

Tubulin Interaction with Kinetochore Proteins: Analysis by In Vitro Assembly and Chemical Cross-linking

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Abstract. The sera from patients with the CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) variation of the autoimmune disease scleroderma contain autoantibodies that specifically recognize the kinetochore by immunofluorescence. Two major antigens of molecular masses 18 and 80 kD are consistently identified by Western blotting of proteins of isolated chromosomes using CREST sera. In this paper, the possible roles that these two proteins play in the interaction of metaphase chromosomes with tubulin and microtubules are examined using two different procedures. In one set of experiments, Chinese hamster ovary (CHO) chromosomes were extracted with 1–2 M NaCl before incubating with phosphocellulose-purified tubulin under in vitro microtubule assembly conditions. After this treatment, the kinetochores of the residual chromosome scaffolds can still initiate the in vitro assembly of microtubules. Immunoblots of the chromosome scaffold proteins demonstrate that the 18-kD protein has been solubilized by the 1–2 M NaCl extraction, suggesting that this protein is not essential for microtubule assembly at the kinetochore. In a second approach,

tubulin was covalently cross-linked to kinetochores of CHO chromosomes using the reversible cross-linking reagent dithiobis (succinimidyl propionate). After DNase I digestion, the chromosomes were solubilized and subjected to anti-tubulin affinity chromatography. Tubulin-kinetochore protein complexes were specifically eluted and analyzed by PAGE and immunoblotting with scleroderma CREST serum. Only a small number of proteins were eluted from the anti-tubulin affinity column as shown by Coomassie Blue-stained gels. In addition to tubulin, an 80-kD polypeptide, bands at 110 and 24 kD, as well as a faint band at 54 kD, can be resolved. Several minor bands can also be seen in silver-stained gels. The 80-kD protein band from whole metaphase chromosomes reacted with scleroderma CREST serum by immunoblotting and therefore probably represents the major centromere antigen CENP-B. This report provides evidence for a specific protein complex on metaphase chromosomes that is contiguous with kinetochore-bound tubulin and may be involved in microtubule-kinetochore interactions during mitosis.

THE kinetochore is a specialized structure, located at the primary constriction (centromere) of metaphase chromosomes, which functions to attach chromosomes to the mitotic spindle. The kinetochore also serves as a focal point through which mitotic forces work to pull chromosomes to the poles at anaphase. Recent studies of mitosis in living cells using immunogold labeling suggest that dynamic assembly and disassembly of tubulin subunits takes place at the plus ends of microtubules, which are attached to the kinetochore (Mitchison et al., 1986). In addition, mitotic motors or force-producing molecules, which translocate chromosomes along stable microtubules in the anaphase spindle (Gorbsky et al., 1987), may actually be located within the structure of the kinetochore (Mitchison et al., 1986; Mitchison and Kirschner, 1985a, b; Gorbsky et al., 1987). Before the mechanisms of such complex functions and interactions can be determined, however, more information

is needed concerning the molecular organization of the kinetochore-microtubule interface.

Although ultrastructural features of the kinetochore have been extensively investigated in a variety of cell types, relatively little information is available on the molecular composition of this important structure. Using human autoantisera, from patients with scleroderma calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia (CREST)¹ which bind specifically to the centromere/kinetochore region (Moroi et al., 1980; Brenner et al., 1981), several polypeptides have been identified with molecular masses ranging from 17 to 140 kD (Cox et al., 1983; Ayer

1. *Abbreviations used in this paper:* CREST, calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia; DSP, dithiobis (succinimidyl propionate); PEM, 80 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, pH 7.2.

and Fritzler, 1984; Nishikai et al., 1984; Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985; McNeilage et al., 1986). One of these, an 80-kD peptide designated CENP-B (Earnshaw and Rothfield, 1985), has recently been cloned and sequenced (Earnshaw et al., 1987). Although this protein is associated with the centromere, its specific localization in the kinetochore and possible interaction with microtubules has not been firmly established.

Here two procedures were used to further characterize kinetochore proteins of mammalian chromosomes. In one, the *in vitro* assembly procedure of Mitchison and Kirschner (1985a) was used to analyze microtubule nucleation from kinetochores of isolated chromosomes after selective extraction of proteins and nucleic acids. In a second approach, proteins contiguous to kinetochore-bound tubulin were identified by chemical cross-linking with Lomant's reagent (di-thiobis[succinimidyl propionate]; DSP) according to the procedure of Tuan and Knowles (1984), as originally reported by Lomant and Fairbanks (1976). Our report provides the first direct evidence that the 80-kD CREST antigen (CENP-B) is indeed a major peptide of the kinetochore and, in combination with a protein complex, is closely associated with bound tubulin. The results of this study also suggest that another CREST antigen, a histone-like 18-kD centromeric protein (Valdivia and Brinkley, 1985; Earnshaw et al., 1984; Earnshaw and Rothfield, 1985; Palmer et al., 1987), is not associated with tubulin and is not essential for microtubule assembly at the kinetochore.

Materials and Methods

Cell Culture and Chromosome Isolation

Chinese hamster ovary (CHO) cells were grown in McCoy's 5A (Hsu's

modification) supplemented with 8% FCS. Indian muntjac cells were grown in Ham's F-10 medium supplemented with 10% FCS plus 2 mM glutamine.

For chromosome isolation, cell cultures were incubated in either 10 μ g/ml vinblastine sulfate for 12 h or 0.1 μ g/ml colcemid for 12–16 h. Mitotic cells were collected and the chromosomes isolated using the methods described by Mitchison and Kirschner (1985a). The isolated chromosomes were collected and frozen in aliquots at -80°C until use.

Chromosome Scaffold Preparation

CHO chromosomes were washed twice in 80 mM Pipes, 1 mM EGTA, 1 mM MgCl_2 , pH 7.2 (PEM), and then incubated for 20 min in 80 mM Pipes, 1 mM EGTA, 5 mM MgCl_2 , 40 μ g/ml DNase I, pH 7.2 at 4°C . After rinsing in PEM, the chromosomes were treated for 10 min at 4°C in PEM made 1 M in NaCl followed by a 20-min treatment at 4°C in PEM made 2 M in NaCl. The residual chromosome scaffolds were then rinsed twice in PEM and used immediately. For microtubule assembly experiments, the CHO chromosomes were cytocentrifuged onto poly-L-lysine-coated coverslips before DNase I digestion and salt extraction.

Tubulin Purification

Tubulin was purified from twice-cycled bovine brain microtubule protein by phosphocellulose chromatography as described by Mitchison and Kirschner (1984). The critical concentration for spontaneous assembly of this tubulin preparation was found to be 1.6 mg/ml (data not shown).

Microtubule Assembly from Kinetochores

The assay for microtubule assembly from kinetochores was essentially the same as described by Mitchison and Kirschner (1985a). Briefly, chromosomes were cytocentrifuged onto coverslips and the coverslips rinsed in PEM to remove residual sucrose. Phosphocellulose-purified bovine brain tubulin was added at 1.7–2.0 mg/ml in PEM with 0.5 mM GTP. The coverslips were incubated at 37°C for 10 min and then fixed for 3 min in 2% glutaraldehyde in PEM followed by postfixation for 5 min in -20°C MeOH. The coverslips were then processed for either anti-tubulin immunofluorescence or anti-kinetochore immunofluorescence using previously published methods (Brinkley et al., 1980, 1984). Coverslips were mounted in Hoechst dye 33258 (50 μ g/ml in 1:1 PBS/glycerol), photographed with Tri-X (Eastman Kodak Co., Rochester, NY), and developed with Acufine (Acufine,

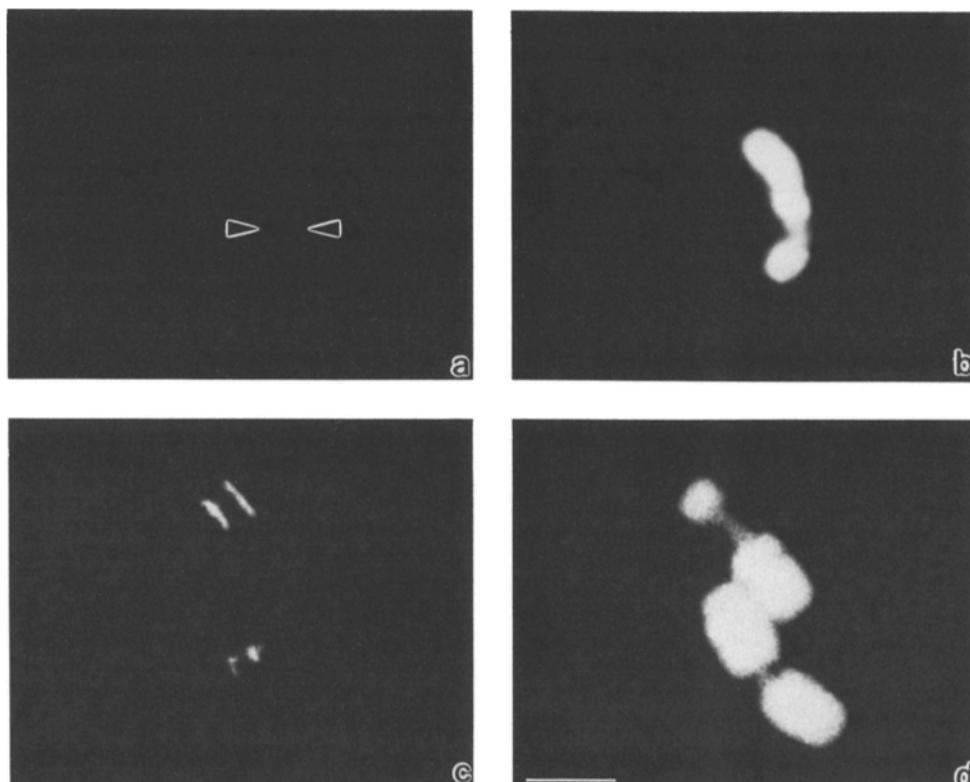


Figure 1. Indian muntjac cells were treated with vinblastine sulfate for 12 h and the chromosomes were isolated as described in the Materials and Methods section. The chromosomes were then processed for anti-tubulin immunofluorescence either before or after incubation with 1.2 mg/ml tubulin for 10 min at 37°C . (a) Anti-tubulin immunofluorescence of an Indian muntjac chromosome that had been isolated from a vinblastine sulfate-treated cell. The arrows point to the centromere region. (b) Hoechst staining of a. (c) Anti-tubulin immunofluorescence of two Indian muntjac chromosomes that have been incubated with 1.2 mg/ml tubulin for 10 min before fixation and processing for anti-tubulin immunofluorescence. (d) Hoechst staining of c. Bar, 5 μ m.

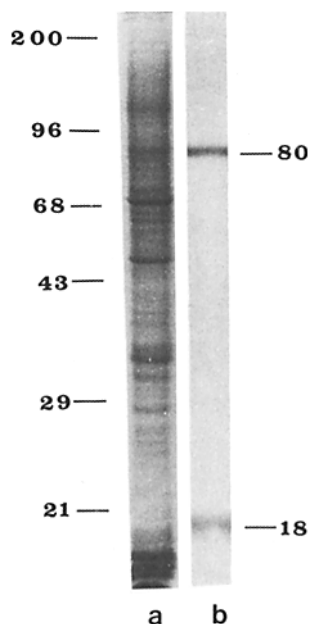


Figure 2. Characterization of scleroderma CREST serum by Western blotting. Lane *a*, a 10% SDS-PAGE of purified CHO chromosomes. Coomassie Blue staining. Lane *b*, the corresponding Western blot of lane *a*. The blot was treated with CREST serum (1:500) in PBS followed by peroxidase-labeled anti-human (1:500) in PBS. The blot was developed with 4-chloro-1-naphthol. The apparent molecular masses of the standard proteins (in kilodaltons) are indicated to the left of lane *a* and the apparent molecular masses of the two antigens recognized by the CREST serum are indicated to the right of lane *b*.

Inc., Chicago, IL). For some experiments, chromosome scaffolds on coverslips were used and microtubule assembly was assayed using the above procedure.

Tubulin Binding to Kinetochores

For binding experiments, whole chromosomes or chromosome scaffolds were incubated with 1.2 mg/ml tubulin in PEM plus 0.5 mM GTP for 10 min at 37°C. The chromosomes were then fixed and processed for immunofluorescence as described above.

Tubulin Cross-linking to Kinetochose Proteins and Purification of Tubulin Kinetochose Protein Complexes

Phosphocellulose-purified 6S brain tubulin at 2.0 mg/ml in PEM + 0.5 mM GTP was added to CHO chromosomes, and microtubules were assembled by incubating the mixture at 37°C for 10 min followed by a 10× dilution in PEM at 4°C to disassemble the microtubules. The chromosomes were pelleted and the pellet was rinsed three times in PEM. Under these conditions no assembled microtubules remained although tubulin is still bound to the kinetochores (Mitchison and Kirschner, 1985a). The chromosomes were resuspended in PEM, and DSP (Pierce Chemical Co., Rockford, IL) was added to a final concentration of 0.4 mM from a freshly made 100× stock (Lomant and Fairbanks, 1976; Tuan and Knowles, 1984). The chromosomes were incubated with the cross-linker for 10 min at 4°C, and the reaction was quenched by the addition of a 50× excess of lysine. The chromosomes were pelleted and rinsed twice in PEM. DNase I (100 µg/ml) in 80 mM Pipes, 1 mM EGTA, 5 mM MgCl₂, pH 7.2, was added to the preparation and the chromosomes digested for 30 min at 4°C. After two rinses in PEM, the digested chromosomes were resuspended in 1% SDS for 30 min at room temperature to solubilize the chromosomal proteins. SDS was removed from the proteins using a slight modification of the procedure of Weber and Kuter (1971) as follows. After dialysis against 6 M urea, the chromosomal proteins were passed over a Dowex AG1 × 2Ac column and eluted with 6 M urea, 50 mM Tris-acetate, pH 7.8. The protein-containing fractions were dialyzed against borate saline buffer and then passed over an anti-tubulin affinity column. Tubulin kinetochose protein complexes were eluted with 4 M MgCl₂ in borate saline buffer dialyzed against dH₂O, and then lyophilized. The protein complexes were heated to 100°C in sample buffer containing 2.5% β-mercaptoethanol before SDS-PAGE to disrupt the disulfide bonds formed by Lomant's reagent. This procedure has also been repeated using chromosome scaffolds as the starting material.

To demonstrate the specificity of the tubulin kinetochose protein binding, the following controls were performed. First, the above experiment was repeated without the addition of cross-linker. Second, the above protocol was repeated with human red blood cell ghosts substituted for CHO chromosomes. Finally, a protein with a similar isoelectric point to tubulin was

incubated with chromosomes. Specifically, human IgG1 (pI 6.6) was incubated with CHO chromosomes. Cross-linker was added, the chromosomes solubilized, and the proteins passed over an anti-human IgG affinity column.

PAGE and Immunoblotting

SDS-PAGE was carried out according to Laemmli (1970). Before electrophoresis, samples were boiled in either sample buffer with 2.5 mM β-mercaptoethanol (reducing conditions) or sample buffer without β-mercaptoethanol (nonreducing). Gels were stained either with Coomassie Brilliant Blue or by silver staining using the methods described by Wray et al. (1981).

Protein transfer to nitrocellulose was performed according to the methods of Towbin et al. (1979). The nitrocellulose blots were probed either with human CREST antiserum (1:500 in PBS) followed by peroxidase-labeled anti-human (1:500 in PBS) or with anti-tubulin (10 µg/ml in PBS) followed by peroxidase-labeled anti-sheep (1:500 in PBS). The preparations were rinsed and developed with 4-chloro-1-naphthol.

Affinity-Chromatography

Affinity columns were prepared by coupling either affinity-purified anti-tubulin (5 mg) or anti-human IgG (2 mg) to CNBr-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO). The columns were run according to the methods of Brinkley et al. (1980).

Patient Sera

All patient sera were obtained from the Comprehensive Arthritis Center at the University of Alabama at Birmingham. The sera were tested for anticentromeric staining properties by indirect immunofluorescence and by immunoblotting against proteins of isolated chromosomes before use in these experiments.

Results

The ability of tubulin to bind to kinetochores is illustrated in Fig. 1. When chromosomes are isolated from vinblastine sulfate-treated Indian muntjac cells, the kinetochores contain virtually no bound tubulin as demonstrated by anti-tubulin immunofluorescence (Fig. 1, *a* and *b*). However, when these chromosomes are incubated with phosphocellulose-purified tubulin, the tubulin binds specifically to the kinetochose as shown in Fig. 1, *c* and *d*. It is clear as observed in the large-sized chromosomes of the muntjac that tubulin binding is indeed confined to the kinetochose and does not include the entire centromere. Identical results (not shown) were obtained when smaller CHO chromosomes were used. It should be noted that microtubule nucleation is not observed under the conditions of these experiments as reported by Mitchison and Kirschner (1985a) (see also Pepper and Brinkley, 1977).

When the proteins of isolated metaphase chromosomes were analyzed by Western blotting using autoimmune sera from several patients with scleroderma CREST, two major antigens with molecular masses of 18 and 80 kD were consistently seen (Fig. 2). In the remaining experiments we attempted to determine (*a*) the localization of these proteins in the kinetochose, and (*b*) whether they play a role in the attachment of metaphase chromosomes to the mitotic spindle. To achieve these objectives, two types of experiments were performed using isolated chromosomes from CHO cells. In the first approach, isolated chromosomes were incubated with exogenous tubulin and the growth of microtubules from kinetochores was analyzed by indirect immunofluorescence after selective extraction of chromosomal proteins with 1–2 M NaCl. In the second experiment, a cleavable, bifunctional cross-linking agent (see Materials and Methods) was used

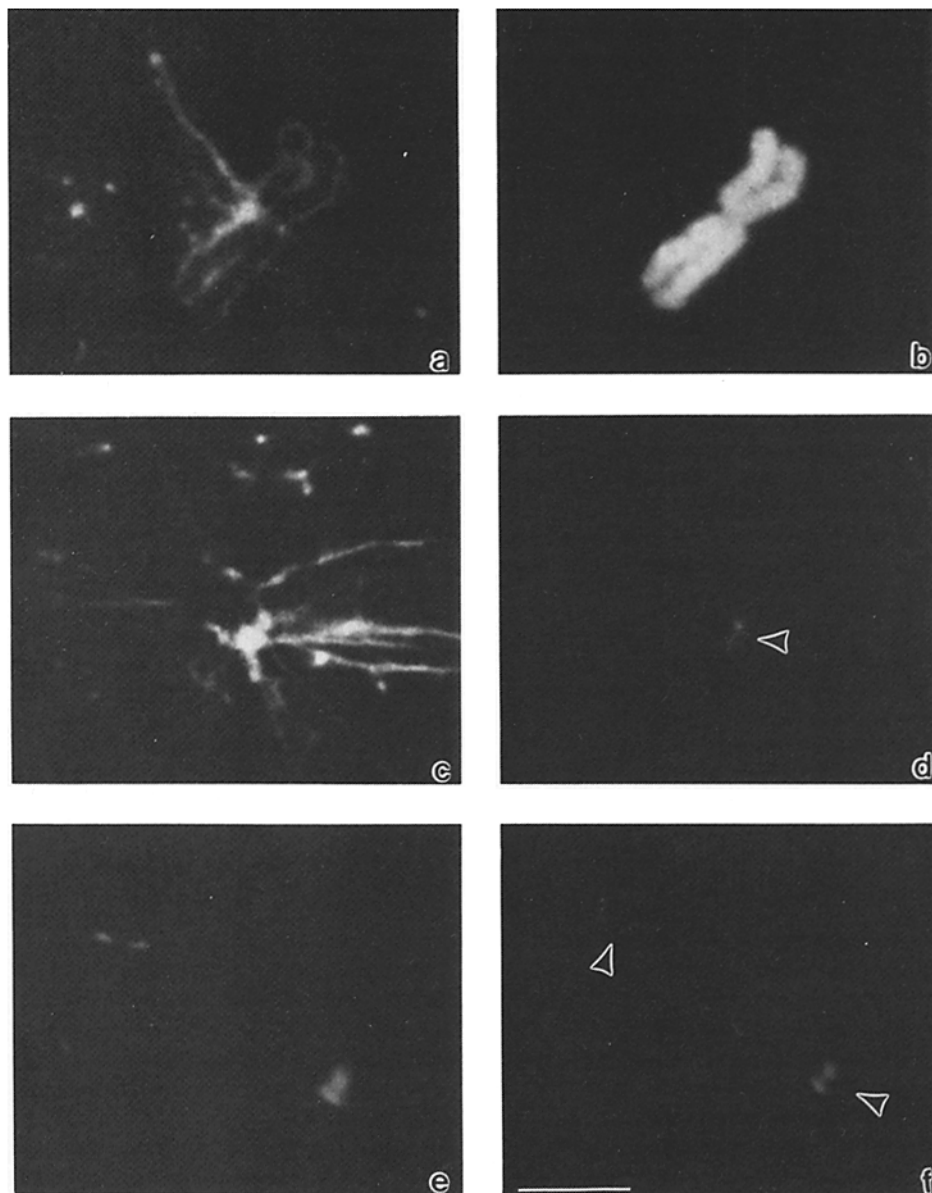


Figure 3. (a and b) The growth of microtubules from a control CHO chromosome. (a) Anti-tubulin immunofluorescence. (b) Hoechst staining of a. (c and d) Microtubule growth from a residual chromosome scaffold. This chromosome had been digested with DNase I and then extracted with 2 M NaCl before incubating with 6S tubulin (1.8 mg/ml). After fixation, the preparation was double-labeled with anti-tubulin (followed by FITC-labeled anti-sheep) and human CREST serum (followed by RITC-labeled anti-human). (c) Anti-tubulin immunofluorescence. (d) Human CREST staining of c. (e and f) Tubulin binding to kinetochores of chromosome scaffolds. Chromosome scaffolds were incubated with 6S tubulin (1.2 mg/ml), fixed, and processed for both anti-tubulin immunofluorescence (e) and CREST immunofluorescence (f). The arrows in f point to the CREST staining residual kinetochores. Bar, 5 μ m.

to chemically couple exogenous tubulin to contiguous kinetochore proteins, which were then solubilized and analyzed by tubulin antibody affinity chromatography and SDS gel electrophoresis.

As shown in Fig. 3, a and b, extensive microtubule growth occurs from untreated, control CHO chromosomes. In our experiments 50–60% of the chromosomes on a coverslip supported the growth of microtubules from kinetochores. Nonspecific, non-microtubule organizing center associated microtubule growth was kept to a minimum (<1%) by incubating the chromosomes in the presence of tubulin concentrations that were very near to the critical concentration necessary for microtubule assembly, as suggested by Mitchison and Kirschner (1985a).

Surprisingly, digestion of chromosomes with DNase I followed by extensive extraction with 1–2 M NaCl has no apparent effect on the growth of microtubules from chromosomes (Fig. 3, c and d). This treatment, which results in the production of chromosome scaffolds, solubilizes much of the

DNA and many of the chromosomal proteins (Fig. 4). That microtubule growth is occurring from residual kinetochores and not from contaminating centrosomes can be inferred from the following evidence. First, Mitchison and Kirschner (1984) have demonstrated that salt extraction abolishes the nucleating capacity of centrosomes. In addition, double immunofluorescent staining of these samples using tubulin antibodies followed by the CREST anti-kinetochore staining procedure demonstrates that microtubule growth is focused on the residual kinetochore (Fig. 3 d).

Chromosome scaffolds, like whole chromosomes, can also bind tubulin at their kinetochores, as shown in Fig. 3, e and f. As with whole chromosomes, kinetochore binding is accomplished at concentrations of tubulin below the critical concentration needed for microtubule nucleation.

The results shown in Fig. 4 demonstrate that the 18-kD protein is solubilized by extracting chromosomes with 2 M NaCl, and that the 18 kD component is not associated with the chromosome scaffold. In Fig. 4, lanes b and c are

Coomassie-stained SDS gels of whole chromosomes and residual scaffolds, respectively. Lanes *d* and *e* are the corresponding Western blots of lanes *b* and *c* probed with CREST serum. In lane *d*, both the 18- and the 80-kD antigens can be seen, whereas in lane *e* only the 80-kD antigen can be identified. This result, taken together with the results shown in Fig. 3, further supports the notion that the 18-kD antigen is a histone-like chromatin protein (Earnshaw et al., 1985; Valdivia and Brinkley, 1985; Palmer et al., 1987), and is not an essential intermediate in the interaction of kinetochores with microtubules. Apparently the proteins that are essential for chromosome-microtubule nucleation are part of the residual chromosome scaffold.

The specificity of the binding reaction of tubulin to kinetochore proteins (Fig. 1) was incorporated into a procedure to directly identify tubulin-binding proteins present in the kinetochore. After incubating chromosomes with exogenous tubulin, the tubulin was covalently cross-linked to the kinetochore using the reversible, disulfide cross-linker DSP (Lomant's reagent). The chromosome proteins were then solubilized and passed over an anti-tubulin affinity column. Tubulin-kinetochore protein complexes were eluted from the column and analyzed by PAGE and Western blotting with CREST serum. Lomant's reagent was chosen as the cross-linker in this experiment for two reasons. First, disulfide bonds formed between contiguous proteins using this reagent can be cleaved by treating the protein complex with β -mercaptoethanol before PAGE. Second, Lomant's reagent works well as physiological pH, whereas other cross-linkers require either acidic or basic pH's, which may denature tubulin and alter its interaction with kinetochore proteins.

The results of the cross-linking experiments are shown in Fig. 5. Lane *b* shows that treatment of chromosomes with 1% SDS results in the solubilization of numerous proteins. When these proteins are passed over an anti-tubulin affinity column, only a small number of proteins are specifically eluted (lane *c*). In addition to tubulin, there is an 80-kD band, a band of 110 kD, a faint band at 54 kD, and a less prominent band between the 80-kD component and the 110-kD band.

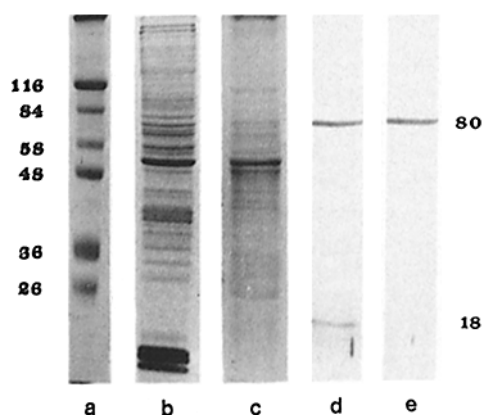


Figure 4. A 10% SDS gel of CHO chromosomes and CHO chromosome scaffolds and the corresponding Western blot with CREST serum. Lane *a*, molecular mass standards (in kilodaltons). Lane *b*, CHO chromosomes. Lane *c*, CHO chromosome scaffolds. Lane *d*, Western blot of whole CHO chromosomes. Lane *e*, Western blot of CHO chromosome scaffolds. The apparent molecular masses of the CREST antigens are given in kilodaltons to the right of lane *e*.

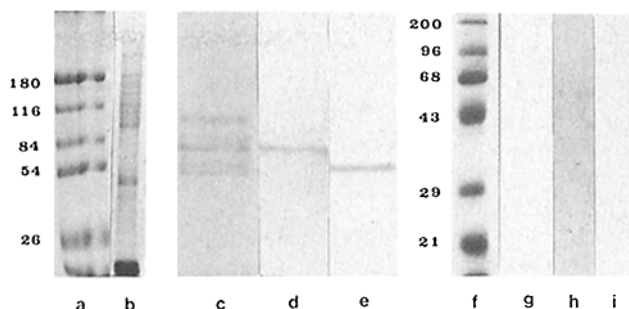


Figure 5. SDS-PAGE and immunoblotting of tubulin-kinetochore protein complexes. Lanes *a-e*, from 10% SDS gels; lanes *f-i*, from 12.5% SDS gels. Lanes *a-c* and *f-i*, Coomassie stained. Lanes *d* and *e*, Western blots with CREST serum and anti-tubulin, respectively. Lane *a*, molecular mass standards (in kilodaltons). Lane *b*, SDS-solubilized proteins from CHO chromosomes that have been cross-linked with 6S tubulin. Lane *c*, chromosomal proteins eluted from an anti-tubulin affinity column after cross-linking of tubulin to kinetochores. Lane *d*, Western blot of lane *c* using CREST serum and peroxidase-labeled anti-human at a dilution of 1:500 in PBS. Lane *e*, immunoblot of lane *c* with anti-tubulin. Anti-tubulin was used at 10 μ g/ml and peroxidase-labeled anti-sheep at a dilution of 1:500 in PBS. Lane *f*, molecular mass standards (in kilodaltons). Lane *g*, an SDS gel of the eluate from an anti-tubulin affinity column after tubulin was cross-linked to human red blood cell ghosts. No proteins can be detected. Lane *h*, proteins eluted from an anti-human IgG affinity column after human IgG1 was cross-linked to CHO chromosomes. Only the heavy chain of IgG can be resolved. Lane *i*, proteins eluted from an anti-tubulin affinity column after tubulin was added to chromosomes without cross-linker (Lomant's reagent).

Bands corresponding to the 140 (CENP-C) and the 17-18-kD (CENP-A) peptides were not seen. The proteins present in the Coomassie-stained bands in lane *c* were purified from the chromosomes isolated from three T-175 flasks of CHO cells.

To determine whether the protein bands present in lane *c* are actually derived from the kinetochore, a Western blot was performed using the scleroderma CREST serum. As lane *d* demonstrates, the CREST serum recognizes the 80-kD region. In addition, a different CREST serum also recognized the same 80-kD band as well as the faint band at 54 kD (result not shown). These results demonstrate that the kinetochore proteins contiguous to tubulin can be purified by cross-linking them to pure tubulin. Unfortunately, since there are multiple bands in lane *c* and because of the nature of the cross-linker, we are still unable to definitively identify the protein(s) of the kinetochore that bind directly to tubulin. For example, it cannot be determined whether all of the bands present in lane *c* bind to tubulin or whether tubulin binds to only one of the proteins which, in turn, is further cross-linked to its nearest neighbor. We have shown by immunoblotting that both tubulin and the CREST-positive protein comigrate in the same high molecular mass band when the kinetochore protein complex is run on nonreducing SDS-PAGE. Moreover, when the unreduced complex was excised, reduced with β -mercaptoethanol and run on reducing SDS gels, a banding pattern identical to that shown in Fig. 5 *c* was observed (data not shown). Experiments are currently underway to characterize the binding of tubulin to kinetochore proteins by nearest neighbor analysis according to the procedure of Wang and Richards (1974).

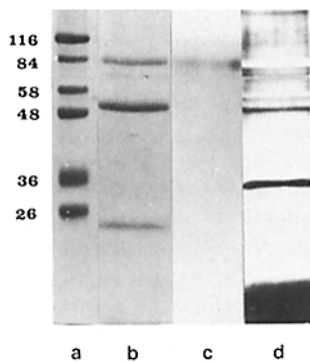


Figure 6. Silver staining of the tubulin-kinetochore protein complexes. Lane *a*, molecular mass standards (in kilodaltons). Lane *b*, the chromosome proteins eluted from an anti-tubulin affinity column after cross-linking of tubulin to kinetochores. This preparation is different from the one in Fig. 5 *c* and shows a prominent band at 24 kD in addition to the bands at 80 and 54 kD. The band at 110 kD cannot be resolved in this preparation.

Lanes *a* and *b* are Coomassie stained. Lane *c*, Western blot of lane *b*. CREST serum and peroxidase-labeled anti-human were both used at a dilution of 1:500 in PBS. The 80-kD CREST antigen is prominent. Lane *d*, silver staining of the tubulin-kinetochore protein complex. The same amount of protein was loaded in this lane as in lane *b*. Because of this overloading of protein, the bands that are prominent in the Coomassie-stained lane (*b*) appear negatively stained in this preparation. Other minor components of the kinetochore complex that become apparent in the silver-stained preparation are bands that migrate in the molecular mass range of the histones as well as a few bands in the 120–150-kD range.

These experiments support the notion that the 80-kD CREST antigen is a kinetochore protein that is closely associated with and possibly bound to tubulin. The absence of an 18-kD band in lanes *c* and *d* suggests that this CREST antigen is not contiguous to tubulin and provides additional evidence that the 18-kD CREST antigen is probably not involved in microtubule interaction with the kinetochore. It should be noted that some variability occurs when kinetochore proteins are extracted by this procedure. Although the 80-kD band was seen consistently in six out of six preparations, the 110-kD band was seen in only three out of six samples. In addition, a discrete band at 24 kD (Fig. 6) was present in approximately half of our preparations.

Three controls were performed to demonstrate the specificity of the binding between tubulin and the kinetochore proteins as well as to demonstrate the importance of the cross-linker in this purification scheme. Fig. 5 *g* shows the results of an experiment in which tubulin was cross-linked to human red blood cell ghosts. Red blood cells were chosen because they have no tubulin cytoskeleton and they are anucleate, and hence do not have kinetochores. As shown in Fig. 5, lane *g*, no erythrocyte proteins were bound to the anti-tubulin affinity column. Lane *h* shows the results of a control experiment in which a protein with a similar isoelectric point to tubulin (IgG1, pI 6.6) was incubated with chromosomes before the addition of cross-linker. This experiment was performed to determine whether the binding of tubulin to the kinetochore proteins was a specific reaction and not simply a charge interaction between tubulin and adjacent chromosomal proteins. The only identifiable band in lane *h* is the heavy chain of IgG. Finally, lane *i* shows the results of a control experiment in which Lomant's reagent was not used. Chromosomes were incubated with tubulin, but no cross-linker was added before SDS solubilization.

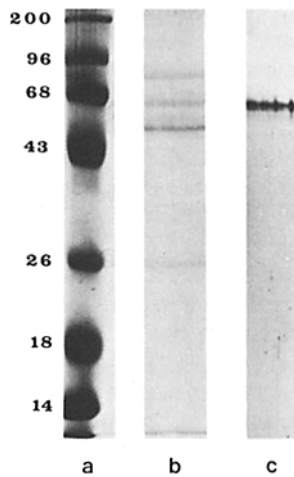


Figure 7. Tubulin cross-linking to chromosome scaffolds. Lane *a*, molecular mass standards (in kilodaltons). Lane *b*, the scaffold proteins eluted from an anti-tubulin affinity column after tubulin was cross-linked to chromosome scaffolds using Lomant's reagent. Note the appearance of a prominent band at 65 kD. Lane *c*, an immunoblot of lane *b*. CREST serum and peroxidase-labeled anti-human were both used at 1:500 in PBS.

The only identifiable bands in lane *i* are faint tubulin bands. The other protein bands that are seen in lane *c* are absent.

To identify minor protein species that eluted with the kinetochore protein complex, more sensitive silver-stained gels were prepared using the same material. A comparison between a Coomassie-stained gel and a silver-stained preparation is shown in Fig. 6. Several additional bands can be seen in the silver-stained gels, including lower molecular mass proteins that probably correspond to histones and a few bands at a higher molecular mass range (120–150 kD). Typically, proteins in greater abundance, such as the 80-kD band, appeared as negative bands in the silver-stained gels.

Chromosome scaffold preparations were also analyzed for a tubulin-kinetochore protein complex by using the same cross-linking procedure as for tubulin-associated proteins of whole chromosomes. As shown in Fig. 7 *b*, a protein complex was extracted from chromosome scaffolds that was similar to that of whole chromosomes, except that a prominent band appears at 65 kD. Although an 80-kD band was still present in the complex, the CREST autoantibody recognized the 65-kD band in immunoblots of scaffold preparations (Fig. 7 *c*).

Discussion

The kinetochore has long been recognized as a site for the attachment of spindle fibers to the centromere and as "... an element of fundamental importance in the movement of chromosomes ..." (Schrader 1953). Although the ultrastructural and cytochemical properties of the kinetochore have been described in a variety of cells (see reviews by Rieder, 1982; Godward, 1985; Brinkley et al., 1985), relatively little is known about the molecular composition of this structure. Our experiments address the question of which proteins make up the kinetochore and how they interact with tubulin and microtubules.

Current knowledge of the proteins that are associated with the centromere/kinetochore region comes from earlier cytochemical studies (Pepper and Brinkley, 1977; see review by Rieder, 1982), and more recently from the use of human autoantibodies from scleroderma CREST patients (Cox et al., 1983; Guldner et al., 1984; Ayer and Fritzler, 1984; Nishikai et al., 1984; Earnshaw and Rothfield, 1985; Earnshaw et al., 1986; Valdivia and Brinkley, 1985; McNeilage et al., 1986).

Collectively these investigations have identified several proteins that may be directly or indirectly associated with the kinetochore or play some role in its activity.

Pepper and Brinkley (1977, 1979) used antitubulin antibodies to identify tubulin in the kinetochore and showed that kinetochore-associated tubulin was essential for microtubule nucleation from chromosomes. Recently improved methods have been developed for investigating the interaction of exogenous tubulin with the kinetochore (Mitchison and Kirschner, 1985a). The latter study suggested that kinetochore-associated tubulin was probably not a structural component of the kinetochore, but was bound tightly to the kinetochore in close proximity to the fibrous corona, a series of fine hair-like projections on the outer face of the kinetochore. As suggested in earlier studies, tubulin binding to the kinetochore was found to be correlated with the enhanced nucleation capacity of the kinetochore *in vitro* (Mitchison and Kirschner, 1985a; Pepper and Brinkley, 1977). Whether tubulin binding to the kinetochore relates to other functions such as microtubule capture and stability (Mitchison and Kirschner, 1985b; Pickett-Heaps et al., 1982) or the dynamic assembly-disassembly of spindle microtubules at their kinetochore ends (Mitchison et al., 1986; Gorbsky et al., 1987) requires further investigation of the molecular composition of the kinetochore.

Studies using human autoantibodies from scleroderma CREST patients that bind to the centromere/kinetochore region (Moroi et al., 1980; Brenner et al., 1981) provided evidence for a family of proteins that may be related to the structure and function of the kinetochore. Earnshaw and Rothfield (1985) identified by immunoblotting three major CREST antigens associated with the centromere. Because of their association with the centromere, these proteins were identified as CENP-A (17–18 kD), CENP-B (80 kD), and CENP-C (140 kD). As mentioned previously, other investigators have used similar procedures to identify peptides ranging in molecular mass from 17 to 140 kD. Two distinct proteins of 18 and 80 kD were identified in an isolated kinetochore fraction (Valdivia and Brinkley, 1985). Recently Earnshaw et al. (1987) cloned and sequenced a cDNA corresponding to CENP-B. This protein was found to be highly acidic and to have an actual molecular mass of 65 kD. This major centromeric antigen contained two large domains enriched in glutamic and aspartic acid.

Before the present study, very little was known about the function or localization of the CREST antigen family. Our study strongly indicates that the 80-kD protein is contiguous with and possibly bound to kinetochore-associated tubulin, which itself is localized on the outer layer of the trilaminar plate as shown by electron microscopic immunogold localization (Mitchison and Kirschner, 1985a). If, in fact, the 80-kD antigen is a tubulin-binding protein it will be useful to identify the amino acid sequences involved, especially since both proteins are highly acidic. However, we cannot rule out the possibility that other members of the protein complex are involved in tubulin binding.

The identity of the other tubulin-associated proteins extracted by our procedure remains to be determined, as does their spatial association with tubulin and the 80-kD component. One of these, a peptide of ~54 kD, was recognized by a CREST serum from one patient in our study (but not by the serum from another patient) and may also be an antigen

in this family of proteins. Kingwell and Rattner (1987) have reported that a 50-kD protein is present in both human and Indian muntjac kinetochores and a 50-kD antigen has been found in the kinetochores of rat chromosomes (Earnshaw et al., 1985).

The variability in the detection of some polypeptides in the kinetochore-protein complex, including the 110- and 24-kD bands, cannot be explained from our data but is under further investigation. Cox et al. (1983) described major centromere antigens in the 20–25-kD range, but these were not identified as proteins of the kinetochore by Earnshaw et al. (1984) or Guldner et al. (1984). Using immunoblotting procedures, Earnshaw and Rothfield (1985) identified antigens in the 20–25-kD range as components of the metaphase chromosome scaffold.

Presently, we cannot explain why the kinetochore protein complex from chromosome scaffolds displays a major CREST-positive band at 65 kD and not at 80 kD as seen in whole chromosomes. It is possible that the 65-kD band is a proteolytic fragment of the 80-kD protein produced during the preparation of chromosome scaffolds. Equally perplexing is the existence of an 80-kD band in the scaffold preparation, which does not bind the CREST antibody. Obviously, the variations observed between tubulin-linked kinetochore proteins of scaffolds and whole chromosomes require further study.

It has not escaped our attention that the 110-kD peptide is within the molecular mass range of kinesin, the major microtubule translocation molecule found in squid axoplasm and bovine brain (Vale et al., 1985). Kinesin, with a molecular mass of 110–120 kD, powers organelles along microtubules. A similar translocator molecule could be involved in anaphase chromosome movement. Further studies are underway to determine possible homologies between the two proteins.

Another CREST antigen, CENP-A (17–18 kD), was not eluted by our purification procedure. Since it can be extracted from chromosomes by heparin or 1–2 M NaCl, we support the notion that it is a histone-like component of centromeric chromatin as suggested by others (Earnshaw et al., 1984, 1985; Valdivia and Brinkley, 1985; Palmer et al., 1987). Its absence from chromosome scaffold preparations, which are fully capable of binding tubulin and initiating the assembly of microtubules at the residual kinetochore, suggests that the 18-kD antigen, and perhaps centromeric chromatin, is not required for either microtubule binding or assembly.

In summary, this investigation has provided new insight into tubulin-linked proteins of the kinetochore, which may be involved in binding of spindle microtubules to the centromere. Further investigations are underway to characterize this kinetochore protein complex.

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References

- Ayer, L., and M. Fritzler. 1984. Anticentromere antibodies bind to trout testis histone I and a low molecular weight protein from rabbit thymus. *Mol. Immunol.* 21:761-770.
- Brenner, S., D. Pepper, M. W. Berns, E. Tan, and B. R. Brinkley. 1981. Kinetochore structure, duplication, and distribution in mammalian cells. Analysis by human autoantibodies from scleroderma patients. *J. Cell Biol.* 91:95-102.
- Brinkley, B. R., S. H. Fistel, J. M. Marcum, and R. L. Pardue. 1980. Microtubules in cultured cells: indirect immunofluorescent staining with tubulin antibody. *Int. Rev. Cytol.* 63:58-95.
- Brinkley, B. R., M. M. Valdivia, A. Tousson, and S. L. Brenner. 1984. Compound kinetochores of the Indian Muntjac: evolution by linear fusion of unit kinetochores. *Chromosoma (Berl.)* 91:1-11.
- Brinkley, B. R., A. Tousson, and M. M. Valdivia. 1985. The kinetochore of mammalian chromosomes: structure and function in normal mitosis and aneuploidy. In *Aneuploidy*. V. L. Dellarco, P. E. Voytek, and A. Hollaender, editors. Plenum Publishing Corp., New York. 243-267.
- Cox, J. V., E. A. Schenk, and J. B. Olmsted. 1983. Human anticentromere antibodies. Distribution, characterization of antigens, and effect of microtubule organization. *Cell* 35:331-339.
- Earnshaw, W. C., and N. Rothfield. 1985. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma (Berl.)* 91:313-321.
- Earnshaw, W. C., N. Halligan, C. Cooke, and N. Rothfield. 1984. The kinetochore is part of the metaphase chromosome scaffold. *J. Cell Biol.* 98:353-357.
- Earnshaw, W. C., C. Bordwell, C. Marino, and N. Rothfield. 1986. Three human chromosomal autoantigens are recognized by sera from patients with anti-centromere antibodies. *J. Clin. Invest.* 77:426-430.
- Earnshaw, W. C., K. S. Sullivan, P. S. Machlin, C. A. Cooke, D. A. Kaiser, T. D. Pollard, N. R. Rothfield, and D. W. Cleveland. 1987. Molecular cloning of cDNA for CENP-B, the major human centromere autoantigen. *J. Cell Biol.* 104:817-829.
- Godward, M. B. E. 1985. The kinetochore. *Int. Rev. Cytol.* 94:77-105.
- Gorbisky, G. J., P. J. Sammak, and G. G. Borisy. 1987. Chromosomes move poleward in anaphase along stationary microtubules that coordinately disassemble from their kinetochore ends. *J. Cell Biol.* 104:9-18.
- Guldner, H. H., H. J. Lakomek, and F. A. Bautz. 1984. Human anti-centromere sera recognize a 19.5 kD non-histone chromosomal protein from HeLa cells. *Clin. Exp. Immunol.* 58:13-20.
- Kingwell, B., and J. B. Rattner. 1987. Mammalian kinetochore structure: a 50kD antigen is present in the mammalian kinetochore. *Chromosoma (Berl.)*. In press.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lomant, A. J., and G. Fairbanks. 1976. Chemical probes of extended biological structure: synthesis and properties of the cleavable protein crosslinking reagent [³⁵S]dithiobis(succinimidyl propionate). *J. Mol. Biol.* 104:243-261.
- McNeillage, J. L., S. Whittingham, C. V. McHugh, and A. J. Barbett. 1986. A highly conserved 72,000 dalton centromeric antigen reactive with autoantibodies from patients with progressive systemic sclerosis. *J. Immunol.* 86:2541-2547.
- Mitchison, T., and M. Kirschner. 1984. Microtubule assembly nucleated by isolated centrosomes. *Nature (Lond.)* 312:232-237.
- Mitchison, T., and M. W. Kirschner. 1985a. Properties of the kinetochore in vitro. I. Microtubule nucleation and tubulin binding. *J. Cell Biol.* 101:755-765.
- Mitchison, T., and M. W. Kirschner. 1985b. Properties of the kinetochore in vitro. II. Microtubule capture and ATP-dependent translocation. *J. Cell Biol.* 101:766-777.
- Mitchison, T., L. Evans, E. Schultz, and M. Kirschner. 1986. Sites of microtubule assembly and disassembly in the mitotic spindle. *Cell* 45:515-527.
- Moroi, Y., C. Peeples, M. J. Fritzler, J. Steigerwald, and E. M. Tan. 1980. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. USA* 77:1627-1631.
- Nishikai, M., Y. Okano, H. Yamashita, and M. Watanabe. 1984. Characterization of centromere (kinetochore) antigen reactive with sera of patients with a scleroderma variant (CREST syndrome). *Ann. Rheum. Dis.* 43:819-824.
- Palmer, D. K., K. O'Day, M. H. Wener, B. S. Andrews, and R. L. Margolis. 1987. A 17kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J. Cell Biol.* 104:805-815.
- Pepper, D. A., and B. R. Brinkley. 1977. Localization of tubulin in the mitotic apparatus of mammalian cells by immunofluorescence and immunoelectron microscopy. *Chromosoma (Berl.)* 60:223-235.
- Pepper, D. A., and B. R. Brinkley. 1979. Microtubule initiation at kinetochores and centrosomes in lysed mitotic cells. Inhibition of site-specific nucleation by tubulin antibody. *J. Cell Biol.* 82:585-591.
- Pickett-Heaps, J. D., D. H. Tippit, and K. R. Porter. 1982. Rethinking mitosis. *Cell* 29:727-744.
- Rieder, C. L. 1982. The formation, structure and composition of the mammalian kinetochore and kinetochore fiber. *Int. Rev. Cytol.* 79:1-58.
- Schrader, F. 1953. Mitosis. The movement of chromosomes in cell division. L. C. Dunn, editor. Columbia University Press, New York. 1-170.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
- Tuan, R. S., and A. Knowles. 1984. Calcium-activated ATPase of the chick embryonic chorioallantoic membrane. *J. Biol. Chem.* 259:2754-2763.
- Valdivia, M. M., and B. R. Brinkley. 1985. Fractionation and initial characterization of the kinetochore from mammalian metaphase chromosomes. *J. Cell Biol.* 101:1124-1134.
- Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force generating protein, kinesin, involved in microtubule-based motility. *Cell* 42:39-50.
- Wang, K., and F. M. Richards. 1974. An approach to nearest neighbor analysis of membrane proteins: application to the human erythrocyte membrane of a method employing cleavable crosslinkages. *J. Biol. Chem.* 249:8005-8018.
- Weber, K., and S. J. Kuter. 1971. Reversible denaturation of enzymes by sodium dodecyl sulfate. *J. Biol. Chem.* 246:4504-4509.
- Wray, W., T. Boulikas, V. P. Wray, and B. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118:197-203.