Identification of Myosin III as a Protein Kinase†

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Received January 24, 1996; Revised Manuscript Received May 8, 1996⊗

ABSTRACT: Drosophila ninaC gene encodes myosin homologous proteins which are classified as myosin III of the myosin superfamily, yet the physiological and biochemical function of myosin III has not characterized. We report here that myosin III does exhibit protein kinase activity. The kinase homologous domain (MYOIIIPK) of myosin III was expressed in the baculovirus expression system and purified to homogeneity. MYOIIIPK phosphorylated a number of proteins including myosin III p132 and smooth muscle myosin regulatory light chain (LC_{20}), suggesting that myosin III is a multifunctional protein kinase. The phosphoamino acid analysis revealed that myosin III is a serine/threonine kinase but not a tyrosine kinase. The observation that MYOIIIPK phosphorylates myosin III suggests that the autophosphorylation might play a role for the regulation of myosin III function. This is the first direct demonstration of kinase activity for the myosin III class.

The photoreceptor cells of Drosophila have a specialized organelle consisting of a stack of microvilli known as a rhabdomere, which contains phototransduction machinery (Tomlinson, 1988). Pak and co-workers (Pak, 1975) pioneered the use of extracellular recording of the electrical activity of the Drosophila eye as a screening assay to study mutants that specifically affect the phototransduction cascade. Drosophila photoreceptors undergo a prolonged depolarization afterpotential (PDA)[1] that persists after cessation of the light stimulus resulting from the stable conversion of rhodopsin to metarhodopsin in response to blue light. During PDA, photoreceptor cells become refractory to subsequent PDA-inducing stimuli, i.e., inactivation. Mutants that are defective for both inactivation and the prolonged depolarization afterpotential are termed neither inactivation nor afterpotential, i.e., nina mutants (Pak, 1979; Pak & Pinto, 1976).

Eight nina genes have been identified from mutations that reduce the amount of rhodopsin in *Drosophila melanogaster* photoreceptor cells (Matsumoto et al., 1987; Smith et al., 1991; Stephenson et al., 1983; Pak, 1979). Among them, the ninaC gene was originally identified on the basis of electroretinogram phenotype (Stephenson et al., 1983). The ninaC locus is one of eight nina complementation groups (ninaA−H) that affect a different gene involved in rhodopsin synthesis, structure, or metabolism (Matsumoto et al., 1987). The ninaC gene was subsequently cloned and found to encode for two photoreceptor specific proteins (NINAC, p132 and p174), both of which contain an amino terminal region with homology to protein kinases followed by a domain with homology to the myosin heavy chain motor domain (Montell & Rubin, 1988). Recent phylogenetic analysis of various myosin-like proteins revealed that NINAC is a member of the myosin superfamily (classified as myosin III), which currently consists of 11 distinct classes (Cheney & Mooseker, 1992; Cheney et al., 1993; Goodson & Spudich, 1993; Mooseker & Chenney, 1995). The ninaC proteins are the only known members of class III of the myosin superfamily so far identified. They also represent the most divergent members of the myosin superfamily (Cheney et al., 1993; Mooseker & Chenney, 1995). NinaC mutants exhibit a characteristic electrophysiological phenotype and light- and age-dependent retinal degeneration (Porter & Montell, 1993; Matsumoto et al., 1987; Porter et al., 1992). Systematic mutagenesis of the ninaC gene has revealed that the kinase homologous domain is necessary for the normal electrophysiological phenotype, whereas the myosin homologous domain is required for localization to, and maintenance of, the structural integrity of the rhabdomeres. Mutants with deletion of the entire kinase homologous domain or with changes in the putative kinase catalytic domain exhibit a retinal electrophysiology which is essentially identical to null ninaC mutants, suggesting that this domain is essential for normal phototransduction (Porter & Montell, 1993), but the actual role of ninaC in this process has yet to be characterized.

So far as we know, there has been no characterization of the biochemical properties of NINAC myosin III and no demonstration of whether it exhibits protein kinase activity or whether it is a motor protein. To address this problem, we have attempted to express the N-terminal kinase homologous domain (MYOIIIPK) and to determine whether it shows

† This work was supported by National Institutes of Health Grants AR 41653, HL 37117, and HL 47530 and by an American Heart Association Grant in Aid.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: S1, myosin subfragment 1; MYOIIIPK, protein kinase domain of myosin III; LC_{20}, 20 000 dalton smooth muscle myosin light chain; PDA, prolonged depolarization afterpotential; SDSPAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; MEK, mitogen activated protein kinase kinase; DMS, dimethyl sulberimidate; MLCK, myosin light chain kinase; PMA, phorbol 12-myristate 13-acetate.

S0006-2960(96)00181-X CCC: $12.00

protein kinase function. The present study provides evidence that myosin III does exhibit protein kinase activity.

MATERIALS AND METHODS

Proteins. Smooth muscle myosin was prepared from frozen turkey gizzards (Ikebe & Hartshorne, 1985b). S1 was prepared from myosin by *Staphylococcus aureus* protease digestion (Ikebe & Hartshorne, 1985b). Calmodulin was prepared from bull testes (Walsh et al., 1983). LC_{20} was prepared as described previously (Kamisoyama et al., 1994). The mutant LC_{20}s were prepared as described (Ikebe et al., 1994a,b). Caldesmon was prepared from frozen turkey gizzards (Bretscher, 1984). Tyrosine kinase specific peptide substrate (RRLIEDAEYAARG), myelin basic protein, casein, protamine, and phosphorylase *b* were obtained from Sigma Chemical Co., (St. Louis, MO). MEK was generously supplied from Dr. H. Karl (Case Western Reserve University Alzheimer's Disease Center).

Production of the Recombinant Baculovirus. cDNA of ninaC p132 was kindly supplied from Dr. Montell (Johns Hopkins University). The cDNA in pBluescript was first modified to create an *Nde*1 site at the 5′ initiation codon. For p132 myosin III expression vector, the vector containing myosin III cDNA was digested with *Nde*1/*Not*1 and the obtained 3.5 kb fragment containing the entire myosin III coding region was subcloned into PET23a (Novagen) using *Not*1/*Nde*1 sites. The cDNA insert was excised from the vector with *Xba*1/*Not*1 and subcloned into the pBluebacM Baculovirus transfer vector using unique *Nhe*1/*Not*1 sites in the multicloning sites which is localized downstream of the polyhydrin promotor. For MYOIIIPK, the pBluescript SKII containing NINAC p132 cDNA was digested with *Nde*1/*Eco*R1, and the resulting 1.2 kb fragment, containing the entire kinase domain, was subcloned into the pT7-7 vector using *Nde*1/*Eco*R1 site. A translational stop codon was created at amino acid residue 328 by site directed mutagenesis strategy as previously described (Deng & Nickoloff, 1992; Yano et al., 1993) so that the protein expressed is terminated at the site selected on the basis of homology alignment of the ninaC protein sequence with other known protein kinases. The MYOIIIPK cDNA from the pT7-7 vector was first shuttled into another *Escherichia coli* expression vector, pET-23a (Novagen), using *Nde*1/*Eco*R1 site. The cDNA insert of myosin III was then excised from the vector with *Not*1/*Xba*1 and subcloned into the pBluebacM Baculovirus transfer vector using unique *Nhe*1/*Not*1 sites. pBluebacM is a modified form of the commercially available pBluebacIII (Invitrogen), and it contains several additional restriction sites in the multicloning site. Following confirmation of the mutation sites by sequencing, the recombinant viruses were generated as described in O'Reiley et al. (1992). In brief, Sf9 insect cells were transfected with a mixture of linearized wild type Baculovirus DNA and the produced transfer vector by liposome mediated transfection. Recombinant viruses were identified by X-gal selection. The recombinant viruses were propagated for several times to increase the titer. The production of the recombinant viruses was confirmed by production of the 38 kDa recombinant protein analyzed by SDSPAGE, and its authenticity was confirmed by western blot analysis as described previously (Higashihara & Ikebe, 1987; Araki & Ikebe, 1991).

Production and Purification of MYOIIIPK. Sf9 cells were infected with the high titer recombinant virus (4 mL of the viral stock for Sf9 cells harvested from three 175 cm^2 culture flasks), and cells were cultured for 72 h at 28 °C in JRH Excell 401 insect cell culture medium (Sigma) supplemented with 5% fetal bovine serum (GIBCO). Cells were harvested by centrifugation (1000*g*) for 10 min. All purification procedures were carried out at 4 °C. Cell pellet (5*g*) was resuspended in 50 mL of extraction buffer (50 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM PMSF, 10 μg/mL leupeptin, 0.34 M sucrose), lysed by sonication (4 × 2 min on ice), and centrifuged (10000*g* for 30 min). The supernatant was subjected to 0−50% ammonium sulfate fractionation. The precipitated proteins were resuspended in 10 mL of buffer A (50 mM Tris-HCl, pH 8.0, 2 mM DTT, 0.2 mM PMSF, 0.34 M sucrose) and dialyzed into the same buffer. The sample was then loaded onto a DEAE-52 ion exchange column equilibrated with buffer A. The column was washed with 15 mL of buffer A and eluted with 150 mL of linear NaCl gradient (0−350 mM) in buffer A, and 1.5 mL fractions were collected. The flow rate was 10 mL/h. Fractions from DEAE-52 containing MYOIIIPK activity were pooled and concentrated by dialysis against saturated ammonium sulfate solution and centrifugation (10000*g* for 30 min). The precipitate was redissolved in 3 mL of buffer A′ (buffer A plus 1.0 M ammonium sulfate) and dialyzed into the same buffer. The sample was then loaded onto a butyl 650M column equilibrated with buffer A′. The column was washed with 10 mL of buffer A′ and eluted with a 100 mL reverse linear ammonium sulfate gradient (1.0 to 0.0 M) in buffer A′. 1.0 mL fractions were collected at a constant flow rate of 7 mL/h. Fractions containing MYOIIIPK activity were pooled and concentrated by ammonium sulfate precipitation and centrifugation (10000*g* for 30 min). The precipitate was resuspended in 1 mL of buffer A, dialyzed into the same buffer and stored at −20 °C in 0.1 mL aliquots.

Biochemical Procedures. (A) Protein Kinase Assay. DEAE-52 and butyl 650M fractions were desalted by a small Sephadex G-50 column as described Penefski (1977) before kinase assays were performed. G-50 treated fractions (20 μL) were added to 100 μL of kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 75 mM NaCl, and 0.2 mg/mL LC_{20}). For characterization of the enzymatic properties of MYOIIIPK, the purified kinase (1 mg/mL) was incubated at 25 °C in the kinase buffer. The kinase reaction was started by adding 40 μM [γ-^{32}P]ATP (600 cpm $pmol^{-1}$) at 25 °C. Data analysis was as previously described (Ikebe et al., 1990). The incorporation of ^{32}P into LC_{20} was determined as described previously (Ikebe & Hartshorne, 1985b).

(B) Phosphorylation of the Various Substrates. The following substrates (0.1 mg/mL concentrations): smooth muscle myosin subfragment 1 (S1), tyrosine kinase specific peptide (RRLIEDAEYAARG), myelin basic protein, LC_{20}, LC_{20} mutants: N-16, and S19A (see text for explanation), MEK, casein, caldesmon, calmodulin, protamine, phosvitin, and, phosphorylase *b* were incubated with MYOIIIPK (0.01 mg/mL) in kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 75 mM NaCl) plus 40 μM [γ-^{32}P]-ATP (600 cpm $pmol^{-1}$). To determine if MYOIIIPK phosphorylates NINAC p132, Sf9 cells infected with recombinant baculovirus expressing NINAC p132 were collected and lysed as previously described in Figure 1. Cell lysates

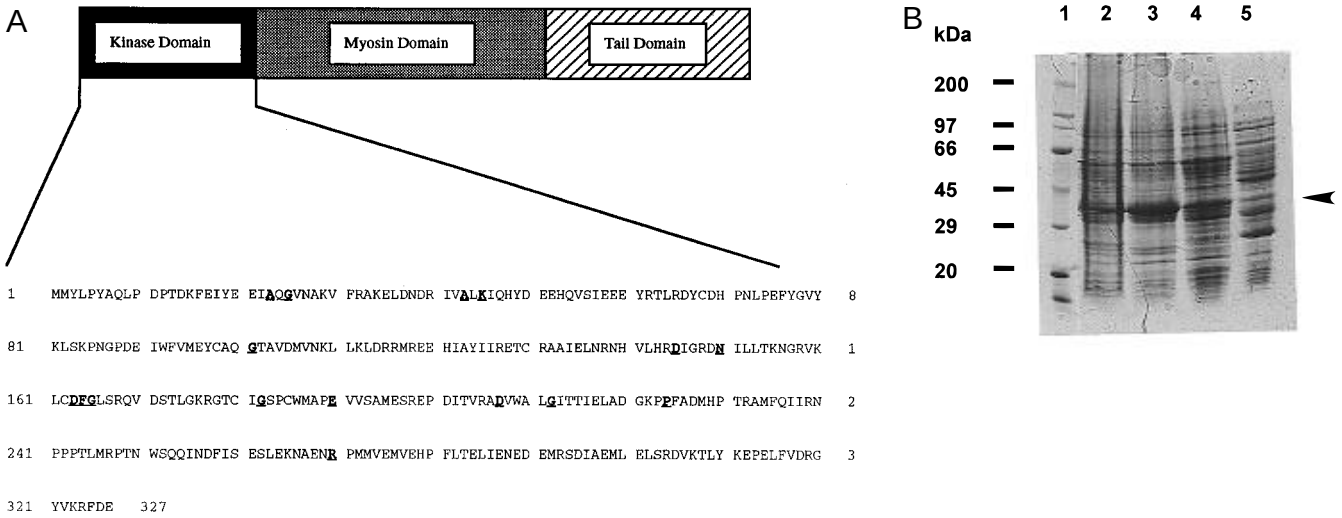

FIGURE 1: Construction and purification of MYOIIIPK. (A) Structure of NINAC myosin III p174 consisting of the amino terminus kinase domain, myosin domain, and tail domain. The deduced amino acid sequence of MYOIIIPK is indicated with 15 consensus residues found in all protein kinases underlined. (B) SDSPAGE of the crude extract and ammonium sulfate fractions of the Sf9 cells expressing the 38 kDa MYOIIIPK. Lane 1, molecular weight standard; lane 2, crude extract of MYOIIIPK expressing cells; lane 3, 0−35% saturated ammonium sulfate fraction; lane 4, 35−50% saturated ammonium sulfate fraction; lane 5, 50−100% saturated ammonium sulfate fraction. Arrowhead indicates 38 kDa MYOIIIPK.

were centrifuged (10000*g* for 30 min). The protein concentration of the supernatants was measured as described by Bradford (1976). Phosphorylation was performed by incubating MYOIIIPK (0.01 mg/mL) with the cell extracts (0.02 mg/mL) or LC_{20} (0.2 mg/mL) in kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 75 mM NaCl) plus 40 μM [γ-^{32}P]ATP (600 cpm pmol^{-1}) at 25 °C for 15 min. The reaction was terminated by addition of SDSPAGE sample buffer and boiling for 3 min.

(C) Production of Polyclonal Antibodies against Myosin III p132. Myosin III p132 cDNA was subcloned into PET23a *E. coli* expression vector, and the recombinant p132 were expressed in BL21 (DEL) strain in the presence of 1 mM IPTG. The cells expressing p132 were solubilized in 4% SDS with boiling and subjected to SDSPAGE. The p132 band was excised from the gel, electroeluted, and used as an antigen. The eluted protein was analyzed by SDSPAGE which confirmed its purity. The isolated p132 was injected into mice using standard procedure, and polyclonal antibodies were obtained. The antibodies were tested for their specificity using western blot analysis. The antibodies recognized p132 but did not react with other *E. coli* proteins. The proteins obtained from the untransformed BL21 (DEL) failed to react with the antibodies. The antibodies also did not react with the untransfected Sf9 cell proteins.

(D) Electrophoresis and Western Blotting. SDSPAGE analysis on 7.5−20% polyacrylamide gels was according to the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue G-250. Immunoblots were performed with anti-NINAC antibody (raised against bacterially expressed NINAC p132).

(E) Phosphoamino Acid Analysis. LC_{20} was phosphorylated by MYOIIIPK or MLCK, and the reaction was terminated by adding trichloroacetic acid (TCA) solution to a final concentration of 10%. The precipitated protein is collected by centrifugation (1000*g* for 10 min) and subjected to acid hydrolysis (6 N HCl at 110 °C for 3 h). Phosphoamino acid analysis was performed as previously described (Ikebe & Hartshorne, 1985a; Ikebe et al., 1987).

(F) Determination of the Molecular Mass. Sephacryl S200 column was equilibrated with 50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, and 0.2 mM DTT. The eluted fractions were assayed for kinase activity and SDSPAGE analysis. The column was calibrated with the following molecular weight standards: smooth muscle myosin (200 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), egg ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and smooth muscle regulatory light chain (20 kDa). The elution volumes of the protein standards were determined by SDSPAGE analysis. DMS cross-linking experiments were performed as previously described (Swaney & O'Brien, 1978).

RESULTS

Expression and Isolation of MYOIIIPK. A *ninaC* mutant cDNA construct encoding only the putative kinase domain (residues 1−327, Figure 1A) was made from *ninaC* cDNA clones obtained from Dr. Craig Montell (Johns Hopkins University, Baltimore) and used to express the kinase domain by the baculovirus expression system (O'Reiley, 1992). Figure 1B shows SDSPAGE of the crude extract of sf9 cells infected by the recombinant virus containing MYOIIIPK cDNA. A significant amount of 38 kDa peptide corresponding to MYOIIIPK was found. Most of the 38 kDa protein was extracted with the buffer containing physiological ionic strength, suggesting that the expressed protein is soluble. The crude extract was subjected to a series of purification steps (see Materials and Methods). Ammonium sulfate fractionation revealed that the 38 kDa peptide was recovered in the 0−50% fraction (Figure 1B); this fraction was applied to column chromatography. Protein kinase activity was monitored by examining the ability of aliquots from the effluent fractions to phosphorylate isolated LC_{20} (the preparation was free from any protein kinase contamination). Figure 2A shows DE52 ion exchange chromatography profile. The protein kinase activity was eluted at about 0.25 M NaCl and was found to coelute with the 38 kDa protein (Figure 2b) The 38 kDa protein was purified to homogeneity with butyl Sepharose chromatography (Figure 3). The protein kinase

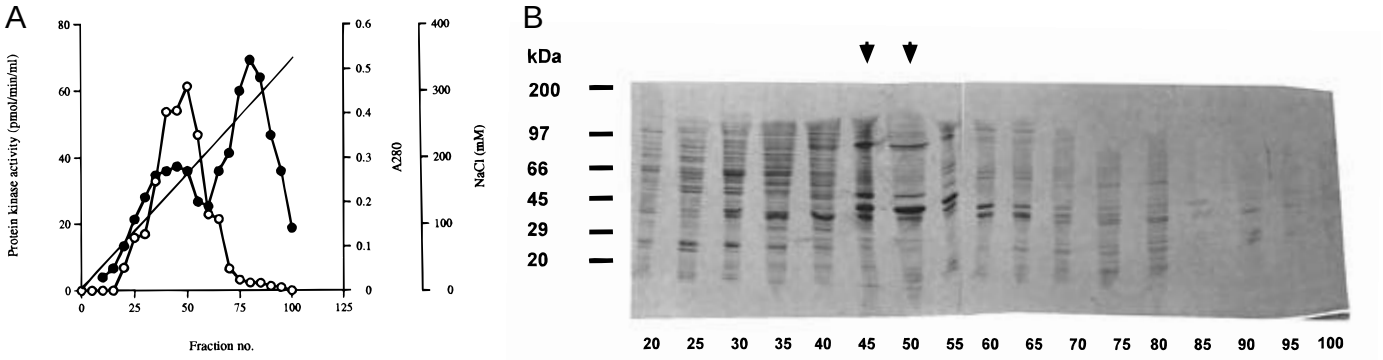

FIGURE 2: Purification of MYOIIIPK by DE52 ion exchange chromatography. (A) Elution profile of DE52 ion exchange chromatography. 0−50% ammonium sulfate fraction was loaded on a DE52 column (2.5 cm × 30 cm) equilibrated as described in Materials and Methods. After the unbound proteins were completely washed out (monitored by the absorbance at 280 nm), a linear NaCl gradient from 0 to 0.35 M (—) was applied to the column. The protein kinase activity of the fractions was measured as follows: 20 μL of each fraction desalted by a Sephadex G-50 column was added to 100 μL of kinase buffer. The reaction was started by adding 40 μM [γ-^{32}P]ATP (600 cpm/pmol) and terminated by adding 5% TCA. ○, protein kinase activity; ●, protein elution profile. The salt gradient is indicated by the diagonal line and the right-hand ordinate. (B) SDSPAGE analysis of DEAE-52 fractions. Arrows indicate peak MYOIIIPK activity fractions. 38 kDa protein was coeluted with protein kinase activity.

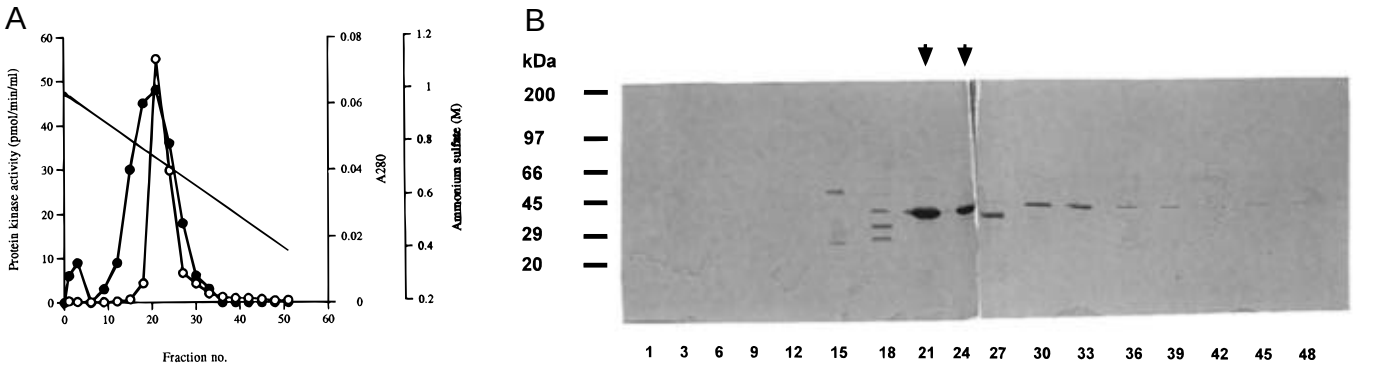

FIGURE 3: Purification of MYOIIIPK by butyl 650 M chromatography. (A) Elution profile of butyl 650M chromatography. DE52 column chromatography fractions containing MYOIIIPK activity were pooled and chromatographed on a butyl 650M column equilibrated with buffer A′. After the unbound proteins were washed out, MYOIIIPK was eluted with reverse ammonium sulfate gradient. ○, protein kinase activity; ●, protein elution profile. The salt gradient is indicated by the straight line and the right-hand ordinate. (B) SDSPAGE analysis of butyl 650M column. Arrows indicate peak MYOIIIPK activity fractions.

activity was coeluted precisely with the 38 kDa MYOIIIPK, suggesting that the kinase activity is associated with MYOIIIPK. To rule out the possibility that the kinase activity was associated with a contaminating endogenous kinase of the Sf9 cells, the protein was also subjected to Sephacryl S200 gel chromatography, and the kinase activity was again found to be associated with the 38 kDa protein (data not shown). Furthermore, the observation that no phosphorylation of LC_{20} was observed with lysates of noninfected Sf9 cells (data not shown) strongly indicates that the expressed MYOIIIPK, but not endogenous Sf9 cell derived protein kinases, is responsible for the phosphorylation of LC_{20}. The authenticity of the 38 kDa protein as MYOIIIPK was examined using anti-myosin III polyclonal antibodies (Figure 4). The antibodies recognized the 38 kDa MYOIIIPK, indicating that the purified 38 kDa peptide is indeed MYOIIIPK.

Characterization of MYOIIIPK. First, phosphoamino acid analyses were performed on LC_{20} phosphorylated by MYOIIIPK to see whether MYOIIIPK is a serine/threonine or tyrosine kinase. The data demonstrate that, with regard to this substrate, MYOIIIPK exhibits only serine/threonine kinase activity since no trace of tyrosine phosphorylation was observed (data not shown). The western blot analysis of phosphotyrosine revealed that phosphotyrosine was not detected with various substrate proteins phosphorylated by MYOIIIPK, suggesting that myosin III is a serine/threonine kinase and not tyrosine kinase in accord with phosphoamino acid analysis of LC_{20}.

The effects of various parameters on the kinase activity of MYOIIIPK was next examined, and the results are shown in Figure 5. NaCl concentration dependence showed biphasic behavior, and the activity decreased markedly above 150 mM NaCl, similar to that observed for other serine/threonine protein kinases. The kinase activity increases with $MgCl_2$ up to 5 mM, indicating that free Mg^{2+} is required for maximum activity since Mg^{2+}-ATP concentration should be constant under the conditions used. The pH optimum of MYOIIIPK activity resides in the alkaline region. K_{ATP} was 15.6 μM which was similar to other known protein kinases, and V_{max} for LC_{20} was 380 nmol/(min·mg).

Unconventional myosins can be classified into two groups based upon their ability to form a dimer. To see whether or not myosin III forms a dimer at its kinase homologous domain, the subunit structure of MYOIIIPK was studied by estimating the native molecular mass of the purified protein. MYOIIIPK was loaded on Sephacryl S200 gel permeation chromatography, and the elution position was determined both by the kinase activity and by SDSPAGE. The estimated molecular mass was 38 kDa (Figure 6), suggesting that MYOIIIPK exists as a monomer. A cross-linking approach was also utilized since chemical cross-linkers have been used

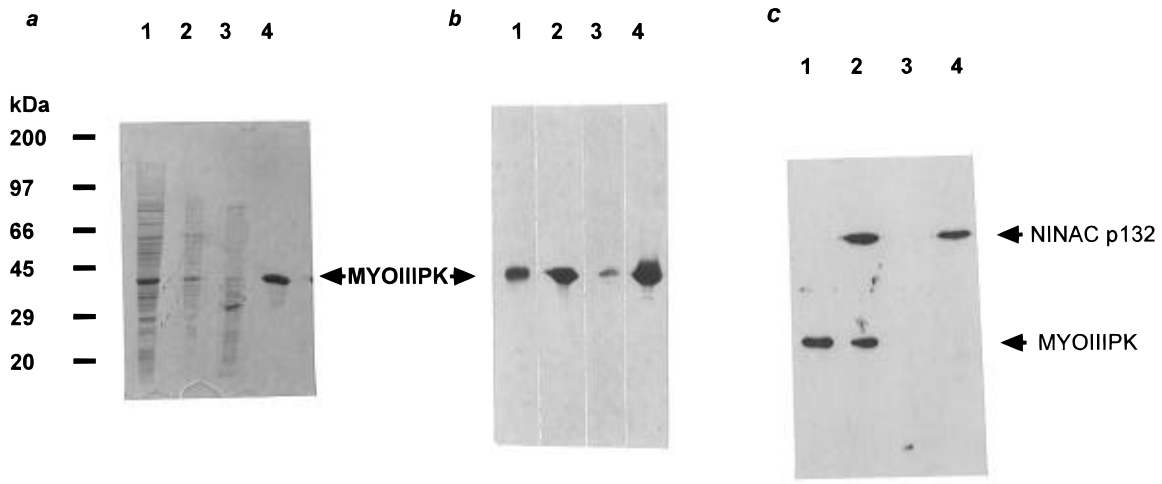

FIGURE 4: Isolation of MYOIIIPK. (a) SDSPAGE analyses of Sf9 cell extract after each purification step. Lane 1, total cell extract; lane 2, ammonium sulfate fraction; lane 3, pooled fraction of DE52 chromatography; lane 4, pooled fraction of butyl 650M chromatography. (b) Immunoblots of A with anti-myosin III antibody. (c) Immunoblots of Sf9 cell extracts with anti-myosin III antibody. MYOIIIPK alone (lane 1), myosin III p132 Sf9 cell extract with purified MYOIIIPK (lane 2), Sf9 cell extract (lane 3), and myosin III p132 Sf9 cell extract (lane 4).

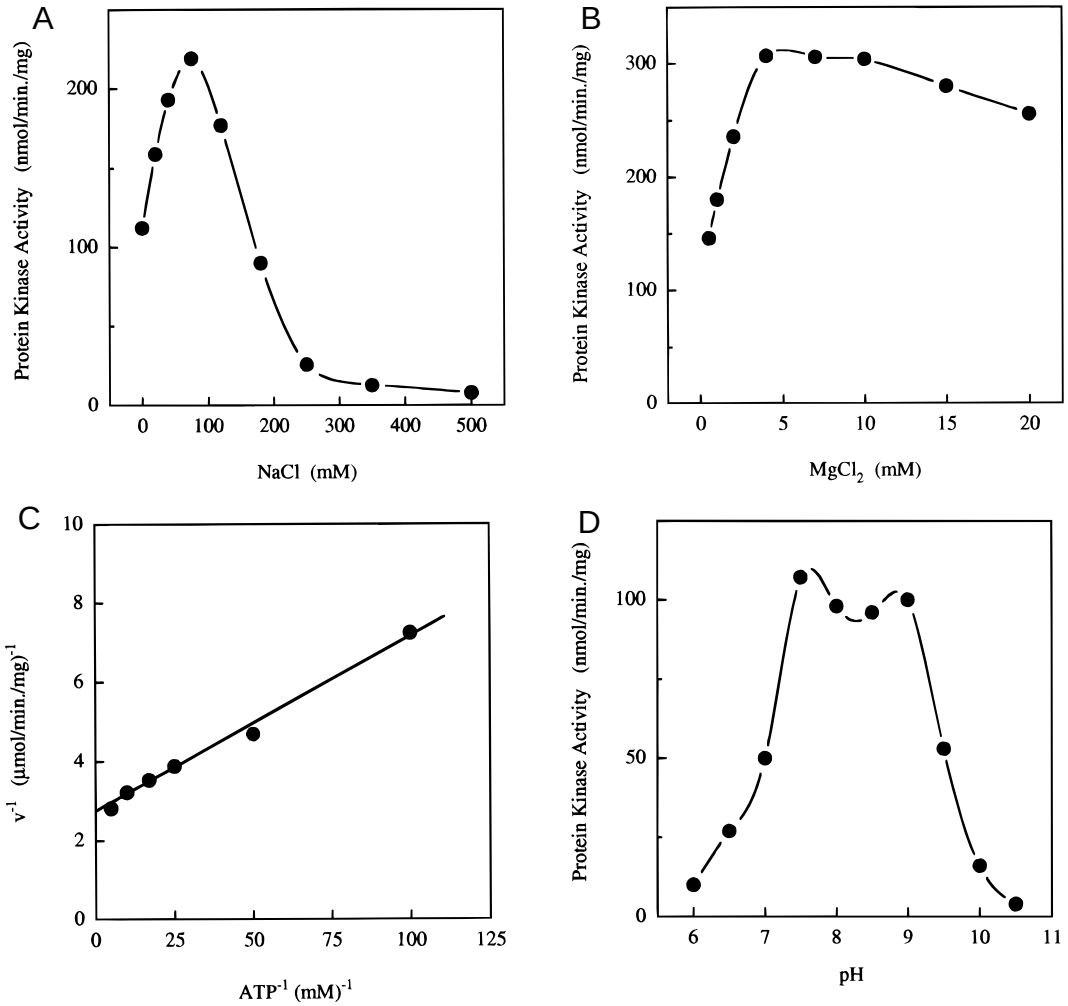

FIGURE 5: Characterization of MYOIIIPK enzyme activity. (A) NaCl dependence. The protein kinase activity was measured as a function of NaCl concentration under the conditions described in Materials and Methods using LC_{20} as a substrate. (B) $MgCl_2$ dependence. Conditions were the same as above except that various concentrations of $MgCl_2$ and 75 mM NaCl were used. (C) ATP dependence. Conditions were the same as above except that various ATP concentrations, 2 mM $MgCl_2$, and 75 mM NaCl were used. (D) pH dependence. Buffers used are imidazole-HCl (pH 6.0−7.0), Tris-HCl (pH 7.5−9.0), and glycine-HCl (pH 9.5−10.5). Conditions are as described in Materials and Methods.

for determining holoenzyme substructure (Swaney & O'Brien, 1978; Wientzeki et al., 1994). The cross-linking experiment failed to detect any multimeric forms of MYOIIIPK, suggesting that MYOIIIPK exists as a monomer with a molecular weight of about 38 kDa consistent with the gel filtration analysis.

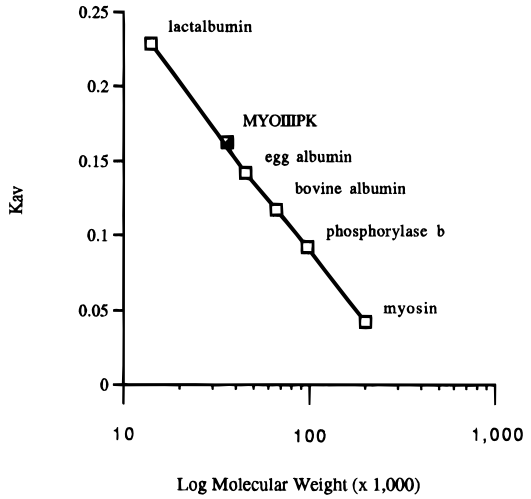

FIGURE 6: Determination of native molecular mass of MYOIIIPK. Purified MYOIIIPK was applied to Sephacryl S200 gel permeation chromatography, and the elution position was determined as described in Materials and Methods.

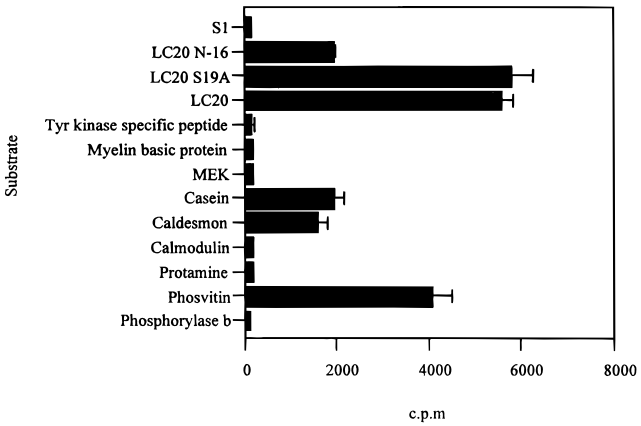

FIGURE 7: Substrate spectrum of MYOIIIPK. Substrate specificity of MYOIIIPK was determined by its ability to phosphorylate various common protein kinase substrates. 0.1 mg/mL of the following substrates: smooth muscle myosin subfragment 1 (S1), tyrosine kinase specific peptide (RRLIEDAEYAARG), myelin basic protein, LC_{20}, LC_{20} mutants: N-16 and S19A (see text for explanation), MEK, casein, caldesmon, calmodulin, protamine, phosvitin, and phosphorylase *b* were incubated with MYOIIIPK (0.01 mg/mL) in kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 75 mM NaCl) plus 40 mM [γ-^{32}P]ATP (600 cpm pmol^{-1}). The reaction was carried out as described in Materials and Methods.

Activation of vertebrate smooth muscle and nonmuscle conventional myosin motor function requires phosphorylation of Ser-19/Thr-18 of LC_{20} by myosin light chain kinase (MLCK) (Ikebe & Hartshorne, 1985a; Ikebe et al., 1986), while phosphorylation at Ser-1, Ser-2, and Ser-19 by either protein kinase C or cdc2 kinase decreases smooth muscle myosin's affinity for actin (Nishikawa et al., 1984; Ikebe et al., 1987; Begnur et al., 1987). Mutant LC_{20} proteins were used to delineate the LC_{20} phosphorylation targets for MYOIIIPK (Figure 7). Ser-19 is not a target for MYOIIIPK since the Ser-19 to Ala-19 mutation in LC_{20} (S19A) did not reduce the extent of phosphorylation. A significant decrease in phosphorylation, however, was observed with the mutant lacking the N-terminal sixteen residues (N-16), suggesting that at least one of the target residues may reside in this segment. Alternatively, a conformational change in LC_{20} accompanying this deletion could alter the accessibility of the normal target residues to the kinase. In contrast to the

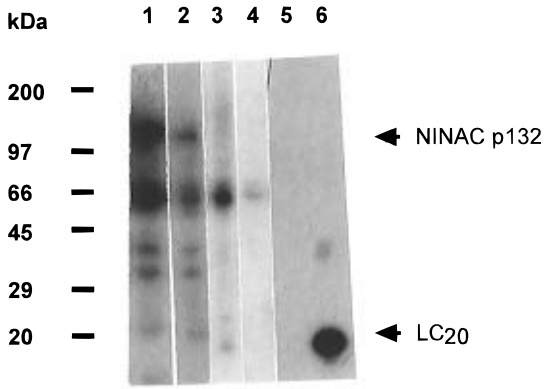

FIGURE 8: Phosphorylation of myosin III by MYOIIIPK. Sf9 cells were infected with recombinant virus expressing p132, and p132 was extracted using the same method for extraction of MYOIIIPK described in Materials and Methods. Phosphorylation was carried out as described in Figure 7. The extract of Sf9 cells expressing p132 (lane 1) or the extract of Sf9 cells without expressing p132 (lane 3) or LC_{20} (lane 5) was incubated with isolated MYOIIIPK in the presence of [γ-^{32}P]ATP. The Sf9 cell extracts expressing p132 (lane 2) or Sf9 cell extract without expressing p132 (lane 4) or LC_{20} (lane 6) was also incubated with [γ-^{32}P]ATP in the absence of MYOIIIPK. The reaction was stopped by 5% TCA, and the precipitates were subjected to SDSPAGE followed by autoradiography using Kodak O-mat (AR) film with intensifying screen. The amount of proteins loaded on the gel was the same for lanes 1−4, and the exposure time for the autoradiography was the same for all six lanes.

behavior observed for MLCK, where both free or heavy chain associated LC_{20} are phosphorylated, MYOIIIPK was unable to phosphorylate heavy chain associated LC_{20} (Figure 7), indicating that the target sites in free LC_{20} are occluded when the light chain is integrated into the myosin molecule and that conventional myosin is unlikely to be a physiological substrate for this enzyme. Substrate specificity of MYOIIIPK was examined using a number of proteins which are known to be substrates of serine/threonine kinases. Among the protein substrates tested, casein, caldesmon, and phosvitin were found to be good substrates for MYOIIIPK (Figure 7). The ability to phosphorylate a number of protein substrates suggests that MYOIIIPK may be a multifunctional protein kinase.

While the endogenous substrate(s) are yet to be characterized, MYOIIIPK can phosphorylate NINAC myosin III p132 (Figure 8). The incubations of Sf9 cell extracts expressing myosin III p132 with MYOIIIPK showed a marked increase in phosphorylation of p132 (Figure 8, lane 1). The lower but detectable levels of myosin III p132 phosphorylation were observed without addition of MYOIIIPK (Figure 8, lane 2), and this probably reflects myosin III p132 autokinase activity since the phosphorylation of several other bands which were not detected in untransfected Sf9 cell extract was also observed for the p132 expressing cell extract (Figure 8, lane 2). The phosphorylated band at the p132 position was completely diminished in untransfected cell extract (lanes 3 and 4), indicating that the phosphorylated protein is p132 myosin III. It should be noted that the phosphorylation of a 66 kDa protein was also increased by addition of MYOIIIPK. MYOIIIPK itself was not autophosphorylated (Figure 8, lane 3), and this was also confirmed in the absence of the cell extract (data not shown).

A number of activators of protein kinases such as cAMP, cGMP, phosphatidylserine/PMA, and Ca^{2+}/calmodulin were examined for their ability to stimulate MYOIIIPK kinase

activity, but none were found to be effective (not shown). The possibility that MYOIIIPK activity is regulated by phosphorylation by other kinases was also tested, but none of the kinases tested (mitogen activated protein kinase, cdc2 kinase, protein kinase C (PKC), cAMP-dependent protein kinase, calmodulin-dependent kinase II) were capable of phosphorylating MYOIIIPK (not shown).

DISCUSSION

The N-terminal 38 kDa domain of myosin III was expressed in Sf9 cells using Baculovirus expression system. The expressed 38 kDa peptide (MYOIIIPK) was isolated by a series of purification steps and found to be a protein kinase. The following results indicate that the kinase activity is associated with MYOIIIPK and not by contaminating protein kinases. (1) In each purification step, the protein kinase activity is precisely associated with the presence of MYOIIIPK. (2) No phosphorylation of LC20 was observed with lysates of noninfected Sf9 cells. (3) The 38 kDa peptide was recognized by anti-myosin III antibodies. (4) The 38 kDa peptide phosphorylated myosin III. The present study is the first report which directly demonstrates myosin III to be a protein kinase.

Protein kinases are classified into two groups according to their substrate specificity, i.e., serine/threonine kinases and tyrosine kinases (Hanks et al., 1988). Phosphoamino acid analysis of LC_{20} phosphorylated by MYOIIIPK revealed that serine residues and not tyrosine residues were phosphorylated. Furthermore, anti-tyrosine antibodies failed to detect phosphotyrosine in the various protein substrates phosphorylated by MYOIIIPK. These results indicate that myosin III is a serine/threonine protein kinase. Hanks et al. (1988) found that subdomains VI and VIII of the catalytic domain of protein kinases contain residues which are conserved in either protein serine/threonine or protein tyrosine kinases. For serine/threonine kinases, DLKPN in subdomain VI and GTSXXYFXAPE in subdomain VIII are found to be consensus sequence while DLAARN or DLRAAN in subdomain VI and PI/VK/RWT/MAPE in subdomain VIII are found in protein tyrosine kinases. The sequences of the corresponding region of MYOIIIPK are DIRGDN and GSPCWMAPE. Although the sequence in subdomain VI does not fit with the consensus sequence proposed by Hanks et al. (1988), the sequence in subdomain VIII fits well with serine/threonine kinase consensus sequence (especially a serine residue in the sequence could be critical). Quite recently, it was reported (Katz et al., 1994) that 97 kDa protein kinase cloned from B cells is most homologous to NINAC protein. This kinase, referred to as GC kinase, was identified to be a serine/threonine protein kinase. The present finding that myosin III is a serine/threonine protein kinase is consistent with this finding.

The fact that MYOIIIPK can phosphorylate various protein substrates suggests that myosin III is likely to be a multifunctional protein kinase. As the two isoforms of myosin III, i.e., p174 and p132, are differently localized in phototransducing cells (Porter et al., 1992), they may phosphorylate distinct proteins in the cells. Of particular interest is that MYOIIIPK phosphorylates myosin III. Because MYOIIIPK does not phosphorylate itself, the phosphorylation sites would be either myosin homologous domain or tail domain. Although the effect of the phosphorylation of myosin III on its function is unclear, this finding opens the possibility that autophosphorylation is involved in the regulation of myosin III function. Autophosphorylation is known to be an important regulatory mechanism of a number of kinases such as receptor tyrosine kinases and calmodulin-dependent protein kinases II and IV (Hanson & Shulman, 1992; Frangakis et al., 1991; Ullich & Schlessinger, 1990), and thus the autophosphorylation of myosin III may function as a regulatory mechanism of its protein kinase activity. Alternatively, the phosphorylation may affect the motor function of myosin III, as the motor functions of various types of myosins are known to be regulated by serine/threonine phosphorylation, such as smooth muscle myosin, regulated by phosphorylation at its regulatory light chain subunit; amoeba myosin II, regulated by the phosphorylation of the heavy chain at the tail domain; amoeba myosin I, regulated by heavy chain phosphorylation at the motor domain. However, in the absence of any defined function for myosin III, the physiological role of myosin III autokinase activity has yet to be determined.

Phosphorylation is an important control mechanisms in signal transduction. The present work, demonstrating that the MYOIIIPK of NINAC is a functional protein kinase, suggests that it may play a role in the phototransduction cascade. Although motor function has yet to be established for myosin III, the demonstration that its MYOIIIPK does exhibit kinase activity and that myosin III can undergo autophosphorylation suggests that phosphorylation regulation of its putative motor function is a realistic possibility.

REFERENCES

Araki, Y., & Ikebe, M. (1991) *Biochem. J. 275*, 679−684.

Bement,W. M., & Mooseker, M. S. (1993) *Nature 365*, 785−786.

Bengur, A. R., Robinson, A. E., Apella, E., & Sellers, J. R. (1987) *J. Biol. Chem. 262*, 7613−7617.

Bradford, M. (1976) *Anal. Biochem. 72*, 248−251.

Cheney, R. E., & Mooseker, M. S. (1991) *Curr. Opin. Cell. Biol. 4*, 27−35.

Cheney, R. E., Riley, M. A., & Mooseker, M. S. (1993) *Cell Motil. Cytoskeleton 24*, 215−223.

Deng, W. P., & Nickoloff, J. A. (1992) *Anal. Biochem. 200*, 81−88.

Frangakis, M. V., Ohmstede, C. A., & Sahyoun, N. (1991) *J. Biol. Chem. 266*, 11309−11316.

Goodson, H. V., & Spudich, J. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 215−223.

Hanks, S. K., Quinn, A. M., & Hunter, T. (1988) *Science 241*, 42−52.

Hanson, P. I., & Schulman, H. (1992) *Annu. Rev. Biochem. 61*, 559−601.

Hicks, J. L., & Williams, D. S. (1992) *J. Cell Sci. 101*, 247−254.

Higashihara, M., Young-Frado, L., Craig, R., & Ikebe, M. (1989) *J. Biol. Chem. 264*, 5218−5225.

Ikebe, M., & Hartshorne, D. J. (1985a) *J. Biol. Chem. 260*, 10027−10031.

Ikebe, M., & Hartshorne, D. J. (1985b) *J. Biol. Chem. 260*, 13146−13153.

Ikebe, M., Hartshorne, D. J., & Elzinga, M. (1986) *J. Biol. Chem., 261*, 36−39.

Ikebe, M., Hartshorne, D. J., & Elzinga, M. (1987) *J. Biol. Chem. 262*, 9569−9573.

Ikebe, M., Reardon, S., Scott-Woo, G. C., Zhou, Z., & Koda, Y. (1990) *Biochemistry 29*, 11242−11248.

Ikebe, M., Ikebe, R., Kamisoyama, H., Reardon, S., Schwonek, J. P., Sanders, C. R., & Matsuura, M. (1994a) *J. Biol. Chem. 269*, 28173−28180.

Ikebe, M., Reardon, S., Mitani, Y., Kamisoyama, H., Matsuura, M., & Ikebe, R. (1994b) *Proc. Natl. Acad. Sci. U.S.A. 96*, 9096−9100.

Kamisoyama, H., Araki, Y., & Ikebe, M. (1994) *Biochemistry 33*, 840−847.
Katz, P., Whalen, G., & Kehl, J. H. (1994) *J. Biol. Chem. 269*, 16802−16809.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Matsumoto, H., Isono, K., Pye, Q., & Pak, W. L. (1987) *Proc. Natn. Acad. Sci. U.S.A. 84*, 985−989.
Montell, C., & Rubin, G. M. (1988) *Cell 52*, 757−772.
Mooseker, M. S., & Cheney, R. E. (1995) *Annu. Rev. Cell Rev. Biol. 11*, 633−675.
Nishikawa, M., Sellers, J. R., Adelstein, R. S., & Hidaka, H. (1984) *J. Biol. Chem. 259*, 8804−8814.
O'Reiley, D. R. *Baculovirus Expression Vectors: a Laboratory Manual*, W. H. Freeman, New York.
Pak, W. L. (1975) Mutants affecting the vision of Drosophila melanogaster, in *Handbook of Genetics* (King, P. C., Ed.) pp 703−733.
Pak, W. L. (1979) Study of photoreceptor function using Drosophila mutants, in *Neurogenetics: Genetic Approaches to the Nervous System* (Breakfield, X. O., Ed.) pp 67−99, Elsevier/North-Holland, New York.
Pak. W. L., & Pinto, L. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 397−448.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.
Porter, J. A., & Montell, C. (1993) *J. Cell Biol. 122*, 601−612.
Porter, J. A., Hicks, J. L., Williams, D. S., & Montell C. (1992) *J. Cell Biol. 116*, 683−693.
Smith, D. P., Stamnes, M. A., & Zuker, C. S. (1991) *Annu. Rev. Cell Biol.* **7**, 161−190.
Stephenson, R. S., O'Tousa, J., Scavarda, N. J., Randall, L. L., & Pak, W. L. (1983) Drosophila mutants with reduced rhodopsin content, in *The Biology of Photoreception* (Cosens, D. J., & Vince-Price, D., Eds.) pp 477−501, Cambridge University Press, Cambridge.
Swaney, J. B., & O'Brien, K. (1978) *J. Biol. Chem. 253*, 7069−7077.
Tomlinson, A. (1988) *Development 104*, 183−193.
Ullrich, A., & Schlessinger, J. (1990) *Cell 61*, 203−212.
Walsh, M. P., Hinkins, S., Dabrowska, R., & Hartshorne, D. J. (1983) *Methods Enzymol. 99*, 279−288.
Wientzek, M., Kay, C. M., Oikawa, K., & Ryan, R. O. (1994) *J. Biol. Chem. 269*, 4605−4612.

BI960181A