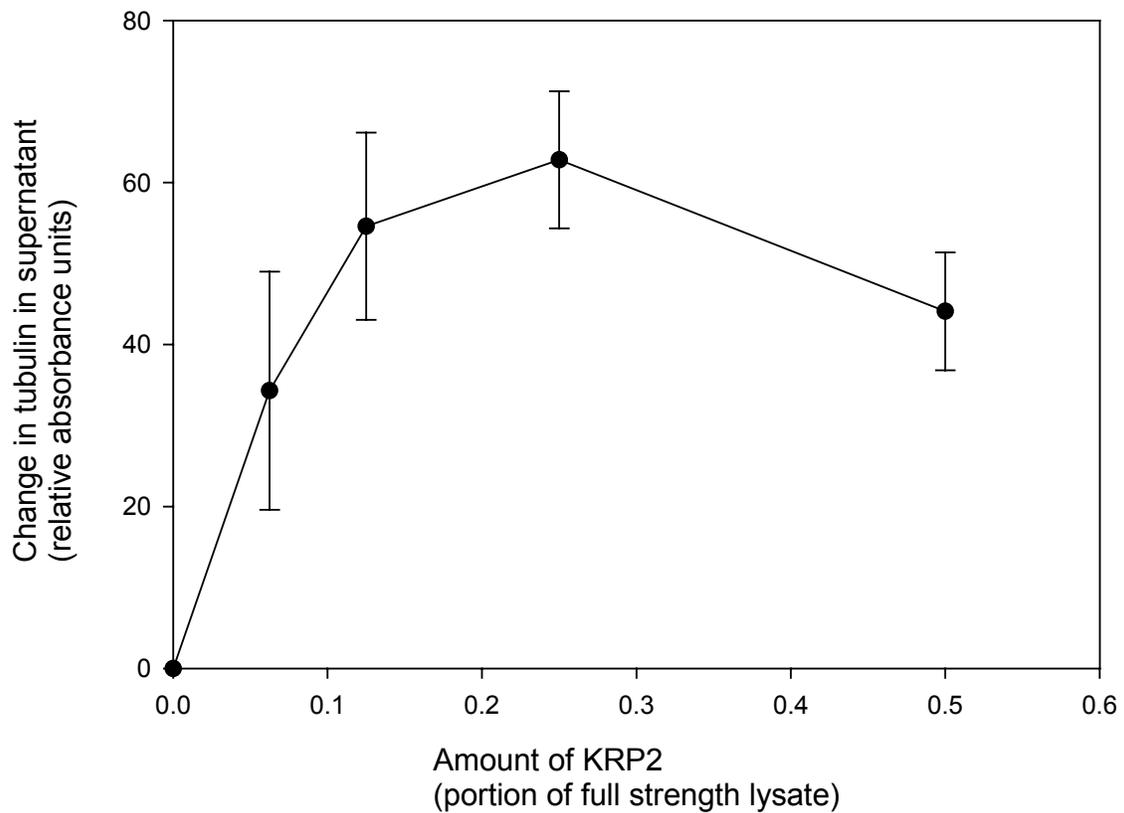
0.7ACR shifted the tubulin content in the favor of the pellets. This is the anticipated result for ACR inhibition of KRP2- induced MT disassembly.

Acrylamide Inhibition of KRP2- Induced MT disassembly- blots



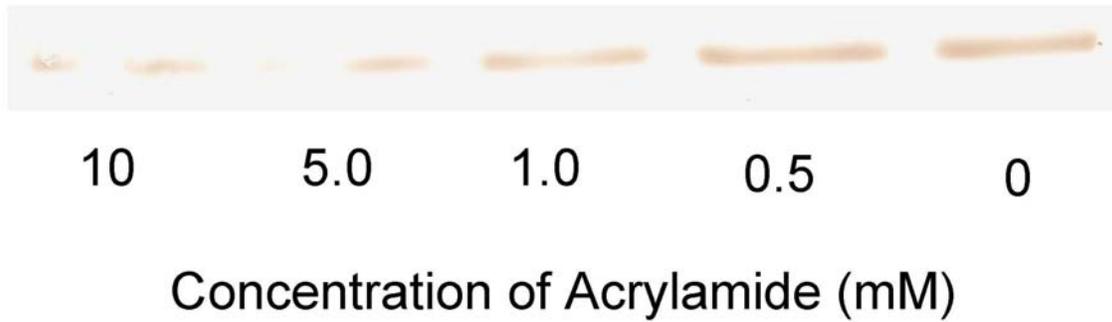
To develop a quantitative assay suitable for detecting a toxicant-induced reduction in KRP2 MT disassembly function, the experiment was repeated using taxol-stabilized MT @ 0.05 mg/ml final concentration and varying the KRP2 concentration by serial dilutions of the bacterial lysate. The following figure shows that amount of tubulin in the supernatant (soluble or disassembled) increased with an increased concentration of KRP2. We concluded that we could measure the activity of KRP2 using this approach. The linear range of the curve was determined to be from 0 to 1/8 (or .125) of the bacterial lysate as shown in the figure below.

Effects of KRP2 concentration on the quantity of disassemble tubulin

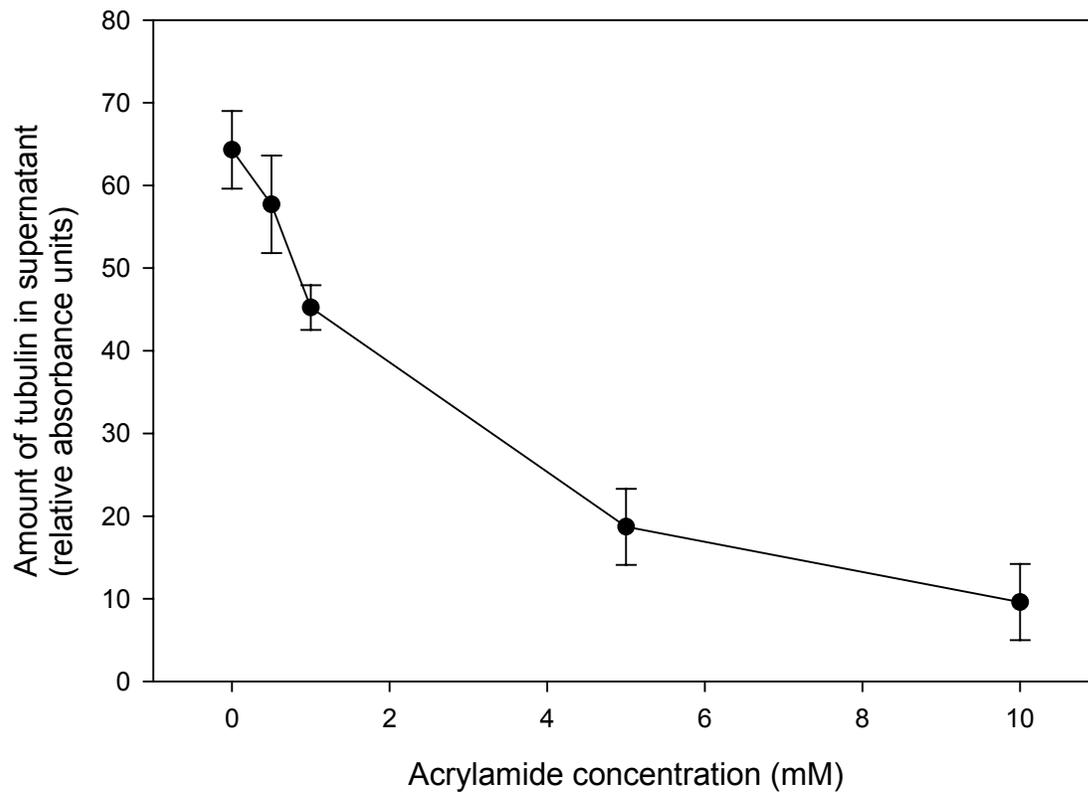


Therefore, a serial dilution of 1/8 of the bacterial lysate was used. Preincubation of this lysate with 0.1 to 1.0mM acrylamide for 10 minutes at RT was followed by mixing with taxol-stabilized MT in buffer and processing as before and analyzing via Western blots the quantity of tubulin in the supernatant fractions. The following figure demonstrates the effect of acrylamide from a representative sample experiment.

KRP2 - alpha-Tubulin Ab



The average quantity of tubulin in the supernatant fractions (depolymerized by KRP2) from the experiments ran in triplicate is shown in the figure below. Acrylamide preincubation with KRP2 produced a dose-dependent reduction in the quantity of tubulin in the supernatant, consistent with inhibition of the depolymerizing activity of this enzyme. However, the dose-range for this effect was higher than previously observed for either bovine brain kinesin or KRP1, the lowest statistically significant concentration was 1mM. This is 10 fold higher than the lowest effective concentration of acrylamide on KRP1. These experiments are currently being repeated to determine the effects of glycidamide and propionamide on KRP2 function.



D. Interpretation

KRP2 is a member of the internal motor subfamily that destabilizes the ends of microtubules (Desai, et al., 1999). Kin I members are microtubule destabilizing enzymes (Cell 96: 69-78). These motors are not conventional motors and have not been shown to glide microtubules (except in one suspect case). KRP2 is closely related to mitosis centromere associated kinesin (MCAK). Another kinesin related protein, called XKCM1, has been identified recently from *Xenopus* with sufficient homology to be placed in the same subfamily with KRP2 (Walczak et al., 1996). Dr. Walczak has had similar difficulty with purification and have identified a MT disassembly function for their protein (Walczak et al., 1996). In fact, XKCM1 has been demonstrated by EM to binds to MT ends and effectively strip individual protofilaments from the tubule. The combination of location and function

suggest this kinesin may be associated with MT disassembly which occurs during anaphase when the chromosomes segregate into the daughter cells. The results from our study indicate that KRP2 is inhibited by ACR in the dose range of 1-10mM. This is very similar to the dose-range of ACR that blocked mitosis in HT1080 cells in vitro with retention of chromosomes at the metaphase plate. This is consistent with the concept that microtubule – depolymerizing kinesins may be inhibited by acrylamide, which results in failure of the migration of chromosomes from the metaphase plate. Lower doses may affect the failure of single chromosomes to migrate properly resulting in the observed aneuploidy following ACR exposure. Therefore, the inhibition of this KRP may represent a mechanism by which action on dividing cells can produce abnormal genetic content, carcinogenicity or altered cell cycle events.