

Immunolocalization of Myosin I Heavy Chain Kinase in *Acanthamoeba castellanii* and Binding of Purified Kinase to Isolated Plasma Membranes

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Abstract. The actin-activated Mg^{2+} -ATPase activities of *Acanthamoeba* myosins I are known to be maximally expressed only when a single threonine (myosin IA) or serine (myosins IB and IC) is phosphorylated by myosin I heavy chain kinase. The purified kinase is highly activated by autophosphorylation and the rate of autophosphorylation is greatly enhanced by the presence of acidic phospholipids. In this paper, we show by immunofluorescence and immunoelectron microscopy of permeabilized cells that myosin I heavy chain kinase is highly concentrated, but not exclusively, at the plasma membrane. Judged by their electrophoretic mobilities, kinase associated with purified plasma membranes may differ from the cytoplasmic kinase, possibly in the extent of its phosphorylation. Purified kinase binds to

highly purified plasma membranes with an apparent K_D of ~ 17 nM and a capacity of ~ 0.8 nmol/mg of plasma membrane protein, values that are similar to the affinity and capacity of plasma membranes for myosins I. Binding of kinase to membranes is inhibited by elevated ionic strength and by extensive autophosphorylation but not by substrate-level concentrations of ATP. Membrane-bound kinase autophosphorylates to a lesser extent than free kinase and does not dissociate from the membranes after autophosphorylation. The co-localization of myosin I heavy chain kinase and myosin I at the plasma membrane is of interest in relation to the possible functions of myosin I especially as phospholipids increase kinase activity.

Two general classes of the mechanoenzyme myosin have been identified in metazoan as well as in protozoan cells. One class, the conventional two-headed myosins, myosins II by the terminology proposed by Korn and Hammer (1988), are found in muscle and nonmuscle cells. The second class, one-headed myosins referred to as myosins I (Korn and Hammer, 1988), have been purified from *Acanthamoeba castellanii*, *Dictyostelium discoideum*, and intestinal brush border (for review see Korn and Hammer, 1988, 1990) and structurally related proteins yet to be characterized functionally have been identified in the photoreceptor cells of *Drosophila melanogaster* (Montell and Rubin, 1988). In addition, several preliminary reports suggest that myosins I and/or other novel myosins are present in brain (Espreafico, E., R. Chaney, F. Spindola, M. Coehlo, D. Pitta, M. Mooseker, and R. Larson. 1990. *J. Cell Biol.* 111: 167a; Li, D., and P. D. Chandler. 1991. *J. Biophys.* 52: 229a), neuronal growth cones (Bahler, 1990), and white blood cells (Atkinson and Peterson, 1991).

The best characterized mechanoenzymes of the myosin I class are those from *Acanthamoeba*. The three *Acanthamoeba* isoenzymes studied thus far, myosins IA, IB, and IC (Maruta et al., 1979; Lynch et al., 1989), contain a single heavy chain with an ~ 80 -kD NH_2 -terminal domain and an ~ 50 -kD $COOH$ -terminal domain. The NH_2 -terminal do-

main closely resembles the subfragment I domain of myosin II (Jung et al., 1987; Jung et al., 1989). This domain contains an ATP-binding site (Lynch et al., 1987; Brzeska et al., 1988) and an ATP-sensitive actin-binding site (Brzeska et al., 1988, 1989a). When proteolytically separated from the rest of the heavy chain, the NH_2 -terminal domain expresses high actin-activated Mg^{2+} -ATPase activity (Brzeska et al., 1988, 1989a). The $COOH$ -terminal domain lacks the α -helical structure of the longer tail domain of myosin II consisting instead of a short globular tail (Jung et al., 1987) which contains an ATP-insensitive actin-binding site (Lynch et al., 1986; Brzeska et al., 1988) and a membrane binding site (Adams and Pollard, 1989; Miyata et al., 1989), neither of which is found in mechanoenzymes of the myosin II class.

The Mg^{2+} -ATPase activities of *Acanthamoeba* myosins IA, IB, and IC are fully expressed only when a single threonine (IA) or a serine (IB and IC) (Hammer et al., 1983; Brzeska et al., 1989b) is phosphorylated by myosin I heavy chain kinase (Pollard and Korn, 1973b; Maruta and Korn, 1977; Hammer et al., 1983). The kinase is a 97-kD protein, whose activity is increased about 50 times by autophosphorylation of multiple sites that have not yet been identified (Brzeska et al., 1990a). Phosphorylation also decreases the electrophoretic mobility of the kinase on sodium dodecyl sulfate polyacrylamide gels with an increase in apparent mo-

lecular mass from 97 to 107 kD (Brzeska et al., 1990a). The rate of autophosphorylation of the kinase is itself stimulated about 20 times in the presence of acidic phospholipids such as phosphatidylserine (Brzeska et al., 1990a,b). Thus, there is a three-step regulatory cascade: acidic phospholipids accelerate the autophosphorylation of myosin I heavy chain kinase, this autophosphorylation increases the rates at which the kinase phosphorylates the myosin I heavy chain, and the myosin I has much greater actin-activated Mg^{2+} -ATPase activity when it is phosphorylated. The role of phospholipids in the regulatory cascade, together with the well-established membrane localization of the amoeba myosins I (Gadasi et al., 1980; Miyata et al., 1989; Baines and Korn, 1990), suggested that the kinase might also be membrane associated.

We have tested this hypothesis in two ways: (a) localization of the kinase in cells by immunofluorescence and immunoelectron microscopy using a kinase-specific polyclonal antibody raised against highly purified kinase; and (b) binding studies using purified preparations of kinase and plasma membranes. The results are consistent with a high concentration, but not exclusive localization, of *Acanthamoeba* myosin I heavy chain kinase at the plasma membrane.

Materials and Methods

Purification of Myosin I Heavy Chain Kinase

The kinase was purified by a recent modification (Lynch et al., 1991) of the original procedure (Hammer et al., 1983). It was stored in buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.01% $NaNO_3$, and 50% (vol/vol) glycerol.

Isolation of Plasma Membranes

Acanthamoeba castellanii (Neff strain) was grown at 30°C in 1-liter flasks as described by Pollard and Korn (1973a). Plasma membranes were isolated according to the method of Clarke et al. (1988). Briefly, the cells were grown to a density of 2×10^6 /ml and were harvested by centrifugation at 250 g for 5 min. The cells were homogenized in 0.35 M sucrose in buffer containing 10 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes), pH 7.4, 1 mg/liter of leupeptin, 10 mg/liter pepstatin and 80 mg/liter of PMSF using a Dounce homogenizer (Kontes Co., Vineland, NJ). The homogenate was centrifuged for 10 min at 250 g. The crude plasma membranes in the supernatant were pelleted at 590 g for 20 min, resuspended in 0.25 M sucrose in 10 mM Tes, pH 6.9, containing 17.5% Percoll, and purified by density gradient centrifugation for 40 min at 48,000 g. The upper fraction was collected, resuspended in 0.15 M KCl in 10 mM Tes, pH 6.9, and pelleted for 20 min at 12,000 g. The pellet was again resuspended and subjected to density gradient centrifugation and then collected as a pellet as before. The final pellet, comprising the purified plasma membranes, was resuspended in 10 mM Tes, pH 6.9. All experiments were performed on freshly prepared plasma membranes.

Preparation of Antibodies

A rabbit polyclonal antiserum was raised against purified *Acanthamoeba* myosin I heavy chain kinase essentially as described before for antibodies against myosins (Baines and Korn, 1990). Purified kinase was subjected to SDS-PAGE, the protein band corresponding to the kinase was excised from the gel after visualization in 4 M sodium acetate (Harlow and Lane, 1988) and the gel strip was homogenized into Freund's complete adjuvant. A female New Zealand white rabbit was primed at the age of 6 mo and received three additional boosts with at least a 4-wk interval between injections. The antiserum was collected 10–12 d after the final immunization. The specificity of the antiserum was assessed by immunoblots (Towbin et al., 1979) of total cell proteins separated by SDS-PAGE. The antiserum recognized the kinase but also recognized another protein of ~55 kD.

The antibody activity toward the unidentified 55-kD polypeptide was re-

1. *Abbreviation used in this paper:* TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

moved in two different ways. First, *Acanthamoeba* extract prepared by the method of Pollard and Korn (1973a) was separated by SDS-PAGE and then transferred to nitrocellulose. The protein bands were visualized by staining with Ponceau red and the band corresponding to the position of the purified kinase standard run on the same gel was excised. The remaining portion of the nitrocellulose was used to adsorb out the cross-reacting antibodies from the antiserum as if performing an immunoblot. Alternatively, the band corresponding to the 55-kD cross-reacting protein was excised from the nitrocellulose and this band was used to adsorb out antibody activity directed against the 55-kD protein.

Binding Assays with Unphosphorylated Kinase

Except when specified otherwise in the text or figure legends, purified kinase (55–220 nM) was mixed with plasma membranes (120 μ g/ml) in buffer containing 50 mM imidazole, pH 7.0, 1 mM $MgCl_2$, 2 mM EGTA, 0.5 mg/ml of BSA, 8 mM KCl, and 8% (vol/vol) glycerol (final concentrations) and the mixture was incubated for 10 min at room temperature. The membranes were then pelleted at 150,000 g for 15 min at 20°C. Usually, the amounts of free and bound kinase were determined by assaying the kinase activity of the total sample before spinning and of the supernatant after spinning using a nine residue synthetic peptide as a substrate. The peptide had the sequence of the phosphorylation site of myosin IC (Brzeska et al., 1989b) (Gly-Arg-Gly-Arg-Ser-Ser-Val-Tyr-Ser) and has been shown to be an excellent substrate for myosin I heavy chain kinase (Brzeska et al., 1990b).

Aliquots of 25 μ l were incubated at 30°C for 1 min in 50 μ l (final volume) of assay solution containing 150 μ M peptide, 2.5 mM [γ - ^{32}P]ATP (50 cpm/pmol), and 60 μ g/ml of plasma membranes in 50 mM imidazole, pH 7.0, 2 mM EGTA, and 3.5 mM $MgCl_2$. The plasma membranes were added to the assay mixture of the supernatant solution after centrifugation so that the conditions of its assay were identical to those of the assay of the total mixture before centrifugation. These assays measured the basal activity of the unphosphorylated kinase as the 1-min incubation time was too short for significant autophosphorylation of the kinase to have occurred.

The reaction was stopped by the addition of glacial acetic acid to a final concentration of 30% (vol/vol), aliquots of the reaction mixture were applied to P-81 ion exchange paper to bind the peptide, the paper was washed to remove all radioactivity except that incorporated into the peptide, and the incorporated radioactivity determined by scintillation counting (Glass et al., 1978). Control samples without the synthetic peptide in the reaction mixture were prepared as blanks. The kinase activity was linear with kinase concentration and proportional to the incubation time in all of the assays.

In some experiments, the amount of membrane-bound kinase was determined independently by densitometric analysis of SDS-PAGE gels (7.5% polyacrylamide) of total samples before spinning and the separated membrane pellets and supernatants obtained after spinning. Coomassie blue-stained gels were quantified by scanning at 580 nm with a scanning densitometer (GS-30; Hoefer Scientific Instruments, San Francisco, CA).

Binding Assays with Phosphorylated Kinase

Kinase (700 nM) was autophosphorylated by incubating in buffer containing 50 mM imidazole, pH 7.0, 2 mM EGTA, 2 mM $MgCl_2$, 20 mM KCl, 20% (vol/vol) glycerol, 0.25 mg/ml of BSA, and 1 mM [γ - ^{32}P]ATP (200 cpm/pmol) at 30°C. After incubation for 3, 10, 20, 40, and 60 min to obtain different levels of phosphorylation, the kinase was added to 120 μ g/ml of plasma membranes in buffer containing (final concentrations) 50 mM imidazole, pH 7.0, 2 mM EGTA, 1 mM $MgCl_2$, 0.5 mg/ml of BSA, 4.0 mM KCl, and 4.0% (vol/vol) glycerol. The final concentrations of kinase and ATP in this mixture were 105 nM and 0.15 mM, respectively. The mixture was incubated for 10 min at room temperature and the membranes were pelleted at 150,000 g for 15 min at 20°C. Under these conditions, further autophosphorylation of the kinase during the binding assay was insignificant. Samples of the total mixture before spinning and of the supernatant after spinning were collected. Each sample was then incubated for 60 min in a buffer containing 50 mM imidazole, pH 7.0, 3.5 mM $MgCl_2$, 0.25 mg/ml of BSA, 2 mM EGTA, and 2.5 mM ATP to obtain complete autophosphorylation. Kinase phosphorylated in the presence and absence of membranes attained the same level of enzymatic activity. The amount of bound kinase could then be determined by assaying for kinase activity as described for the assays of bound unphosphorylated kinase except that the specific activity of the ATP was 180 cpm/pmol.

The extent of phosphorylation of the kinase was determined by separating aliquots of known volume by SDS-PAGE (7.5% polyacrylamide). The gels were stained with Coomassie blue and the ^{32}P -labeled kinase was visualized by autoradiography using XAR film (Eastman Kodak Co., Rochester,

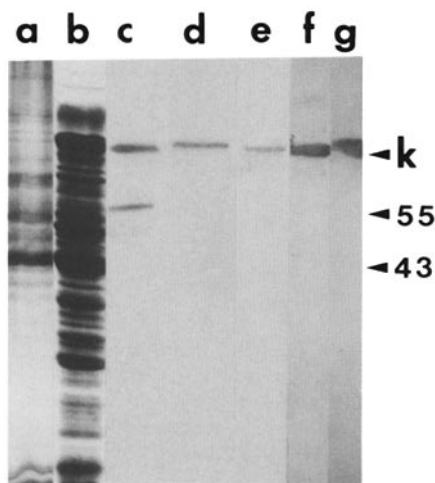


Figure 1. Characterization of the antiserum to myosin I heavy chain kinase by immunoblots of purified kinase and *Acanthamoeba* plasma membranes, cell extract, and total cell proteins of *Acanthamoeba castellanii*. (Lanes a and b) Coomassie blue-stained SDS-PAGE of (a) purified plasma membranes (40 μ g of proteins) and (b) cell extract (80 μ g). (Lanes c–e) Immunoblots obtained with the unpurified antiserum of (c) cell extract (80 μ g), (d) purified plasma membranes (40 μ g), and (e) purified kinase (0.3 μ g). (Lanes f and g) Immunoblots obtained with the purified antiserum of (f) cell extract (100 μ g) and (g) total cell proteins. The antiserum was used at a dilution of 1:800 (which corresponded to a serum protein concentration of 41 μ g/ml) for all immunoblots.

NY). The bands corresponding to phosphorylated kinase were excised and incubated overnight at 50°C in a solution containing 200 μ l of H₂O and 2 ml of Protosol. The resulting solution was neutralized with 70 μ l of glacial acetic acid and counted in 15 ml of scintillation mixture.

Immunolocalization of Myosin I Heavy Chain Kinase

Amoebae were grown in either 1-liter culture flasks to a density of 10⁶ cells/ml, as described by Pollard and Korn (1973a), on either a plastic substrate in 750-ml culture flasks (Falcon Plastics, Cockeysville, MD) or a glass substrate in eight chamber Lab-Tek tissue culture slides (Nunc Inc., Naperville, IL). Cells grown in contact with the plastic or glass substrate were much flatter than cells grown in suspension thus improving the resolution obtained in the immunofluorescence studies.

The methods used for immunofluorescence and immunoelectron microscopy were described previously (Baines and Korn, 1990). Briefly, cells were fixed with 3% formaldehyde and 0.25% glutaraldehyde in growth medium for 45 min at room temperature and then permeabilized with either 0.1% saponin for 10 min, 0.2% saponin for 15 min, or 0.5% saponin for 45 min at room temperature (mild, intermediate, and extensive permeabilization, respectively). Cells were washed in PBS, pH 7.4, after fixation and again after permeabilization and treated with 1 mg/ml of sodium borohydride in PBS for 10 min to reduce the free aldehydes. To block nonspecific binding of antibodies, cells were incubated in 1.0% BSA and 50 mM L-lysine in PBS, pH 7.4. Cells were incubated with the primary and secondary antibodies in the BSA/lysine/PBS buffer with five washes with PBS between incubations.

Other Methods

All protein concentrations were estimated by the method of Bradford (1976) using BSA as a standard. SDS-PAGE was performed according to the method of Laemmli (1970).

Reagents

Pepstatin was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). [γ -³²P]ATP and Protosol were purchased from Du Pont New England Nuclear Research Products (Boston, MA) and scintillation mixture from Re-

search Products International Corp. (Mount Prospect, IL). Ion exchange paper P-18 was obtained from Whatman Ltd. (Maidstone, Kent, England), Ponceau red and saponin from Sigma Chemical Co. (St. Louis, MO), and Freund's complete adjuvant from Difco Laboratories Inc. (Detroit, MI). Leupeptin, BSA, FITC-conjugated goat anti-rabbit IgG and HRP goat anti-rabbit IgG were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Goat anti-rabbit antibodies conjugated to gold particles were obtained from Janssen Life Sciences Products (Beerse, Belgium). All other chemicals were reagent grade.

Results

Characterization of the Antibody

In addition to a band at the same position as the kinase (Fig. 1, lane e), the initial antiserum recognized a 55-kD protein on immunoblots of cell extract (Fig. 1, lane c) and total cell proteins (not shown). As the 55-kD protein was not present in the purified plasma membranes (Fig. 1, lane a), the initial antiserum detected only a single protein band, corresponding to the kinase, on immunoblots of plasma membranes (Fig. 1, lane d). The kinase band detected by the antibody on immunoblots of plasma membranes (Fig. 1, lane d) migrated slightly more slowly than the kinase band detected by the antibody in cell extracts (Fig. 1, lane c). This may have been because of the kinase in the plasma membrane being more extensively phosphorylated (or otherwise modified) than the total cell kinase (Brzeska et al., 1990a).

After purification, as described in Materials and Methods, the antiserum recognized only a single band on immunoblots of cell extracts (Fig. 1, lane f), which co-migrated with purified unphosphorylated kinase (Fig. 1, lane e), indicating successful removal of antibody activity directed at the 55-kD protein. On immunoblots of total cell protein (Fig. 1, lane g), the purified antibody recognized two very closely migrating, poorly resolved bands; these bands probably corresponded to the single bands detected in the separate samples of cell extract and plasma membranes (Fig. 1, lanes c and d) supporting the possibility that these two bands represented two forms of kinase.

The antiserum could detect 2 μ g of pure kinase even when diluted 1:1,500, which corresponds to a serum protein concentration of 22 μ g/ml. Preimmune serum at dilutions of 1:400 to 1:1,000, corresponding to a serum protein concentration of 81–32.5 μ g/ml, did not detect any bands on immunoblots of total cell proteins (data not shown).

Indirect Immunofluorescence Localization of Kinase

Indirect immunofluorescence microscopy clearly demonstrated a strong localization of myosin I heavy chain kinase at the plasma membrane (Fig. 2 b). Fluorescence was also observed in the cortical region of some cells apparently, as inferred from through-focus observations, just below the plasma membrane (Fig. 2 b, arrowheads). Identical results were obtained when cells were fixed in 100% methanol, instead of the mixture of aldehydes, and when cells were permeabilized by 100% acetone or any of the saponin protocols described in Materials and Methods. Cells that had not been permeabilized to allow entry of the antibody did not show any fluorescence above background (Fig. 2 d) nor did cells in which incubation with antibody was replaced by incubation with preimmune serum (Fig. 2 f) or omitted entirely.

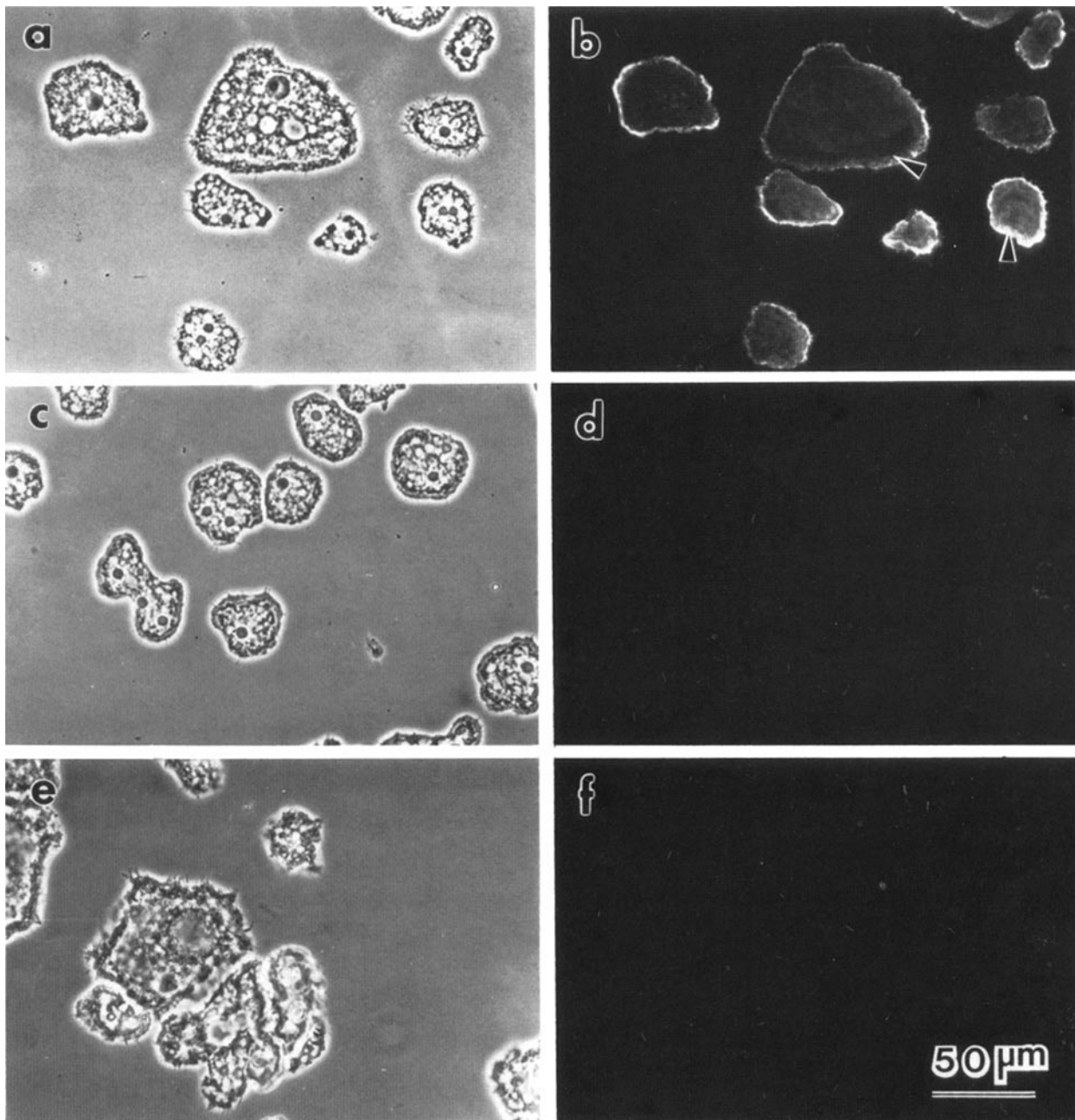


Figure 2. Indirect immunofluorescence localization of myosin I heavy chain kinase. (a) Phase contrast and (b) fluorescence microscopy of cells treated with purified immune serum (0.66 mg/ml) and FITC-linked goat anti-rabbit IgG after intermediate permeabilization; (c) phase contrast and (d) fluorescence microscopy of cells treated with immune serum (0.66 mg/ml) and FITC-linked goat anti-rabbit IgG without permeabilization; (e) phase contrast and (f) fluorescence microscopy of cells treated with preimmune serum (0.66 mg/ml) and FITC-linked goat anti-rabbit IgG after intermediate permeabilization.

Figure 3. Immunoelectron microscopy localization of myosin I heavy chain kinase. (a) A mildly saponin-permeabilized cell treated with purified immune serum (0.66 mg/ml) and goat anti-rabbit IgG coupled to 5 nm gold. Only the plasma membrane was labeled by the antibody (arrows). (b) A cell after intermediate permeabilization treated as in a. The plasma membrane was labeled by the antibody (arrows) and some label was observed in the cell cortex beneath the plasma membrane. (c) A cell after intermediate permeabilization treated as in a but with preimmune serum. Although labeling was minimal, occasional gold particles were observed both on the plasma membrane and in the cell cytoplasm (arrows). (d) An extensively permeabilized cell treated as in a but in which the contractile vacuole is apparent. The plasma membrane was labeled by the antibody (arrows) but no labeling of the contractile vacuole membrane was detected. (e) An extensively permeabilized cell treated as in a showing gold label associated with a large internal vacuole (arrows), the plasma membrane (small arrowheads), and a small vesicle (large arrowhead). (f) An extensively permeabilized cell treated as in a. The plasma membrane was still labeled even after extensive permeabilization (arrows).

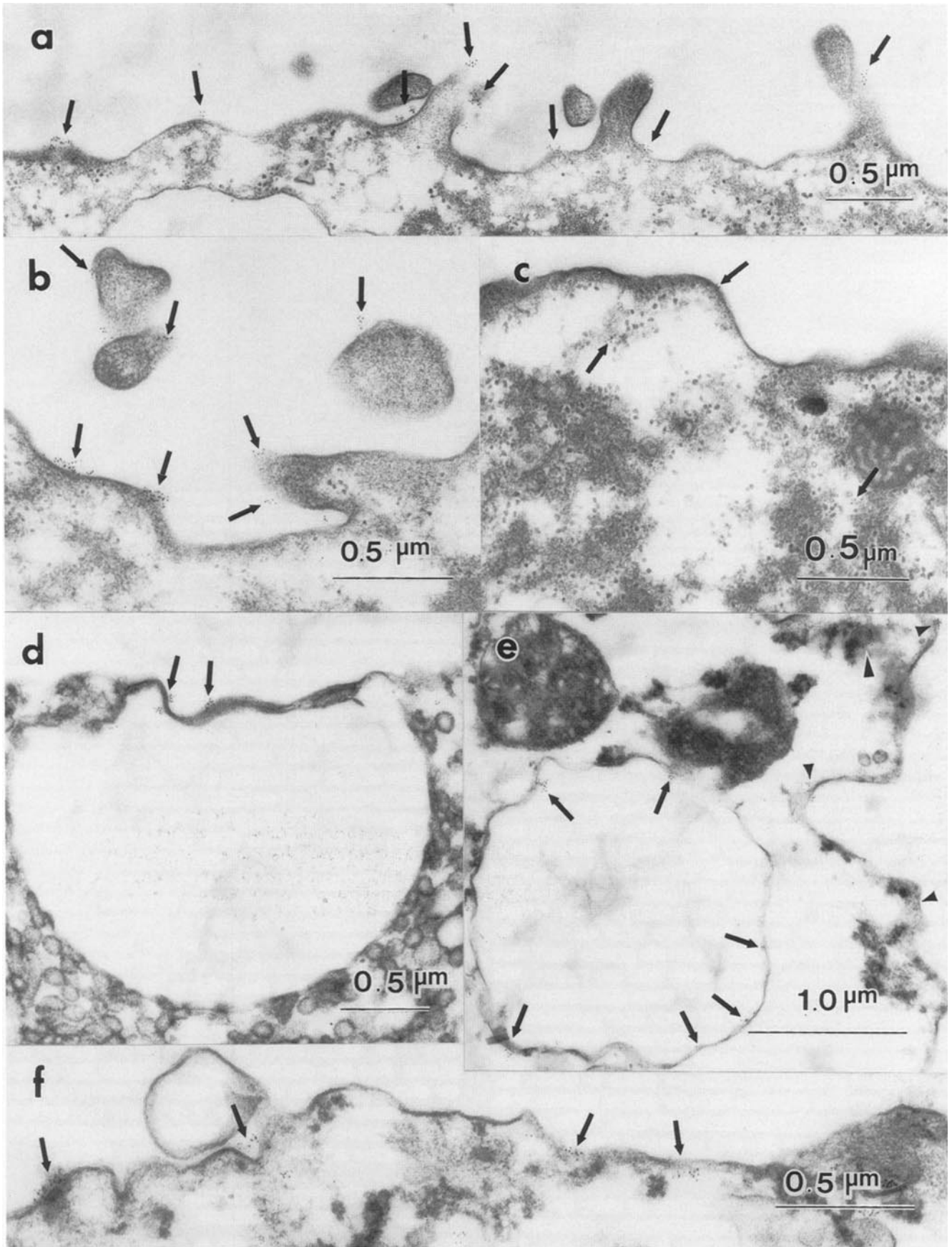


Table I. Immunoelectron Microscopic Localization of *Acanthamoeba* Myosin I Heavy Chain Kinase

	Particles/ μm	Particles/ μm^2	Percent on inner face	Total particles	Total area μm^2
Light permeabilization					
Plasma membrane					
Immune	12.9 \pm 4.2	1,290	43 \pm 15	1,754	1.38
Preimmune	0.36 \pm 0.35	36	0	29	0.68
Intermediate permeabilization					
Plasma membrane					
Immune	12.47 \pm 5.2	1,247	37 \pm 22	1,200	0.97
Preimmune	0.34 \pm 0.3	34	0	30	0.79
Cytoplasm					
Immune		18.0 \pm 4.9		1,660	96
Preimmune		2.2 \pm 0.6		36	19
Extensive permeabilization					
Plasma membrane					
Immune	10.83 \pm 3.3	1,083	69 \pm 22	741	0.69
Preimmune	0	0	0	0	0.23
Large internal vacuoles*					
Immune	10.7 \pm 2.1	1,070	47 \pm 17	461	0.4
Preimmune	0	0	0	0	0.26

These data were derived from measurements performed on a minimum of 10 cells from three different sample preparations. For statistical analysis, each cell was treated as a single sample and a mean was calculated for each. Thus, the numbers given in the table represent a mean of means. Membrane area was calculated as membrane length \times 10 nm. Membrane-associated gold particles included all particles within a zone 10 nm on either side of the membrane.

* Only seven of eleven vacuoles were labeled. Those seven were labeled to similar extents while the four others were completely unlabeled suggesting that the labeled and unlabeled vacuoles did not represent the same organelle. However, they could not be distinguished morphologically, possibly because of the poor preservation of structure after the extensive permeabilization that was required to label them. The data reported are mean values for the seven labeled vacuoles.

Immunogold Localization of Kinase

The presence of myosin I heavy chain kinase at the plasma membrane was confirmed at the higher resolution obtainable by immunogold EM (Fig. 3, *a, b, e, and f, arrows*). The gold label was closely associated with the plasma membrane (Fig. 3, *arrows*). Assuming that an average cell occupied a volume of 2,540 μm^3 and had a plasma membrane volume of 15.24 μm^3 (Clarke et al., 1988), the quantification of the distribution of gold label given in Table I could be used to calculate the approximate partition of myosin I heavy chain kinase between cytoplasm and plasma membrane. The cytoplasm contained \sim 16 particles/ μm^2 which with a section thickness of 80 nm translates to 200 particles/ μm^3 ; the plasma membrane contained about 1,200 particles/ μm^2 or 1.5×10^4 particles/ μm^3 . Therefore, in an average cell of volume 2,540 μm^3 , \sim 30% of the total gold label would be expected to have been associated with the plasma membrane which comprised only \sim 0.6% of the total cell volume. The cytoplasmic gold label appeared to occur predominantly in the cortical region of the cell. However, some gold label was also associated with large internal vacuoles; of eleven vacuoles studied, four were unlabeled while the remaining seven were labeled to \sim 1,000 particles/ μm^2 (Table I). Unlike myosin IC, which is extracted from the plasma membrane by extensive permeabilization (Baines and Korn, 1990), myosin I heavy chain kinase was still associated with the plasma membrane after extensive permeabilization (Fig. 3 *f* and Table I).

In any quantitative immunolocalization study there is an inherent risk that cytoplasmic antigen might be preferentially extracted and, thus, underestimated. To minimize error from this source, a number of different conditions were used. After mild saponin permeabilization, the antibody did not penetrate into the cell and the cytoplasm was more heavily la-

beled after immediate permeabilization than when extensive permeabilization conditions were used. The relatively large standard deviations in the data in Table I may be because of experimental variability but, alternatively, could reflect genuine variations dependent on the motile states of individual cells. An alternative approach, immunolocalization after low-temperature embedment, gave an unacceptably low signal to noise ratio. Also, attempts to support the immunolocalization data by determining the amount of sedimentable (membrane-associated) and nonsedimentable (cytoplasmic) kinase in cell homogenates gave highly variable results, possibly because bound kinase dissociated relatively easily.

The gold label at the plasma membrane was approximately equally distributed between the inner and outer faces (Table I). The observed label at the outer surface is probably, in part, a consequence of the level of resolution of the immunolocalization procedure and, in part, due to lability of the membrane-associated kinase to the detergent treatment. Antigen is not normally accessible at the outer cell surface as no labeling was detectable in nonpermeabilized cells.

No gold label was associated with the contractile vacuole (Fig. 3 *b*), mitochondria (Fig. 3 *e*) or the nucleus (not shown), even after extensive saponin permeabilization (data not shown). Cells that had been incubated with preimmune serum instead of antibody (Fig. 3 *c*), or that had not been permeabilized or had not been incubated with a primary antiserum (data not shown) showed very little labeling.

Binding of Unphosphorylated Kinase to Isolated Plasma Membranes

Because the extent of binding of kinase to plasma membranes was to be quantified by comparing the total kinase activity that remained in the supernatant after pelleting plasma membranes to that of the original mixture of membranes and

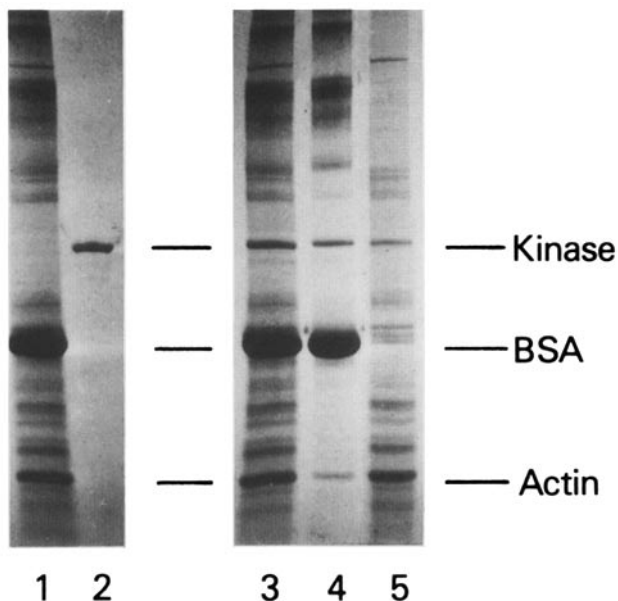


Figure 4. Binding of unphosphorylated myosin I heavy chain kinase to plasma membranes as evaluated by SDS-PAGE. Purified kinase (210 nM) was incubated with purified plasma membranes (120 $\mu\text{g/ml}$) and the mixture pelleted. Equivalent aliquots of the purified plasma membranes (lane 1), the purified kinase preparation (lane 2), the original mixture before centrifugation (lane 3), the supernatant after centrifugation (lane 4), and the pellet (lane 5) were separated by SDS-PAGE and stained with Coomassie blue. BSA was added to all samples that contained membranes. For other details, see Materials and Methods.

kinase, it was necessary to determine whether the enzymatic activity of the kinase was affected by the presence of plasma membranes. It was found that the presence of 120 $\mu\text{g/ml}$ of plasma membranes (the concentration present in the binding

assays) had no significant effect on the enzymatic activities of unphosphorylated or phosphorylated myosin I heavy chain kinase. Thus the enzymatic assay could be used to obtain relatively precise estimates of the affinity and capacity of the plasma membranes for kinase.

The binding of myosin I heavy chain kinase to plasma membranes is illustrated by SDS-PAGE in Fig. 4. All of the samples to which BSA had been added to prevent non-specific binding contained, in addition to the BSA, minor bands not seen in either the purified plasma membranes or kinase alone; these were minor contaminants in the BSA. The complete absence of BSA from the pelleted fraction (Fig. 4, lane 5) showed that the kinase in the pellet could not have been derived from trapped supernatant solution.

The kinase bound to plasma membranes in a saturable manner with high affinity (Fig. 5). The apparent K_D for binding (evaluated from the double reciprocal plot) was 17 nM and, at saturation, 0.8 nmol of exogenously added kinase was bound/mg of plasma membrane protein. However, binding of kinase to plasma membranes was not a simple equilibrium reaction in as much as the bound kinase was not released when the membranes were diluted (data not shown). The apparent binding constants for the kinase are very similar to those that had been obtained previously by Miyata et al. (1989) for the binding of myosins IA and IB to KI-extracted plasma membranes.

Although endogenous kinase was sometimes detectable on immunoblots of purified plasma membranes (Fig. 1, lane *d*), the concentration of endogenous membrane-bound kinase was always too little to detect by Coomassie blue staining (for example Fig. 4, lane 1) and much too low to have any effect on the quantification of the binding of exogenous kinase. Also, in control studies, it was found that kinase does not bind to F-actin nor does F-actin affect the autophosphorylation of kinase (not shown). Thus, the membrane-bound ac-

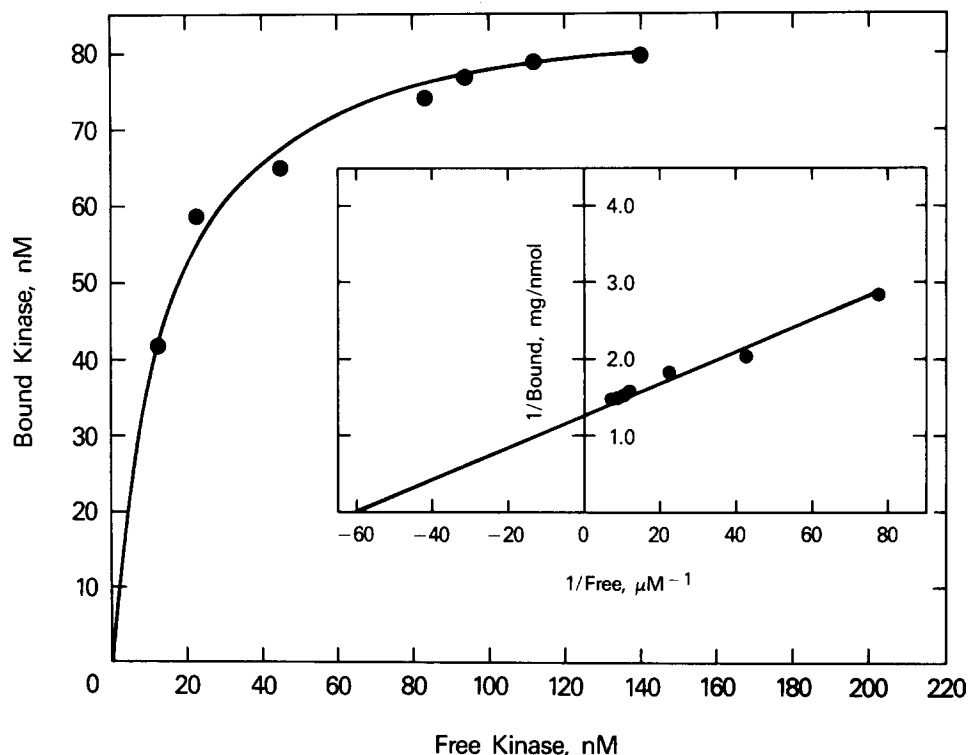


Figure 5. Quantitative analysis of the binding of unphosphorylated myosin I heavy chain kinase to plasma membranes. Purified kinase, at the concentrations shown, was incubated with purified plasma membranes (120 $\mu\text{g/ml}$), the membranes were pelleted by ultracentrifugation and the amount of bound and free kinase were quantified by measuring the kinase activity of the original mixtures before centrifugation and the supernatant solutions after centrifugation. Even though plasma membranes had no demonstrable effect on the kinase assay, membranes were nevertheless added at a concentration of 60 $\mu\text{g/ml}$ when the membrane-free supernatants were assayed for kinase activity so that all samples were identical in the assay. For further experimental details, see Materials and Methods. The inset shows the double reciprocal plot of the binding data from which the apparent K_D and binding capacity of the membranes were estimated (see text).

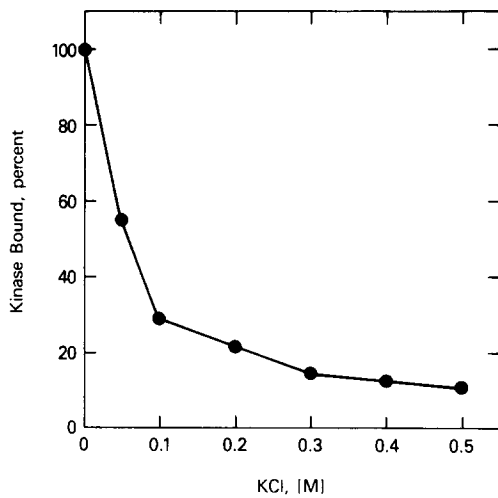


Figure 6. Influence of KCl on the binding of myosin I heavy chain kinase to plasma membranes. Purified kinase (140 nM) was incubated for 10 min with plasma membranes (120 $\mu\text{g}/\text{ml}$) in the presence of increasing amounts of KCl. The original sample and the supernatants and pellets obtained by ultracentrifugation were analyzed by SDS-PAGE and the amount of kinase bound to the plasma membranes was quantified by densitometric analysis of the gels and calculated as percent of that bound in the absence of KCl (100% = 0.61 nmol of kinase/mg of plasma membrane proteins).

tin would not have affected the binding studies. In other studies (data not shown), it was found that about the same amount of exogenous kinase bound to plasma membranes after treatment of the plasma membranes with either 1 M carbonate/bicarbonate buffer, pH 10.5, or 0.6 M KCl in the usual buffer,

both of which extracted about half of the membrane-associated proteins.

Effect of Ionic Strength and Kinase Phosphorylation on Binding of Kinase to Plasma Membranes

The effect of ionic strength on the binding of unphosphorylated kinase to plasma membranes is shown in Fig. 6. Binding was 50% inhibited by addition of ~ 55 mM KCl, but 2.5 mM ATP (substrate concentration) had no effect on binding (data not shown). Similarly, addition of 55 mM KCl to membranes with bound kinase resulted in the release of $\sim 50\%$ of the kinase but 1 mM ATP had no effect (see below and Fig. 10).

To test the effect of phosphorylation of the kinase on its ability to bind to plasma membranes, kinase was allowed to autophosphorylate and aliquots removed at 0, 3, 5, 10, 20, 40, and 60 min were assayed both for the extent of phosphorylation of the kinase and for the affinity of the kinase for plasma membranes. By 40 min, the kinase had been maximally phosphorylated, as determined by the fact that by SDS-PAGE it had shifted from an apparent mass of 97 to 107 kD (Fig. 7 A) and that the amount of ^{32}P incorporated was essentially the same after 40, 60, and 90 min of incubation (data not shown).

There was an apparently linear relationship between the amount of kinase that bound to plasma membranes and the level of phosphorylation of the kinase (Fig. 8). However, this is probably an oversimplified interpretation of the data because, as was reported previously (Brzeska et al., 1990a) and can be seen in Fig. 7 A, the partially phosphorylated ki-

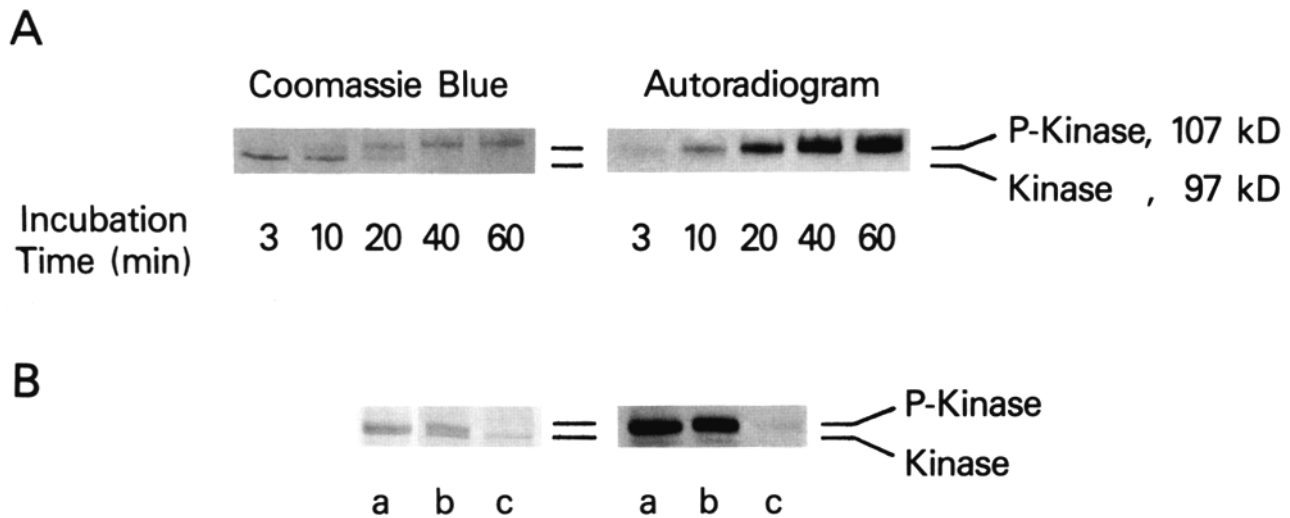


Figure 7. Influence of autophosphorylation of myosin I heavy chain kinase on its SDS-PAGE mobility and ability to bind to plasma membranes. (A) Samples of kinase that had been allowed to autophosphorylate for 3, 10, 20, 40, or 60 min (see Materials and Methods) were analyzed by SDS-PAGE. The gels were stained with Coomassie blue and then subjected to autoradiography. Only the portion of the gel showing the kinase band is reproduced; as highly purified kinase was used, there were no other detectable components on the gel. Phosphorylation caused a progressive increase in apparent mass until the final value of 107 kD was reached. The mean phosphorylation after incubation for 10 min was ~ 4 mol of P/mol of kinase and at 60 min ~ 15 mol of P/mol of kinase. (B) Kinase was autophosphorylated in the absence of plasma membranes (see Materials and Methods) to the extent of ~ 4 mol of P/mol. Then, phosphorylated kinase (310 nM) was added to plasma membranes (120 $\mu\text{g}/\text{ml}$) and the membranes were pelleted. Coomassie blue-stained and autoradiogram of SDS-PAGE of the original sample (a), supernatant (b), and pellet (c); only the regions corresponding to the kinase bands are shown. Note that the plasma membrane preferentially bound unphosphorylated kinase and that $\sim 20\%$ of the total kinase was bound (~ 0.5 nmol of kinase/mg of membrane protein).

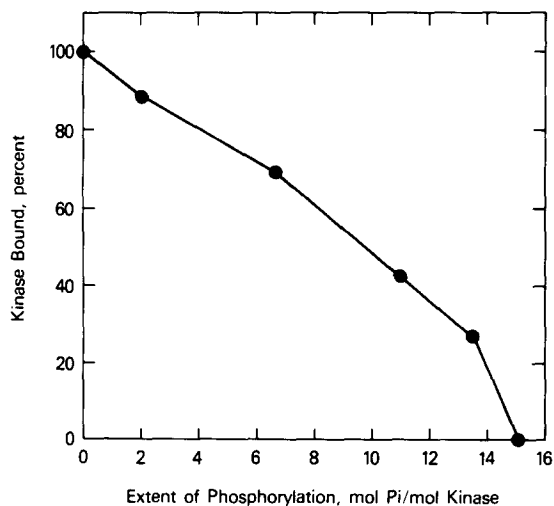


Figure 8. Effect of autophosphorylation of myosin I heavy chain kinase on its ability to bind to plasma membranes. Plasma membranes (120 $\mu\text{g/ml}$) were incubated with kinase (105 nM) that was either unphosphorylated or that had been phosphorylated for various periods of time as in the experiment described in Fig. 7. After incubation for 10 min, the plasma membranes were pelleted and the amount of bound kinase calculated from the kinase activity remaining in the supernatants. Other samples of the phosphorylated kinase were analyzed for the extent of phosphorylation. The data are plotted as percent of the amount of unphosphorylated kinase that bound to the membranes (100% = 0.52 nmol of kinase/mg of membrane protein).

nase consisted of a mixture of unphosphorylated molecules, with an apparent mass of 97 kD, and phosphorylated molecules, whose apparent masses increased to a final value of 107 kD. Note that after 10 and 20 min of incubation only some of the kinase had altered electrophoretic mobility and that the position of the higher band is intermediate between that of the unphosphorylated (0 min) and fully phosphorylated forms (60 min). The totally unphosphorylated molecules bound preferentially to the plasma membranes (Fig. 7 B); therefore, the binding curve shown in Fig. 8 probably does not signify a direct correlation between the extent of phosphorylation of a kinase molecule and its affinity for plasma membranes.

The relative inability of exogenously added phosphorylated kinase to bind to the plasma membranes was of interest in view of the demonstrated plasma membrane association of the kinase in situ. Therefore, we examined the effect of autophosphorylation on kinase that had been previously bound to membranes in the unphosphorylated state. Unexpectedly, the maximal extent of phosphorylation of membrane-bound kinase was much lower never exceeding (in three separate experiments) ~ 2 mol of phosphate/mol of kinase (Fig. 9). Significantly, the kinase did not dissociate from the membranes after it was autophosphorylated, even following prolonged incubation (Fig. 10). Thus, neither this level of phosphorylation nor substrate concentrations of ATP cause previously bound kinase to dissociate from the membranes. These results also indicate that kinase interacts with membranes differently than with phospholipid vesicles because in the latter case autophosphorylation is much more extensive (Brzeska et al., 1991a).

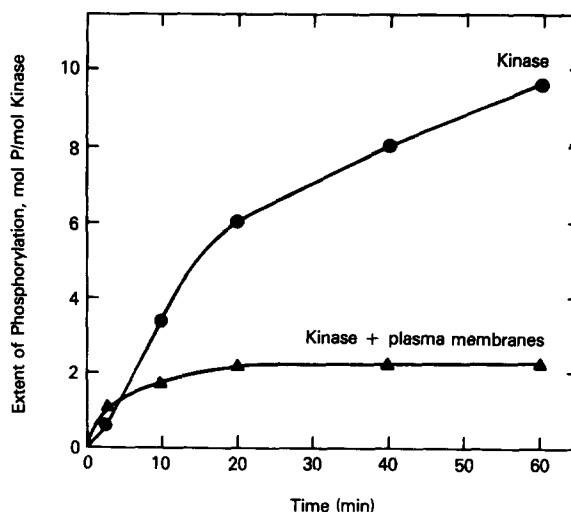


Figure 9. Autophosphorylation of plasma membrane-bound myosin I heavy chain kinase. Plasma membranes (120 $\mu\text{g/ml}$) were incubated with unphosphorylated kinase (101 nM) as described in the legend to Fig. 5. The membranes were pelleted, washed to remove unbound kinase, and resuspended at the original concentration of 120 $\mu\text{g/ml}$ in buffer containing 50 mM imidazole, pH 7.0, 2 mM EGTA, 2 mM MgCl_2 , 1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (200 cpm/pmol), and BSA (0.5 mg/ml). The concentration of membrane-bound kinase was determined to be 60 nM. An identical concentration of free kinase was incubated in the same phosphorylation buffer. Aliquots were removed from both samples at the indicated times and the extent of phosphorylation of the kinase was determined as described in Materials and Methods.

Discussion

The high density in situ of myosin I heavy chain kinase in the plasma membrane of *Acanthamoeba* is particularly interesting in light of the previous evidence that its substrate, myosin I, is also heavily concentrated at the plasma membrane of both *Acanthamoeba* (Gadasi and Korn, 1980; Baines and Korn, 1990; Miyata et al., 1989) and *Dictyostelium* (Fukui et al., 1989), in the latter case predominantly at the leading edges of locomoting, dividing, and phagocytic cells suggesting that at least one myosin I isoform may have a role in pseudopod extension. However, there is considerably more myosin I heavy chain kinase than myosin IC in the cytoplasm (compare data in Table I to data in Table I of Baines and Korn, 1990). Possibly, this cytoplasmic kinase can phosphorylate the myosin IC in contractile vacuole membranes (Baines and Korn, 1990), as these membranes appear to contain little if any kinase. In addition, recent experiments (I. C. Baines and E. D. Korn, unpublished results) indicate the presence of substantial myosin IA in the actin-rich cortex and the association of myosins IA and IB with intracellular vesicle membranes. Myosin I in these locations may be substrates of the cytoplasmic myosin I heavy chain kinase.

The apparently different electrophoretic mobilities of the plasma membrane-associated and cytoplasmic kinase fractions raises the possibility that the former may be in the phosphorylated, activated state while the latter is unphosphorylated and relatively inactive. Unfortunately, there was too little endogenous kinase associated with the purified plasma membranes to allow extraction of the kinase and determination of its specific enzymatic activity.

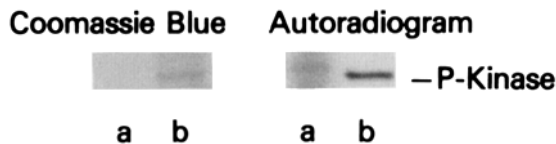


Figure 10. Nondissociation of membrane-bound myosin I heavy chain kinase after phosphorylation. Unphosphorylated kinase was bound to plasma membranes as described in the legend to Fig. 9 except that the initial kinase concentration was 250 nM. The membrane-bound kinase was then incubated for 40 min under phosphorylation conditions as described in the legend to Fig. 9. The concentration of plasma membranes was 120 $\mu\text{g}/\text{ml}$ and of membrane-bound kinase 78 nM; the final level of phosphorylation was 2 mol of P/mol of kinase. The membranes were then pelleted and the supernatant (a) and pellet (b) were subjected to SDS-PAGE and analyzed by Coomassie blue staining and autoradiography. Only the regions corresponding to the positions of the kinase bands are shown. The trace amount of phosphorylated kinase detectable in the supernatant by autoradiography represents the small amount of unbound kinase that was carried along from the initial binding step.

There is some similarity between the apparent affinities of myosin I heavy chain kinase and its substrate myosin I for isolated plasma membranes. The apparent K_D for the kinase, 17 nM, is similar to the K_D for myosin IA and IB, 30 and 50 nM, respectively, (Miyata et al., 1989), and the capacity of the isolated plasma membranes for kinase, 0.8 nmol/mg of membrane protein, is similar to their capacity for myosins IA and IB, 0.65 and 0.85 nmol/mg of membrane protein, respectively (Miyata et al., 1989). Myosin I can be dissociated from plasma membranes by elevated ionic strength and the binding of the kinase to membranes is also inhibited by increasing the ionic strength to >50 mM KCl and by prior autophosphorylation of the kinase, which might, similarly, be reflections of an electrostatic interaction between kinase and the membrane. Myosin I has been shown to bind with similar, although somewhat lower, affinity to liposomes containing acidic phospholipids (Adams and Pollard, 1989), suggesting that its binding to plasma membranes may be, at least in part, mediated in this way. The fact that phosphatidylserine and phosphatidylinositol greatly enhance the rate of autophosphorylation of myosin I heavy chain kinase (Brzeska et al., 1990a,b) suggests the possibility that the binding of kinase to plasma membrane may also be mediated through its affinity for acidic phospholipids. The lack of ready dissociation of bound kinase when the membranes were diluted might suggest, however, that secondary events occurred after binding, such as aggregation of acidic phospholipids at the initial binding sites or association of kinase with membrane proteins. Additional interactions such as these might explain the limited extent of autophosphorylation of membrane-bound kinase and the observation that it remains bound to the membrane even after phosphorylation.

Speculatively, then, one could imagine a chain of events in which binding of an extracellular or intracellular ligand to the plasma membrane would induce phospholipid stimulation of autophosphorylation of membrane-bound myosin I heavy chain kinase thus increasing its ability to phosphorylate the heavy chain of membrane-bound myosin I which would then exhibit the actin-activated Mg^{2+} -ATPase activity necessary to support motile activity. Protein phosphatase activity would be necessary to reverse the activation of both the myosin I and the kinases. Cytoplasmic kinase might be simi-

larly regulated by cytoplasmic phospholipids or intracellular membranes and then enhance the actin-activated Mg^{2+} -ATPase activity of cytoplasmic and intracellular vesicle-associated myosin I.

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