The GTP-binding protein Ran/TC4 is required for protein import into the nucleus

Mary Shannon Moore & Günter Blobel

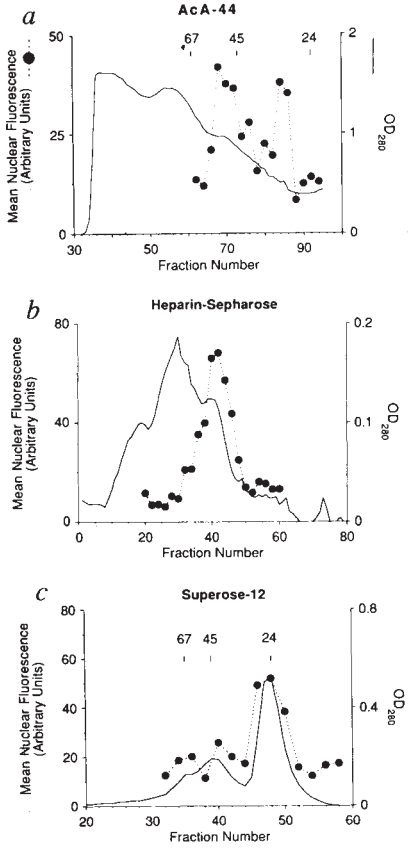
Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

Two cytosolic fractions (A and B) from *Xenopus* oocytes are sufficient to support protein import into the nuclei of digitonin-permeabilized cells[1]. Fraction A recognizes the nuclear localization sequence (NLS) and binds the import substrate to the nuclear envelope, whereas fraction B mediates the subsequent passage of the bound substrate into the nucleus. Here we report that two interacting components are required for full fraction-B activity, purify one of these components to homogeneity, and show that it is the highly abundant GTP-binding protein Ran (Ras-related nuclear protein)/TC4.

A polypeptide of M_r 25K was purified from *Xenopus* ovarian cytosol on the basis of its ability to stimulate import of an NLS-containing substrate into the nuclei of permeabilized[2] cells in the presence of fraction A (Fig. 1). A 103-fold increase in specific activity (Fig. 1*d*) was sufficient to obtain a pure protein (Fig. 1*e*). On the first gel filtration column (AcA-44; Fig. 1*a*) two peaks of B activity were observed, one eluting with an apparent M_r of 50–60K, as previously reported for the active B component[1], and the other eluting at about 30K. On the last purification step (Superose-12; Fig. 1*c*), however, the activity eluted at an apparent M_r of 25K, corresponding to the size of the single polypeptide present.

Two internal peptide sequences (VATLGVEVHPLVF and FNVWDTAGQEK; single-letter amino-acid code) from the 25K polypeptide cleaved with Lys-C (ref. 3) were identical to predicted amino acids 40–52 and 61–71 of the human protein Ran/TC4 (refs 4–6). Immunoblotting of the purified 25K protein with an anti-Ran/TC4 (human) peptide antiserum confirmed that it was the *Xenopus* homologue of Ran/TC4 (Fig. 2).

Although this protein was isolated on the basis of its ability to support nuclear import (with fraction A), this ability seemed to vary with different preparations and to deteriorate over days at 4 °C during the purification. The purified protein, although able to stimulate the import seen with the A fraction alone, was less effective than crude fraction B[1], indicating that a stimulatory factor had perhaps been lost during the purification. Either *Xenopus* Ran/TC4 or recombinant human Ran/TC4 were therefore incubated with increasing concentrations of crude fraction B (Fig. 3*a*) in the presence of fraction A. At levels of crude B that



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Step	Protein (mg)	Activity (units)	Specific Activity (units mg^{-1})	Fold Purification	Yield (%)
1) 146,000 x g sup.	2304	120,080	52	-	100%
2) 25-50% $(NH_4)_2SO_4$	696	72,000	103	2	60%
3) AcA-44	70.4	30,800	438	8	26%
4) Heparin-Sepharose	3.75	5000	1333	26	4.2%
5) Butyl-Sepharose	2	4280	2140	41	3.6%
6) Superose-12	0.323	1728	5350	103	1.4%

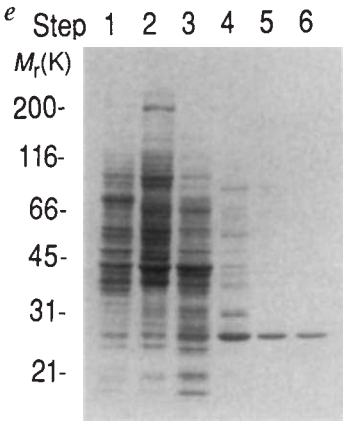

FIG. 1 Purification of the B activity. *a*–*c*, Column profiles of steps 3, 4 (gradient elution fractions only) and 6 of the purification (see *d*). The fractions were assayed for B activity (in the presence of 0.8 mg ml^{-1} fraction A) as described in the legend to Fig. 3. The elution positions of the calibration standards BSA (67K), ovalbumin (45K), and chymotrypsinogen A (24K) are indicated in *a* and *c*. *d*, Summary of purification; units of B activity are as described[1]. *e*, Coomassie-blue-stained 6–15% SDS–PAGE gel showing the pooled active fractions of each stage of the purification. The lane numbers (e) correspond to the purification steps (*d*); the following amount of protein was precipitated with trichloroacetic acid and loaded on each lane: 30 µg, lanes 1, 2, 3; 10 µg, lane 4; 5 µg, lane 5; and 2 µg, lane 6. The migration positions of M_r standards are shown at the left.

METHODS. Isolation of *Xenopus* ovarian cytosol in buffer A (20 mM HEPES–KOH, pH 7.3, 2 mM magnesium acetate, 2 mM DTT) and ammonium sulphate precipitation (25–50%) were as described[1]. The pellet (~350 mg protein) was resuspended to 15 ml in buffer A containing 80 mM potassium acetate, the suspension was filtered (0.2 µm), and loaded on a 510 ml AcA-44 (Fisher) gel filtration column (2.5 × 104 cm) equilibrated in the same buffer at a flow rate of 30 ml h^{-1}. Fractions (4 ml) were collected and assayed for B activity. The active fractions (66–88 in *a*) from two separate runs were pooled and diluted with an equal volume of buffer A containing 0.01% Nikkol[25]. This solution was loaded on a 50 ml heparin–Sepharose (Pharmacia) column (2.5 × 10.2 cm) equilibrated in buffer A containing 40 mM potassium acetate and 0.005% Nikkol at a flow rate of 100 ml h^{-1}. The column was washed with 200 ml of column buffer and eluted with a 500-ml gradient of 40–600 mM potassium acetate in buffer A containing 0.005% Nikkol. Fractions (6 ml) were collected, concentrated (Centricon-10; Amicon), and assayed for B activity. The peak active fractions (37–46 in *b*) were pooled, brought to 25% saturation with solid ammonium sulphate (13.4 g per 100 ml) and loaded on a 2 ml (1.4 × 1.3 cm) butyl-Sepharose (Pharmacia) column equilibrated in buffer A containing 1.2 M ammonium sulphate at a flow rate of 40 ml h^{-1}. The column was washed with 20 ml of column buffer and eluted with buffer A containing 100 mM potassium acetate. The protein-containing eluate fractions were pooled (4 ml) and concentrated to 200 µl; 100 µl was injected (in 2 successive runs) on a Pharmacia Superose-12 (FPLC) gel filtration column equilibrated in buffer A containing 100 mM potassium acetate at a flow rate of 0.4 ml min^{-1}. Fractions (200 µl) were collected and assayed. The active fractions (45–51 in c) were pooled, concentrated to 1 mg ml^{-1}, frozen in liquid nitrogen, and stored at −80 °C. The starting material for this purification was the ovarian tissue obtained from 38 frogs (~575 ml settled ovary).

alone sustained little or no import, added *Xenopus* Ran/TC4 produced a marked increase in nuclear import. Ran/TC4 (50 μg ml^{-1}), together with 0.5 mg ml^{-1} crude B, sustained import at levels comparable to those achieved with crude B at saturation (2 mg ml^{-1}). Unlike *Xenopus* Ran/TC4, recombinant human Ran/TC4 showed no activity when added with fraction A alone (Fig. 3*a*; compare the values at [B] = 0 mg ml^{-1}) but 50 μg ml^{-1} increased import activity slightly relative to crude B. A concentration curve showed that the specific activity of the recombinant Ran/TC4 in this assay was about half that of the *Xenopus* Ran/TC4 (Fig. 3*b*), perhaps reflecting species differences or, more likely, differences in the purification procedures. Another small GTP-binding protein (p21$^{H\text{-}ras}$) had no effect in the assay, indicating a specific requirement for Ran/TC4.

Fluorescence micrographs of the import assay done in the presence of the various factors show that fraction A alone (Fig. 4*A*, *d*) gives a characteristic peripheral nuclear staining and a small amount of import as previously described[1], whereas either Ran/TC4 (Fig. 4*A*, *c*) or a low concentration of crude B alone (Fig. 4*A*, *b*) had no effect. Fraction A plus Ran/TC4 increased import (Fig. 4*A*, *f*) but a high level of import required the combination of A, Ran/TC4, and a low concentration of crude B (Fig. 4*A*, *g*). Import was abolished by wheat-germ agglutinin (WGA) (Fig. 4*A*, *h*), a known inhibitor of nuclear pore complex function[7,8], confirming that the observed increases in intranuclear fluorescence were due to import and not to a disruption of the nuclear envelope.

To investigate whether Ran/TC4 has to be in the 'active', GTP-bound state to mediate nuclear import, GTP, GDP-βS, or GMP-PNP were added to import reactions containing fraction A and a low concentration of crude B either with or without Ran/TC4. None of these nucleotides affected the rim staining and the small amount of import obtained with fraction A and crude B alone (Fig. 4*B*, *a*, *c*, *e*), but both GDP-βS and GMP-PNP inhibited the increased import mediated by Ran/TC4. In the presence of GDP-βS, Ran/TC4 had no effect (Fig. 4*B*, *c*, *d*), whereas in the presence of the non-hydrolysable GTP analogue GMP-PNP the rim staining and small amount of import obtained in the absence of Ran/TC4 were reversed (or pre-

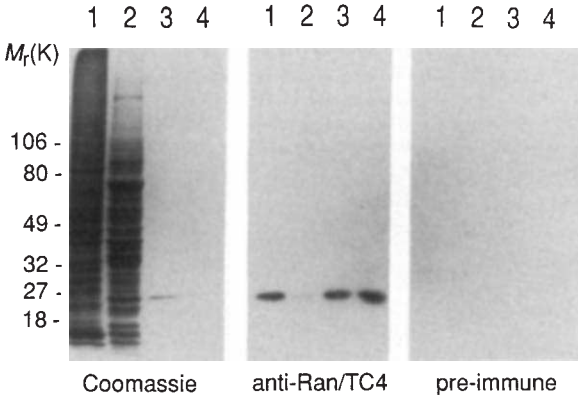

FIG. 2 The purified 25K protein is Ran/TC4. Lanes are: 1, 50 μg total HeLa cell lysate; 2, 50 μg *Xenopus* ovarian cytosol (146,000*g* supernatant); 3, 500 ng *Xenopus* protein isolated in the purification shown in Fig. 1; and 4, 250 ng of human recombinant Ran/TC4. Proteins were separated by SDS–PAGE and either stained with Coomassie blue (left panel) or transferred to nitrocellulose and probed with an antiserum to a human Ran/TC4 peptide (middle panel) or preimmune serum (right panel). Shown at the left are the migration positions of M_r standards.
METHODS. The peptide (C)QYEHDLEVAQTT (corresponding to amino acids 196–207 of the human Ran/TC4 sequence[5] plus an amino-terminal cysteine for coupling purposes) was synthesized, coupled to keyhole limpet haemocyanin with MBS (Pierce), and used to produce antibodies in rabbits. Protein samples were subjected to SDS–PAGE and transferred to nitrocellulose. Immunoblotting was as described [5], except that rabbit sera were diluted 1:500 and the primary incubation was done overnight at 4° C. To obtain the HeLa cell lysate, washed and pelleted HeLa cells were incubated with 1% SDS, boiled (5 min), sonicated and microfuged. The resulting supernatant was used for loading. Human recombinant Ran/TC4 was a gift from E. Coutavas, M. Rush and P. D'Eustachio (NYU Medical Center, New York). The methods for the expression and purification of recombinant Ran/TC4 from *Escherichia coli* will be presented elsewhere (E. Coutavas, M. Ren, J. D. Oppenheim, P. D'Eustachio and M. G. Rush, manuscript in preparation).

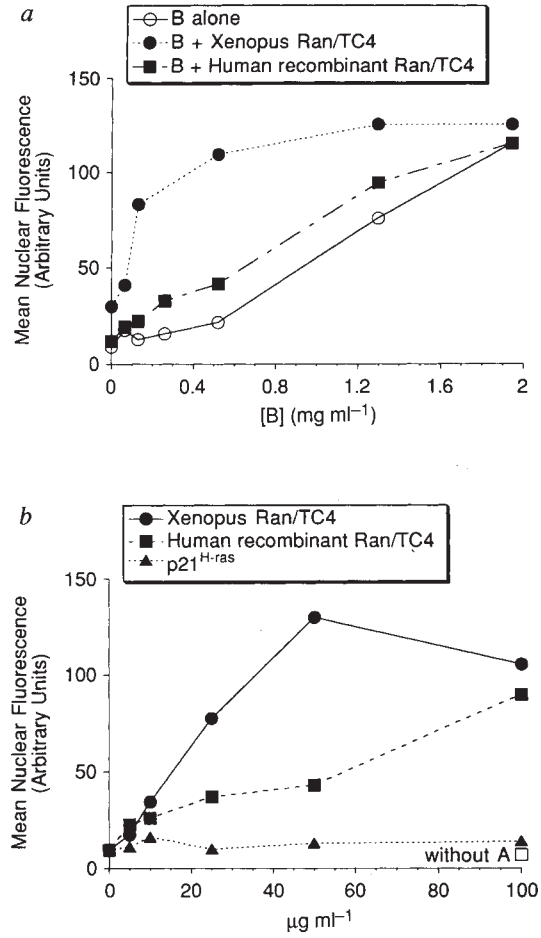

FIG. 3 Ran/TC4 requires a stimulatory factor for full activity. *a*, Increasing concentrations of crude fraction B were assayed (in the presence of fraction A) for import activity either alone (open circles), with 50 μg ml^{-1} purified *Xenopus* Ran/TC4 (solid circles), or with 50 μg ml^{-1} human recombinant Ran/TC4 (squares). *b*, Crude fraction B (0.4 mg ml^{-1}) was incubated with fraction A and increasing concentrations of either *Xenopus* Ran/TC4 (circles), human recombinant Ran/TC4 (solid squares) or recombinant p21$^{H\text{-}ras}$ (triangles). The empty square shows the value obtained with human recombinant Ran/TC4 assayed with crude fraction B but without fraction A.
METHODS. Digitonin-permeabilized buffalo rat liver (BRL) cells were incubated in the standard assay mixture (1 mg ml^{-1} BSA, 1 mM ATP, 5 mM phosphocreatine, and 20 U ml^{-1} creatine phosphokinase in buffer A containing 110 mM potassium acetate and 1 mM EGTA) for 15 min at room temperature before washing and fixation[1,2]. The import substrate (rhodamine-labelled human serum albumin coupled to peptide containing the nuclear localization sequence of the SV40 T antigen)[1] was added at 5 μg ml^{-1}. *Xenopus* fraction A[1] was added at a concentration of 0.8 mg ml^{-1} to all samples in *a* and *b* except where noted. The crude fraction B was obtained by ammonium sulphate precipitation (60%) from the DE-52 flow-through fractions obtained during the separation of fractions A and B as described[1]. Quantification was as described[1] and between 10 and 30 nuclei were scanned per point. The p21$^{H\text{-}ras}$ (ref. 26) was a gift from M. Brown and J. Goldstein.

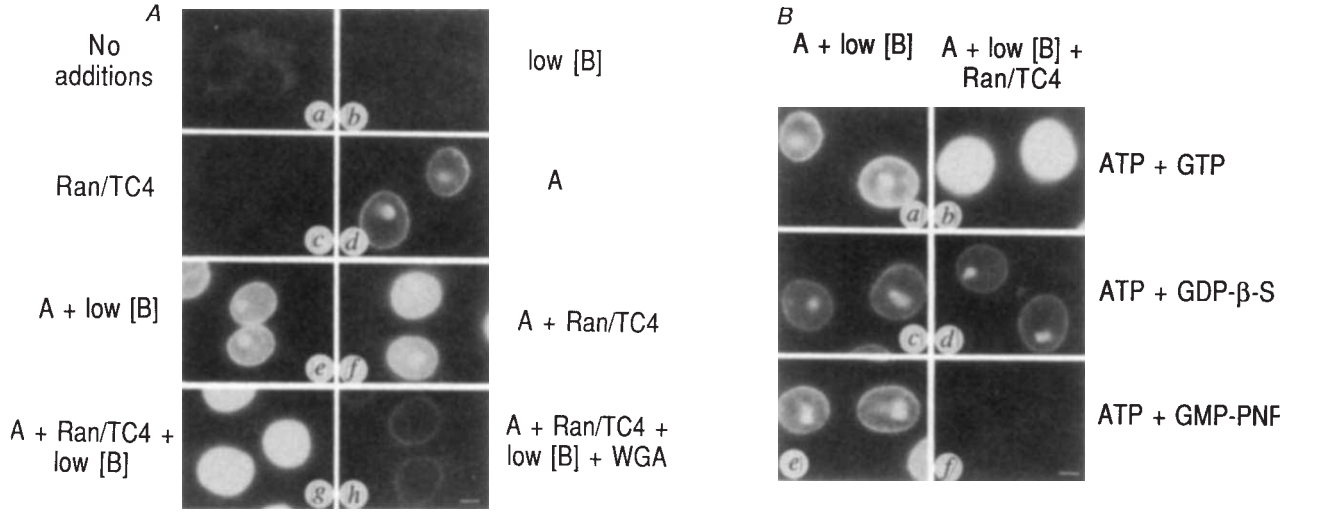

FIG. 4 Fluorescence microscopy of the import assay under various conditions. *A*, Import conditions were as described in the legend to Fig. 3. Where indicated, the following components were incubated together: fraction A (0.8 mg ml^{-1}), low [B] (crude fraction B at 0.4 mg ml^{-1}), and purified *Xenopus* Ran/TC4 (50 μg ml^{-1}). Wheat-germ agglutinin (WGA; Vector) 0.5 mg ml^{-1}. Scale bar, 10 μm. *B*, Fraction A, low [B], and *Xenopus* Ran/TC4 were the same as in *A*. The ATP regenerating system was deleted in this assay and the incubation included 1 mM ATP plus either 1 mM GTP, 1 mM GDP-β*S*, or 1 mM GMP-PNP (all from Boehringer Mannheim). All of the micrographs in each panel were from the same experiment and were exposed and printed for the same length of time. Scale bar, 10 μm.

vented) (Fig. 4*B*, *e*,*f*), giving a staining pattern identical to samples that had never received fraction A (Fig. 4*A*, *a*–*c*). GTP hydrolysis is thus required for nuclear import, but it is still unclear whether it is performed by Ran/TC4 or another GTPase.

Ran/TC4 represents 0.36% of the total protein in HeLa cells[6], of which about 80% is nuclear and is not released on permeabilization of cells with digitonin[5]. This nuclear Ran/TC4 is apparently unable to substitute for the cytoplasmic pool in the import assay, although the small amount of import seen with the A fraction alone (ref. 1; and Fig. 4*A*) might be due to this nuclear pool.

Ran/TC4 forms a complex with an abundant chromatin-binding protein called RCC1 (regulator of chromosome condensation) which catalyses nucleotide exchange on Ran/TC4 *in vitro*[6,9,10]. RCC1 affects numerous nuclear events, including DNA replication, the cell cycle, maintenance of nuclear structure, the mating response (in yeast), and RNA transcription, processing, and export[5,11–21] (reviewed in ref. 22). These pleiotropic effects might reflect a role for RCC1 in maintaining the active GTP-bound form of Ran/TC4, and the stimulatory activity that we have observed in the crude B fraction might be due to a cytoplasmic protein serving the same function. If some of the purified Ran/TC4 was bound to GTP, this might explain its limited activity without crude B (unlike recombinant Ran/TC4). The B fraction contains no Hsc70 (refs 23 and 24, and data not shown) and the 50–60K peak of B activity seen in Fig. 1*a* is too small to be the RCC1–Ran/TC4 complex[6]; it may represent a complex between the putative exchange protein and Ran/TC4.

The precise function of Ran/TC4 in nuclear import remains unclear. It may shuttle between the nucleus and the cytoplasm in association with carriers that function as receptors for cargo to be imported into or exported from the nucleus, with GTP-Ran/TC4 facilitating the cargo–carrier association and GDP-Ran/TC4 facilitating dissociation. This cargo would thus consist of proteins or ribonucleoproteins (and perhaps deoxyribonucleoproteins in the case of viruses) and the A fraction would contain at least one of the carriers. Cargo–carrier dissociation could then be controlled by localized GTPase-activating proteins (GAPs), whereas association would be mediated by proteins like RCC1. Such a mechanism would allow deposition of import substrate by localized GAPs and acquisition of export substrate at suitable points along the import/export pathway. □

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