primary cells[3], oncogene co-operation may involve the unblocking of two regulating steps on the same signalling pathway. It should be possible to establish the role of other transcription factors (Fos, Ets, Myb, Rel, Myc, SRF; see ref. 7) in signal transduction and the transformed phenotype using mutants similar to Δ9. These reagents have the particular advantage that they are downstream in the pathways, having the potential to cause a wide range of transformed cells to revert. □

Received 17 April; accepted 8 July 1991.

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ACKNOWLEDGEMENTS. We thank C. Marshall for reagents, C. Benoist, F. Bibollet and J. M. Limacher for help with the nude mice, members of the laboratory for discussion, and the cell culture, oligonucleotide, animal house, secretarial and photographic services staff for their help, and CNRS, INSERM, ARC and FNCLCC for financial assistance. A. L. was a recipient of EMBO and EEC cancer training fellowships.

Characterization of the rough endoplasmic reticulum ribosome-binding activity

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THE rough endoplasmic reticulum membranes of mammalian cells contain specific ribosome-binding sites[1]. A purification to apparent homogeneity of a negatively charged protein (ERp180) of relative molecular mass 180,000 (180 K) was reported which was proposed to function as a rough endoplasmic reticulum ribosome receptor[2]. We report here that ribosome-binding site activity quantitatively solubilized from rough endoplasmic reticulum membranes does not cofractionate with ERp180. By contrast, ribosome-binding site activity fractionates as a much smaller, positively charged protein.

The density of ribosome-binding sites present in canine pancreatic rough microsomal membranes (RM) was determined after dissociation of ribosomes from RM with high salt after the nascent proteins were released with puromycin. Ribosome-binding sites on the stripped RM were quantitated by rebinding ^{125}I-labelled ribosomes[1]. Scatchard analysis indicated that the density of sites on the RM was 350 fmol per equivalent (or 120 nmol per g membrane protein; not shown), in good agreement with previous estimates[3].

Ribosome-binding sites were solubilized using a two-step procedure modified from Yoshida *et al.*[4]. Endoplasmic reticulum lumenal proteins were released by lysing RM in a nonionic detergent in 50 mM KCl. Membrane proteins were then solubilized using the detergent CHAPS under high-salt conditions. The ribosome-binding activity of the extracts was assayed after reconstituting the membrane proteins into liposomes. Figure 1 shows that the number of binding sites recovered increased as the ratio of CHAPS to protein was increased during solubilization. At a ratio of detergent to protein of 12.5 μg CHAPS per mg protein or greater, a maximum yield of 320 fmol ribosome-binding sites per equivalent of RM was obtained. Hence, more than 90% of the sites present in RM were solubilized and functionally recovered after reconstitution into liposomes. The extraction and reconstitution procedure allowed us, therefore, to account quantitatively for the ribosome-binding sites present in the starting RM fraction.

We fractionated the CHAPS extract by velocity sedimentation (Fig. 2) or by ion-exchange chromatography (Fig. 3). The peak of ribosome-binding activity sedimented near the top of the sucrose gradient (<4S) (Fig. 2*a*). Most (80%) of the sites did not bind to the DEAE resin (Fig. 3*a*). The fractionation on the DEAE column resulted in a large increase in the specific activity in the flow-through fraction and a concomitant decrease in the eluate fraction (Fig. 3*a*). Scatchard analysis of ribosome-binding activity after DEAE fractionation indicated a dissociation constant (K_d) of 12 nM, experimentally indistinguishable from ribosome binding to RM (data not shown; ref. 3). In addition, the salt and protease sensitivity of ribosome binding to these proteoliposomes and RM fractions were indistinguishable (data not shown). Thus, the ribosome-binding activity recovered in the flow-through fraction of the DEAE column had all the properties characteristic of that of the starting RM fraction.

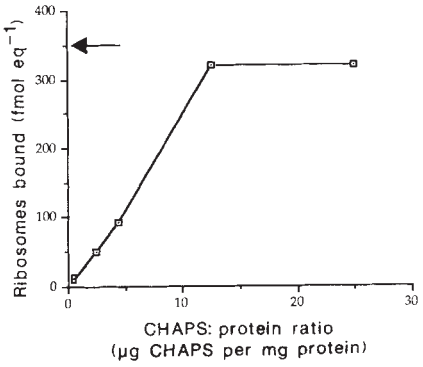

FIG. 1 Quantitative solubilization of ribosome-binding activity. A CHAPS extract of RM was prepared at different detergent-to-protein ratios. The ribosome-binding activity in the extracts was determined after reconstitution into liposomes. The arrow indicates the binding activity present in an equivalent amount of ribosome-stripped RM.
METHODS. Dog pancreatic endoplasmic reticulum membranes (1 eq μl^{-1}; defined in ref. 8), isolated as described previously[8], were extracted initially using 1.5% Emulgen-913 and 50 mM KCl in buffer A containing 50 mM Tris, pH 7.5, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 1.0 μg ml^{-1} each of antipain, leupeptin, chymostatin and pepstatin for 30 min at 0 °C. Membranes were pelleted through a 0.6 M sucrose cushion in buffer A containing 1.5% Emulgen-913 and 50 mM KCl in a Beckman 50.2 Ti rotor at 40,000 r.p.m. for 2 h at 4 °C. Pellets were resuspended by homogenization at final protein concentrations (Bio-rad protein assay) varying from 0.8 to 40 mg ml^{-1} (0.1–5.0 eq μl^{-1}) in buffer A containing 2% (w/v) CHAPS, 10% sucrose and 500 mM KCl and allowed to equilibrate at 0 °C for 30 min. CHAPS extracts were centrifuged in a 50.2 Ti rotor at 50,000 r.p.m. for 3 h at 4 °C. Supernatants were adjusted with buffer A containing 2% CHAPS, 10% sucrose and 500 mM KCl to a final concentration of 0.1 eq μl^{-1}. To 100 μl (10 eq) of each of the supernatants, 0.5 mg of egg phosphatidylcholine, solubilized in 50 mM Tris, pH 7.5, 50 mM KCl, 0.25 mg sodium cholate and 0.1 mg CHAPS, was added and reconstituted by dialysis against 1,000 volumes of 50 mM Tris, pH 7.5, and 50 mM KCl for 16–40 h with one change of the dialysis buffer. Proteoliposomes (50 μl, 5 eq) were assayed for ribosome binding as previously described[1] with the modification that ^{125}I-labelled rat liver ribosomes at a concentration of 50 nM were used as the substrate. Rat liver ribosomes were prepared as described[1] and iodinated using Bolton–Hunter reagent for 1 h at 0 °C and subsequently purified on a 10–30% sucrose gradient containing 50 mM Tris, pH 7.5, 50 mM KCl and 5 mM $MgCl_2$.

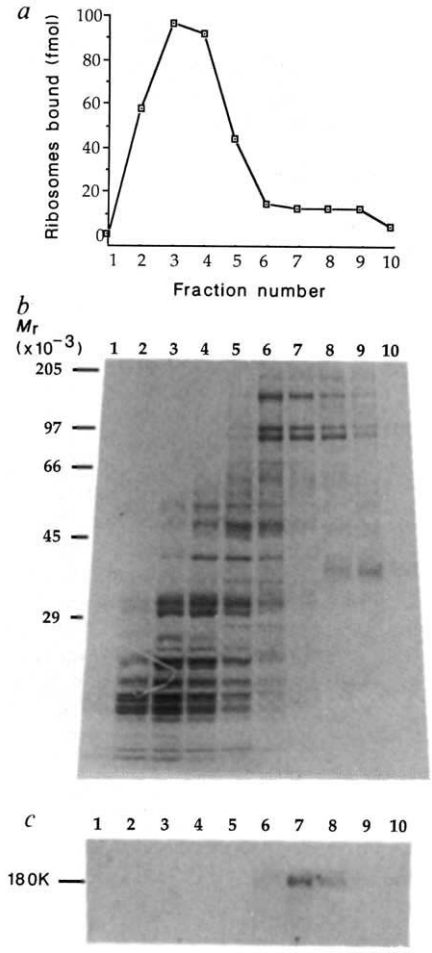

FIG. 2 Sucrose gradient centrifugation of CHAPS-solubilized extract. A CHAPS extract of RM was prepared at the optimal detergent to protein ratio (Fig. 1) and fractionated by sucrose gradient centrifugation. Sedimentation was from left to right. *a*, Fractions were analysed for ribosome binding after reconstitution into liposomes (Fig. 1). The recovery of ribosome-binding activity after fractionation approached 100%. *b*, *c*, Fractions were analysed by SDS-PAGE and staining with Coomassie blue (*b*) or immunoblotting with anti-180K serum (*c*).

METHODS. CHAPS extract was prepared as described in Fig. 1 at a protein-to-CHAPS ratio of 12.5 μg CHAPS per mg protein. CHAPS extract (200 μl, 40 eq) was layered on a 2.0 ml 5–20% sucrose gradient in buffer A containing 0.5% CHAPS and 500 mM KCl and centrifuged in a Beckman TLS55 rotor at 55,000 r.p.m. for 12 h at 4 °C. Gradients were fractionated into 200 μl aliquots. Each fraction (100 μl) was analysed for ribosome-binding activity as described in Fig. 1, with the exception that 4 nM ^{125}I-labelled ribosomes were used in the assay. Separate 100 μl aliquots were analysed by SDS-PAGE, followed by either Coomassie-blue staining or immunoblotting for the 180K protein. For western blotting, proteoliposomes were subjected to SDS-PAGE and transferred onto nitrocellulose. Blots were incubated with rabbit antiserum raised by injection of SDS-PAGE purified ERp180 (D.L.Z. and P.W., unpublished results). Immune complexes were visualized using ^{125}I-labelled goat anti-rabbit IgG and autoradiography.

Savitz and Meyer reported the identification of ERp180 which they proposed to be a rough endoplasmic reticulum ribosome receptor[2]. They identified a 160K proteolytic fragment which was able to inhibit ribosome binding to RM. Antibodies raised against this proteolytic fragment were used to purify the parent protein, ERp180. Both the sedimentation behaviour and the charge properties of ERp180 are different from the ribosome-binding activity characterized in Figs 2 and 3.

To address this discrepancy, we used antibodies raised against ERp180 purified by SDS-PAGE. The antiserum recognized a 180K membrane protein and a 160K soluble fragment released from RM after treatment with thermolysin (data not shown), indicating that it was specific for ERp180. The antiserum was used to follow ERp180 during sucrose gradient sedimentation

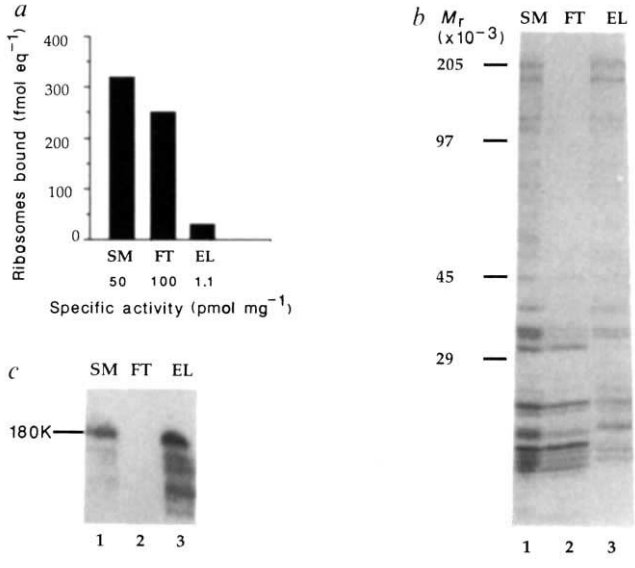

FIG. 3 DEAE chromatography of CHAPS-solubilized extract. A CHAPS extract of RM (as in Fig. 2) was fractionated on DEAE-Sepharose. The initial extract (lane 1, 'SM'), a low-salt flow-through fraction (lane 2, 'FT'), and a high-salt eluate fraction (lane 3, 'EL') were collected. *a*, Equivalent amounts of each fraction were analysed for ribosome binding. The bar graph displays the total ribosome-binding activity recovered in the respective fractions; the specific activity for each fraction calculated as pmol ribosome-binding site per mg protein is indicated. *b*, *c*, Equivalent amounts of each fraction were analysed by SDS-PAGE and staining with Coomassie blue (10 eq per lane, *b*) or immunoblotting with anti-180K serum (5 eq per lane, *c*). During fractionation on the DEAE column some degradation of the 180K protein was observed in the eluate fraction.

METHODS. CHAPS extract (1 ml, 200 eq) was diluted 10× and adjusted to 0.5% CHAPS, 50 mM KCl with buffer A and a 15% CHAPS stock solution. The extract was passed over a 2 ml DEAE-Sepharose Cl-6B column equilibrated with buffer A, containing 0.5% CHAPS, 50 mM KCl. The column was washed with three column volumes of the equilibrating buffer, which was combined with the flow-through fraction. The column was eluted with buffer A, containing 0.5% CHAPS, 500 mM NaCl. For saturation binding analysis, 500 μl of the DEAE flow-through fraction was reconstituted into liposomes by the addition of 2.5 mg egg phosphotidylcholine, solubilized in 50 mM Tris, pH 7.5, 50 mM KCl, 1.25 mg sodium cholate and 0.5 mg CHAPS and by dialysis as described in Fig. 1. Fractions (29 μl, 0.57 eq) were assayed for ribosome-binding activity using ribosome concentrations ranging from 10 to 100 nM as described in Fig. 1.

(Fig. 2*c*) or during the DEAE-Sepharose fractionation (Fig. 3*c*) of the CHAPS extract. Note that in both cases ERp180 did not cofractionate with the ribosome-binding activity.

These discrepancies could be due to differences between the solubilization and reconstitution procedures described here and those used by Savitz and Meyer, but given the nearly quantitative solubilization and reconstitution of the ribosome-binding activity we observed, this explanation is unlikely. Moreover, under our conditions ERp180 was incorporated into the proteoliposomes efficiently and in the proper orientation (data not shown).

Chemical crosslinking studies with endogenous membrane-bound ribosomes have demonstrated that some ERp180, in addition to other endoplasmic reticulum membrane proteins including ribophorins, could be found in proximity to membrane-bound ribosomes[5]. Ribophorins were previously proposed to function in ribosome binding[6], but were subsequently shown not to be required[3,4]. Recently, crosslinking of ribosomes to a 34K membrane protein was observed with reconstituted proteoliposomes which exhibited ribosome-binding activity[7]. Thus, on the basis of crosslinking studies the environment of the membrane-bound ribosome seems complex and, formally, the possibility remains that ERp180 contacts ribosomes. But the data presented here demonstrate that the ribosome-binding activity of RM, as biochemically defined by a quantitative binding assay, resides in a protein that still awaits purification. □

Received 13 May; accepted 2 July 1991.

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ACKNOWLEDGEMENTS. We thank C. Nicchitta, J. Miller, P. Collins and R. Gilmore for helpful discussions. This work was supported by the American Cancer Society (to J.M.N.), the Lucille P. Markey Charitable Trust (to D.L.Z.), the Howard Hughes Foundation (to S.C.O.) and the NIH and Alfred P. Sloan Foundation (to P.W.).

Multiple nucleotide-binding sites in the sequence of dynein β heavy chain

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AXONEMAL dyneins have two or three globular heads joined by flexible tails to a common base, with each head/tail unit consisting of a single heavy-chain polypeptide of relative molecular mass >400,000. The sizes of the components have been deduced by electron microscopy[1–3]. The isolated β heavy chain of sea urchin sperm flagella, which is immunologically identical to that of the embryo cilia (data not shown; ref. 4), is of particular interest as it retains the capability for microtubule translocation *in vitro*[5,6]. Limited proteolysis of the β heavy chain divides it into two fragments, A and B, which sediment separately at 12S and 6S, and possibly correspond to the head and tail domains of the molecule[7]. Dynein ATPase is the energy-transducing enzyme that generates the sliding movement between tubules that underlies the beating of cilia and flagella of eukaryotes, and possibly also other large intracellular movements[8,9]. Here we report that the deduced amino-acid sequence of the β heavy chain of axonemal dynein from embryos of the sea urchin *Tripneustes gratilla* has 4,466 residues and contains the consensus motifs for five nucleotide-binding sites. The probable hydrolytic ATP-binding site can be identified by its location close to or at the V1 site of vanadate-mediated photocleavage[10]. The general features of the map of photocleavage and proteolytic peptides reported earlier have been confirmed, except that the map's polarity is reversed. The predicted secondary structure of the β heavy chain consists of an α/β-type pattern along its whole length. The two longest regions of potential α helix, with unbroken heptad hydrophobic repeats 120 and 50 amino acids long, may be of functional importance. But dynein does not seem to contain an extended coiled-coil tail domain.

Progress in analysing the structure and function of dynein has been hindered by the lack of primary sequence information resulting from the difficulty of cloning a complementary DNA encoding such a large polypeptide. We have now used a polymerase chain reaction (PCR)-directed procedure to obtain the complete sequence of the β heavy chain of dynein from sea urchin embryos (Fig. 1). In the region of overlap our sequence shows about 98% identity to two partial clones of dynein β heavy chain from a different species of sea urchin examined by Ogawa[12,13], but no significant similarity to two other partial sequences[14,15].

The relative positions of the N-terminal microsequences of the tryptic peptides (relative molecular masses 130,000 (130K) and 124K) on the L13-14 parent clone and a short upstream extension of it indicated early that the polarity of the peptide chain must be reversed from what had been deduced previously by assay of acetyl residues in the photocleaved β chain[11]. Further sequence data showing the positions of the other microsequenced peptides, as well as the probable position of the V1 vanadate-mediated photocleavage site[10], unambiguously confirm the new polarity. A revised map is shown in Fig. 2.

A striking feature of the sequence is the presence of five copies of the consensus A motif[16] of nucleotide-binding sites (A/G)XXXXGK(T/S) (single-letter amino-acid code; Figs 1 and 3*a*). The principal hydrolytic ATP-binding site seems to be the GPAGTGKT sequence (GKT2) at residues 1,852–1,859, for this position 660 amino acids downstream from the N terminus of the 215K tryptic peptide corresponds closely to the expected location of the V1 photocleavage site on the peptide map (Fig. 2). By analogy to the known location of vanadate-mediated photocleavage in myosin and in adenylate kinase[17,18], proline 1,853 in the proposed hydrolytic ATP-binding site of the β chain is a likely candidate for the site of scission in V1 photocleavage. The sequence around the hydrolytic ATP-binding site of the β chain does not closely resemble that of the other known cytoskeletal motor proteins; in particular, the flexible glycine-rich loop extends upstream beyond the GXXXXGKT motif and is longer than that in most other ATP-binding proteins (Fig. 3, and ref. 19).

A second GKT nucleotide-binding motif (GKT1) occurs at residues 154–161, and two GKS motifs are located at 2,133–2,140 and 2,460–2,467. A modified GKQ motif occurs at 2,805–2,812 (Figs 1 and 3*a*). Although the function of GKT2, the hydrolytic ATP-binding site at 1,852–1,859 is clear, the roles of the two GKS sites, the GKQ site and the GKT1 site are much less so. The almost identical sequence (GNAGXGKS) of the two GKS motifs (Fig. 3*a*) suggests that they constitute parts of two nucleotide-binding sites that have been conserved in evolution and represent an important functional aspect of the dynein β chain. Possible roles include the regulation of dynein crossbridge activity which is required for the oscillatory beating of cilia and flagella, or involvement in the inhibitory action of high ATP concentrations on axonemal dynein ATPase activity[20]. The sizes of the peptides formed by cleavage at the unique site of Fe(III)-mediated photocleavage[21] indicate that it lies close to the GKS2 site. Similarly, the roughly 100K mass of the peptide formed by double photocleavage at the V1 and V2 sites[22] makes serine