

In addition, we find that DND41 cells do not transcribe α -TCR, because a ^{32}P -labelled $\text{C}\alpha$ probe failed to detect messenger RNA transcripts in northern blots (Fig. 3a, lanes 4 and 5). Indeed, the $J\alpha$ region is in germline configuration on both chromosomes in this cell line^{23,24}. Karyotype analysis of DND41 cells revealed no evidence for translocations or inversions involving either chromosome 7 (containing the β - and γ -TCR loci) or chromosome 14 (containing the δ - and α -TCR loci), indicating that hybrid receptor chains should not occur in these cells (data not shown). Taken together, these results indicate that the heterodimer on DND41 cells is composed of normal β - and δ -TCR subunits and does not include either γ -TCR or α -TCR chains.

We then determined whether the $\beta\delta$ TCR-CD3 complex could be functional and transduce an activation signal to the cell. Indo-1-loaded DND41 cells were labelled with anti-TCR δ 1 or anti-CD3 antibodies and their TCRs crosslinked using mouse immunoglobulin-specific antiserum while continuously monitoring the cytoplasmic free Ca^{2+} concentration. Both antibodies induce a transient increase in the internal concentration [Ca^{2+}]_i in the presence of crosslinking antiserum (Fig. 1b). There was no such change in [Ca^{2+}]_i after crosslinking of CD1a molecules or transferrin receptors (Fig. 1b, and data not shown).

The association of β -TCR with δ -TCR we describe here was unexpected, because to date only α -TCR has been found with β , and γ -TCR with δ on T-cell surfaces^{25,26}. In fact, in PEER cells it has been shown that when β -, γ - and δ -TCR are synthesized in the same cell, only the $\gamma\delta$ TCR pair is expressed on the cell surface^{5,27}. We assume that β -TCR is able to physically pair with δ because of certain structural features shared by δ -TCR and α -TCR, such as their constant domain sizes and their spacing of basic residues in the proposed transmembrane region¹³. However, the pairing of β - with δ -TCR (rather than with α) may occur only in the absence of their preferred partners. There is a similarly preferred association for isotypically matched major histocompatibility complex class II A α A β and E α E β pairs, isotype-mixed A β E α dimers probably occurring only when the preferred partners are unavailable²⁸. During T-cell development δ -, γ - and β -TCR may be expressed at the same time, before α -TCR rearrangement²⁶. It is possible that developing thymocytes which fail to productively rearrange their γ -TCR may synthesize β - and δ -TCR subunits and express these stably on the cell surface, as happens on DND41 cells (Fig. 4).

The issue of whether $\gamma\delta$ -TCR and $\alpha\beta$ -TCR result from sequential rearrangements of the TCR loci²⁹ or as separate lineages^{12,30} is controversial. The potential occurrence of a $\beta\delta$ -TCR provides a further level of complexity in these T-cell lineage relationships. Nevertheless, as a δ -TCR containing heterodimer, the events which operate to separate δ -TCR and α -TCR expression are likely to apply similarly to $\gamma\delta$ - and $\beta\delta$ -bearing cells, both of which require the α -locus to be in germline configuration and the δ -locus to be rearranged. The ability of δ -TCR and α -TCR to pair with β -TCR seems to be managed during development. Mechanisms controlling gene expression, the organization of the α/δ -TCR locus, and physical properties that govern chain pairing together are crucial features in regulating the frequency of expression of $\alpha\beta$ TCR, $\gamma\delta$ TCR and potentially $\beta\delta$ TCR. Further studies will be necessary to determine the occurrence and function of cells expressing the $\beta\delta$ TCR.

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Binding of myosin I to membrane lipids

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THE single-headed myosins called myosin-I were first isolated from the protozoan *Acanthamoeba*¹ and subsequently identified in other cells (reviewed in ref. 2). We previously reported evidence that myosin-I is responsible for the movement of membranes, extracted from *Acanthamoeba*, along actin filaments *in vitro*³. Here we show for the first time that myosin-I can bind directly to NaOH-extracted membranes isolated from *Acanthamoeba* and to vesicles of pure lipids with an affinity sufficient for extensive binding in the cell. Membrane-bound myosin-I may provide a mechanism for many cellular movements previously thought to involve filamentous myosin-II.

Although myosin-I was originally isolated as a soluble enzyme, four lines of evidence suggest a membrane association. First, fluorescent-antibody localization⁴⁻⁶ showed *Acanthamoeba* myosin-I concentrated near the plasma membrane. Second, the radial spoke between the actin filament core and the surrounding plasma membrane in intestinal microvilli⁷ has been identified as myosin-I (see ref. 8 for a review). Third, membranous vesicles isolated from *Acanthamoeba* can move along actin filaments via an attached motor tentatively identified as myosin-I by antibody inhibition³. Fourth, substantial amounts of *Acanthamoeba* myosin-I are associated with membranes fractionated by density-gradient centrifugation³.

To look directly for myosin-I binding to membranes we have performed reconstitution assays using purified myosin-IA and -IB from *Acanthamoeba* and NaOH-extracted membranes^{9,10} from *Acanthamoeba* organelles. Extraction of peripheral membranes proteins, particularly actin¹¹⁻¹³ and myosin-I itself, from the membranes was considered a necessary first step to the demonstration of a direct binding site for myosin-I.

In a buffer containing physiological concentrations of ATP and salt, purified myosin-IA and -IB bind to membranes

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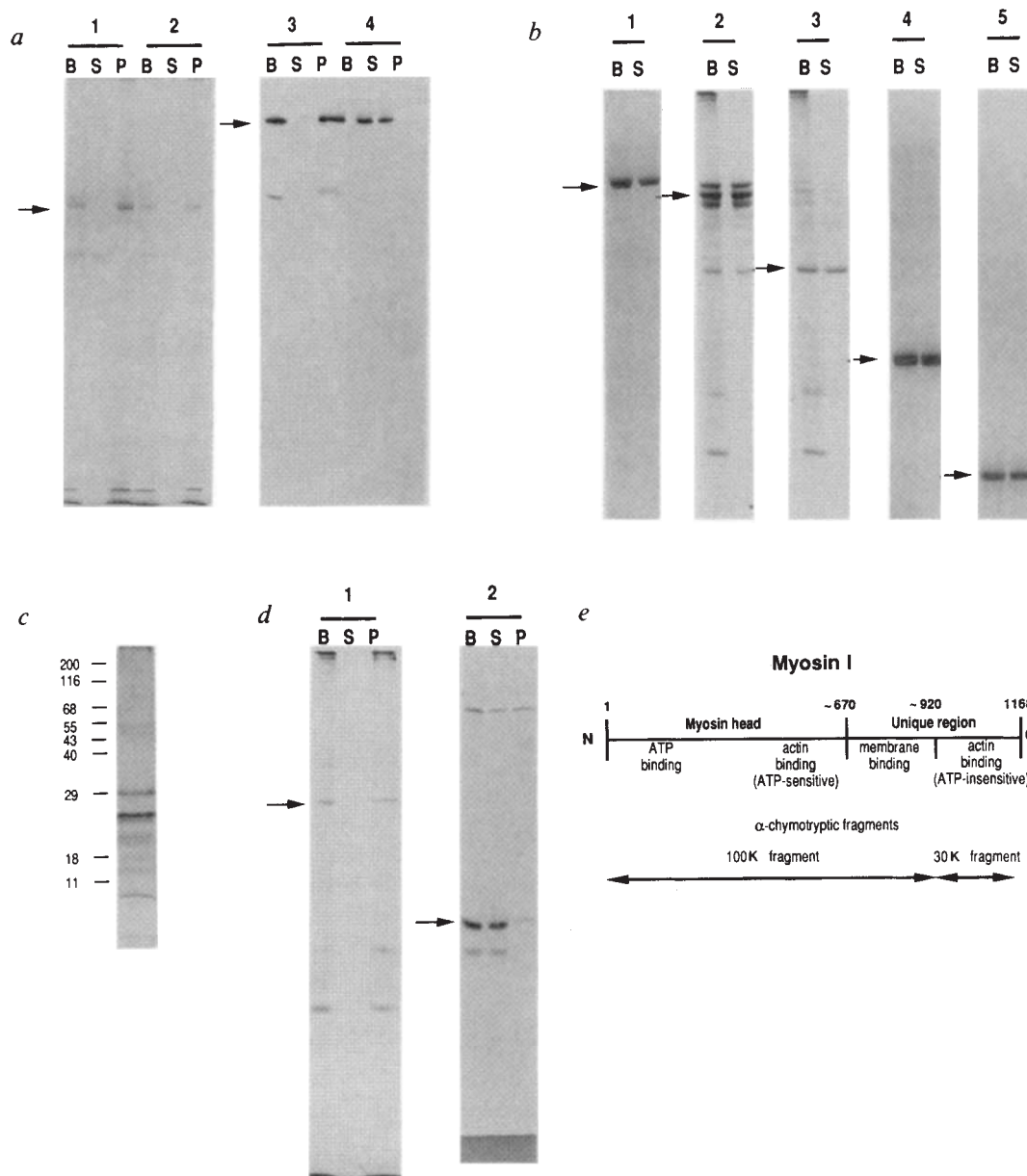


FIG. 1 *Acanthamoeba* myosins-IA and -IB bind to extracted membranes, whereas other purified proteins do not. Bound myosin-I was separated from free myosin-I by centrifugation. B, samples before centrifugation; S, supernatant after centrifugation; P, pellet. These fractions were analysed by SDS polyacrylamide gel electrophoresis²³. A membrane concentration equivalent to ~100 μ g of membrane protein was used in these experiments. *a*, Binding to membranes. The gels in 1 and 2 were stained with coomassie blue. 1, myosin-IA pellets with membranes; 2, myosin-IB pellets with membranes. Gels in 3 and 4 were immunoblots prepared by transfer onto nitrocellulose, stained with a monoclonal antibody to myosin-I (ref. 5) and visualized with peroxidase-conjugated goat anti-mouse immunoglobulin. 3, myosin-IB pellets with membranes; 4, myosin-IB alone remains in the supernatant. *b*, Reaction of other purified proteins with extracted membranes. Gels stained with coomassie blue. 1, *Acanthamoeba* myosin II; 2, rabbit skeletal muscle heavy meromyosin; 3, rabbit skeletal muscle myosin subfragment 1; 4, bovine serum albumin and 5, ovalbumin. *c*, Proteins of NaOH-extracted membranes stained with silver. A heterogeneous mixture of proteins are present. Myosin-I and actin are minor and variable components. *d*, Reaction of chymotryptic fragments of myosin-IA (ref. 14) with extracted membranes. 1, Coomassie blue stain. The 100 K chymotryptic fragment pellets with the membranes. The 30 K fragment is not visible on this 8% gel. 2, An immunoblot of a 12.5% gel shows that the 30 K fragment remains in the supernatant (S) when the membranes pellet. *e*, Schematic of myosin-I heavy chain based on the nucleotide sequence published for *Acanthamoeba* myosin-IB (ref. 15) and proteolytic fragments¹⁴ of myosin-IA.

METHODS. The use of the method described in ref. 14 routinely yielded a

total 20–30 mg of the two similar isozymes named myosins-IA plus -IB from 1 kg of *Acanthamoeba*. Rabbit skeletal muscle myosin was prepared by the method of Kielley and Harrington²⁴. Ovalbumin, bovine serum albumin (BSA), were purchased from Sigma. Heavy meromyosin and subfragment-1 of rabbit skeletal muscle myosin were prepared by the methods of Margossian and Lowey²⁵. Chymotryptic fragments of myosin-IA were prepared by the method described in ref. 14. Membranous organelles were prepared at 0–4 °C from ~20 g of *Acanthamoeba*. Cells were suspended in 2 volumes of extraction buffer (60 mM potassium glutamate, 10 mM imidazole-HCl, pH 6.4, 2 mM EGTA, 2 mM MgCl₂) supplemented with 4 mM ATP, 1 mM PMSF (phenylmethylsulphonyl fluoride) and 18 μ g ml⁻¹ benzamidine, broken with 20 strokes of a tight-fitting Dounce homogenizer and centrifuged for 3 min at 3,000 r.p.m. in a Beckman JA 20 rotor. The supernatant was further centrifuged for 90 min at 38,000 r.p.m. in a Beckman Ti 45 rotor. The brown membrane pellet was resuspended in homogenization buffer with 1 mM ATP; care was taken only to resuspend the membrane pellet, not the clear poly-ribosome pellet. Extracted membranes were made using the method of Luna⁹, as follows. Membranes were washed by dilution to 50 ml in extraction buffer with 1 mM ATP and centrifuged for 60 minutes as above. The membrane pellet was resuspended in 50 ml of 0.1 M NaOH with 1 mM dithiothreitol homogenized and then sonicated for 30 s. This extract was centrifuged as above for 30 min. The pellet was homogenized in 50 ml extraction buffer and re-centrifuged for 30 min. The pellet was finally homogenized in 10 ml extraction buffer and frozen in liquid nitrogen and stored at -80 °C for several weeks. Pelleting assays were performed as described in Fig. 2.

(Fig. 1a) stripped of all actin, myosin-I and other peripheral proteins with 100 mM NaOH (Fig. 1c). No myosin-I pellets in the absence of membranes, and none of various other purified proteins, including other myosins isozymes, pellets with stripped membranes under these conditions (Fig. 1b). Myosin-I still binds to stripped membranes after they are digested extensively with α -chymotrypsin (data not shown). Binding of both myosin-IA and -IB to extracted membranes is inhibited by ~50% by the addition of 100 mM NaCl and by almost 100% by 250 mM NaCl.

The concentration dependence of the binding of purified myosin-I to stripped membranes is consistent with a dissociation constant of 140 nM and saturates at ~0.2–1.0 mg myosin-I per mg of membrane protein (Fig. 2). Given the heterogeneity of the membrane polypeptides (Fig. 1c), the binding capacity of the membranes for myosin-I far exceeds the molar concentration of any single membrane protein.

Experiments with proteolytic fragments of myosin-I (ref. 14) tentatively localize the membrane binding site to a part of the heavy chain between residues 670 and 920 (Fig. 1d, e). First, a 30 K (relative molecular mass, M_r) C-terminal fragment containing the ATP-insensitive binding site¹⁴ does not bind to the stripped membranes (Fig. 1d). Second, a 100 K N-terminal fragment, consisting of a typical myosin-head sequence up to residue 670 (ref. 15), binds to membranes (Fig. 1d). Because neither *Acanthamoeba* myosin-II nor head fragments of muscle myosin bind to membranes and because the head domain of myosin-I is unlikely to bind to any undetected actin in the membrane fraction under the conditions tested, the region of previously unknown function distal to residue 670 is probably

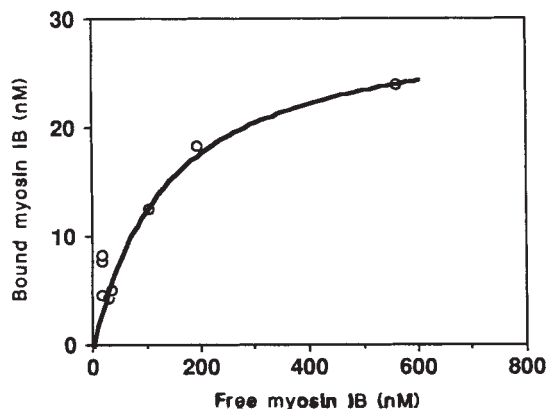


FIG. 2 Concentration dependence of myosin-IB binding to NaOH-extracted membranes. The solid line is a theoretical plot for $K_d = 139$ nM. A second experiment gave $K_d = 137$ nM. The binding capacity was 0.2–1.0 mg myosin-1 mg^{-1} membrane protein, depending on the protein assay used, because the Bradford²⁶ and Lowry²⁷ assays (with or without SDS) give different estimates of membrane protein.

METHODS. Various concentrations of myosin-I were mixed with membranes containing ~5 μg of protein 0.2 ml extraction buffer containing, 1 mM ATP and 1 mg ml^{-1} (BSA) and pelleted as described below. The samples were run on a gel with a range of myosin-IB internal standards. Proteins were transferred onto nitrocellulose then reacted with an iodinated²⁸ monoclonal antibody (M1.8) against myosin-I (ref. 5). An autoradiogram of the blot was used as a guide to cut out individual myosin-I bands which were then counted in a γ -counter. The standard curve obtained from the internal standards was fitted with a polynomial curve and the experimental values interpolated from this curve. Pelleting assays were performed in a Beckman TL100 centrifuge. Myosin-I was mixed with organelles in a total volume of 0.2 or 0.4 ml in homogenization buffer plus 1 mM ATP. In all quantitative experiments this buffer contained 1 mg ml^{-1} BSA to minimize nonspecific interactions with the walls of the tube. Samples (20 μl) were taken of the starting mixture before centrifugation. In a number of experiments a cushion of 15% sucrose in homogenization buffer was added below the sample; this allowed cleaner recovery of the pellets. The tubes were centrifuged at 30,000 r.p.m. for 10 min at 20 °C. The supernatant was then removed. The pellet was resuspended in SDS sample buffer²³. Binding was followed by western blots of samples electrophoresed in SDS on 8% polyacrylamide gels²³.

responsible for binding. We do not exclude acylation of this region.

Myosin-I binds to purified membranes in a gel-filtration assay (Fig. 3). Free myosin-I elutes near the salt volume and is well separated from myosin-I associated with vesicles in the void volume (Fig. 3, band 2). Similar results are obtained with vesicles of *Acanthamoeba* lipids, pure phosphatidyl serine or pure phosphatidyl inositol 4,5-bisphosphate $\text{PtdIns}(4,5)\text{P}_2$ (Fig. 3, band 3–5). Myosin-I does not bind to vesicles of pure phosphatidyl choline under these conditions (Fig. 3 band 6). These results suggest that myosin-I binds to negatively charged surfaces provided by anionic phospholipids.

Reconstruction of the binding of myosin-I to membranes stripped of peripheral proteins occurs under the same physiological conditions where a motile myosin-I–membrane complex was originally isolated from the cell³. The dissociation constant of the complex is consistent with extensive binding of myosin-I to membranes within the cell. Although only lipids are required to form this complex *in vitro*, an interaction with lipids is unlikely to impart much specificity. It seems likely that intrinsic or peripheral proteins, including actin, limit the lateral mobility of myosin-I in the plane of the membrane and lead to local differences in concentration¹⁶. The ATP-insensitive, actin-binding site near the C terminus of myosin-I is a particularly attractive candidate for such an anchor because the affinity ($K_d = 0.12$ – 0.34 μM) is sufficient for binding to actin, but weak enough

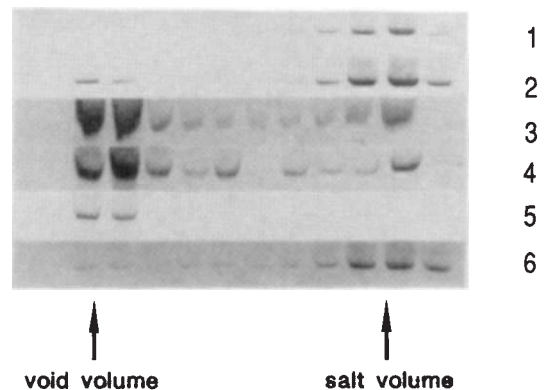
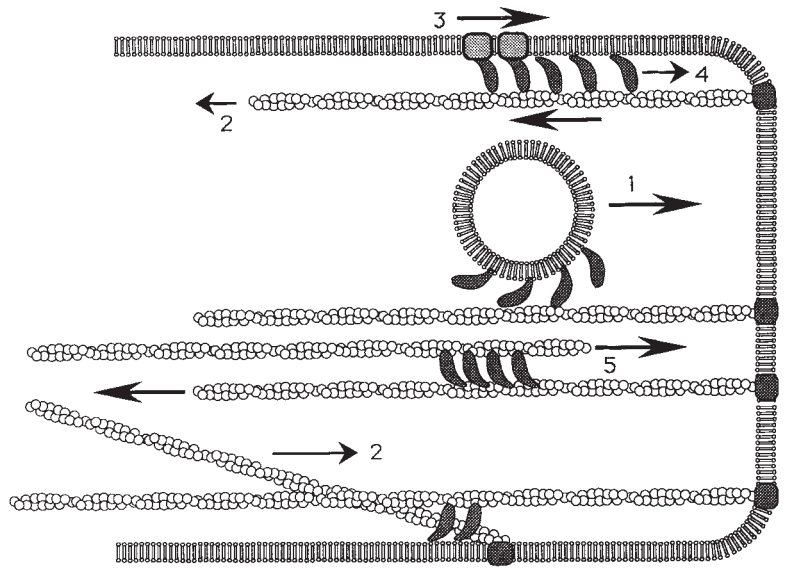


FIG. 3 Gel-filtration assay for myosin-I binding to membranes and lipid vesicles. A 0.7×19.0 cm column of Sepharose-cl 4B was run in homogenization buffer with 1 mM ATP and 1 mg ml^{-1} BSA. A 0.5-ml sample was loaded onto the column followed by 1 ml column buffer. The sample was then followed directly with the same buffer plus 0.3 M NaCl. This step was necessary because free myosin-I tended to stick to the column matrix² when not bound to other surfaces. Membrane-bound myosin-I passed unhindered through the column, ahead of the salt wash. Fractions of 0.6 ml were collected and the myosin-I visualized by western blotting from 8% gels. The column was loaded with myosin-IB mixed with (1) buffer alone; (2) NaOH-extracted membranes; (3) vesicles of *Acanthamoeba* lipids; (4) vesicles of pure phosphatidyl serine; (5) vesicles of pure $\text{PtdIns}(4,5)\text{P}_2$; and (6) vesicles of pure phosphatidyl choline.

METHODS. Lipids were prepared from *Acanthamoeba* by organic extraction²⁹. Briefly, 4.8 g of cells (washed three times) in 30 ml of 50 mM NaCl were extracted for 30 min after vortexing in 37.5 ml chloroform and 75 ml methanol. To this mixture was added 37.5 ml of H_2O plus 37.5 ml chloroform and the resulting suspension centrifuged to separate the organic and aqueous phases. The upper, aqueous phase was aspirated off to allow the lower, organic phase containing the extracted lipids to be dried under a steady stream of nitrogen gas. The lipid residue left was resuspended in chloroform and stored at -20 °C. To prepare these lipids for use in binding experiments, the solvent was again evaporated off with a stream of nitrogen gas and the residue resuspended in water by sonication. Protein was not detectable in this extract by a Bradford protein assay²⁶ or SDS-PAGE²³. Purified lipids (Sigma) were prepared for use by evaporating off the organic solvent used for storage and resuspended in water with extensive sonication at a concentration of 1 mg ml^{-1} . In the case of $\text{PtdIns}(4,5)\text{P}_2$ the lipid was added to homogenization buffer (containing divalent cations) used for the binding experiments at least 30 min before use. This permitted the conversion of the lipid from micelles to vesicles favoured under these conditions.

FIG. 4 Schemes for myosin-I-membrane interactions within the cell. 1, Membrane-bound myosin-I moves organelles along actin filaments within the cytoplasm; 2, myosin-I tethered in the membrane by association with other membrane proteins or a second actin filament produces a contraction on actin filaments. This could cause contraction of actin filaments tethered to cell structures such as the plasma membrane^{30,31}, or concentrate actin filaments into an area of activity; 3, myosin-I causes the movement of the plasma membrane relative to actin filaments in the cytoplasm. The direction of movement would be determined by the polarity of the actin filaments. At the cell periphery, in microvilli or filopodia the direction of this movement would be expected to be centrifugal. 4, Myosin-I and associated membrane molecules may be free to diffuse in the plane of the membrane. An interaction of myosin-I with tethered cortical actin filaments may serve to move myosin-I along the actin filaments, concentrating them in a region of activity. 5, Myosin-I has already been shown to cause contraction of actin filaments relative to each other, *in vitro*^{14,21,22}. A network of actin filament in the cytoplasm could be contracted in this way.



to allow dynamic rearrangements. Furthermore, it could be that if PtdIns(4,5)P₂ contributes to the binding site in the cell, phosphoinositide metabolism modulates the amount of myosin-I bound to membranes.

The continuation of many movements in cells depleted of myosin-II by genetic manipulations^{17,18} or by microinjection of inhibitory antibodies^{19,20} suggests that myosin-I powers various movements. *In vitro* studies^{14,21,22} show that myosin-I can cross-link and contract networks of actin filaments (Fig. 4, number 5). In addition, the association of myosin-I with membrane lipids provides potential mechanisms for at least four other types of movement (Fig. 4): (1) organelles along actin filaments in the cytoplasm; (2) membranes relative to actin filaments in the cytoplasm; (3) the plasma membrane relative to a tethered actin filament gel; and (4) membrane molecules relative to cortical actin filaments.

Coincident with the completion of this work, a paper from Miyata *et al.*³² has replicated some of the results described here and shows that myosin-I binds to purified plasma membranes. □

Leucine zippers of *fos*, *jun* and GCN4 dictate dimerization specificity and thereby control DNA binding

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THE products of the *fos* and *jun* protooncogenes form a stable heterodimer which binds to the TPA-responsive element (TRE) TGACTCA with high affinity¹⁻⁹. These two proteins, together with the yeast GCN4 protein, belong to a growing family of transcription factors, including FosB, Fra1, JunB and JunD, whose members share a highly conserved DNA-binding domain¹⁰⁻¹⁴. This domain is composed of two structures: a basic motif, which is thought to bind directly to DNA; and a leucine zipper¹⁵, which provides a dimerization interface. Although this domain is highly conserved in Fos, Jun and GCN4, each of these three proteins has very different relative affinities for the TRE. To understand these differences, we used 'domain-swapping' experiments designed to test the relative contributions of the basic motif and the leucine zipper to TRE-binding affinity. Here we show that *fos*, *jun* and GCN4 have different affinities for the TRE due to differences in the hetero- or homo-dimerization capacity of their leucine zipper domains; the basic motifs of these three proteins have comparable DNA binding potential. These results indicate that leucine zippers control the types of protein complexes which can associate with a TRE and regulate gene expression.

We have previously shown¹ that the 92-residue Fos-core peptide (Fig. 1), which includes the Fos basic motif and the Fos leucine zipper, like Fos, cannot bind to the TRE alone, but can do so when in a dimeric complex with Jun. The lack of intrinsic DNA-binding activity of the Fos-core peptide may result from inability to form dimers⁴⁻⁷, or from the inability of the Fos basic motif in a Fos/Fos homodimer to interact with the TRE. To distinguish between these alternatives, we first asked whether the Fos-core peptide can dimerize with the full-length Fos

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