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Supporting Methods

Derivation of the Rate of Reverse Export from the Cytoplasmic Part of the RanGTP Gradient (J_C). The solution of Eq. **1** for *Xenopus* oocytes is given by a simplification because of the large radius of the cytoplasm (L = 0.4-0.6 mm) in the context of realistic parameter values.

$$r = \frac{J_R}{K_R \cdot \sqrt{kD}} \exp\left(-\sqrt{\frac{k}{D}} \cdot x\right) \quad \textbf{[6]}$$

The height of the RanGTP gradient ($r[x = 0]$) increases linearly with the flux rate (J_R), whereas the rate of RanGTP hydrolysis and diffusion of RanGTP have an effect on both the height of the gradient and its slope.

J_R reflects the unknown activity and partitioning of accessible RanGTP and corresponds to a certain fraction of the total RanGTP flow through the nuclear pore complex (NPC); estimated value: 250 RanGTP molecules/NPC/s (1). The exact rate of *in vivo* RanGTP hydrolysis cannot be determined because many parameters that affect this rate have not been measured (2). In spite of this, the influence of the main parameters on reverse export behavior can be analyzed.

The slope of the RanGTP gradient is primarily influenced by the RanBP2/RanGAP complexes located on the cytoplasmic filaments of the NPC because their local concentration is around 200-400 times higher than that of RanBP1 and RanGAP in the cytoplasm (3). We therefore model a one-dimensional random walk of export complexes within the NPC and along the cytoplamic filaments using one-dimensional reaction-diffusion equations. Our modeling and experimental data confirm that a considerable proportion of export complex dissociation occurs within the distance of the NPC encompassed by the cytoplasmic filaments.

To calculate the probability of export complex (c) formation in the cytoplasmic part of the RanGTP gradient, Eqs. **1** and **6** were extended by Eq. **7**.

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2} + k_n r - k_B c \qquad \left[\frac{dc}{dx}\right]_{x=0} = \frac{1}{\eta}\cdot\frac{c}{l} \qquad \left[\frac{dc}{dx}\right]_{x=L\approx\infty} = 0 \qquad [7]$$

The solution of the system of Eqs. **1** and **3** for steady state gives the concentration of export complexes at the cytoplasmic face of the NPC ($x = 0$) (Eq. **8**). The flux of export complexes (J_C) into the NPC from the cytoplasm is given by Eq. **9**:

$$[c]_{x=0} = \frac{J_R}{K_R D}\cdot\frac{1-\sqrt{\frac{k_B}{k_B+k_n}}}{\sqrt{\frac{k_B}{D}}+\frac{1}{\eta l}} \qquad [8]$$

$$J_C = DK_C\frac{1}{\eta}\frac{[c]_{x=0}}{l} = \frac{K_C J_R}{K_R}\left(\frac{1-\sqrt{\frac{k_B}{k_B+k_n}}}{\eta\sqrt{\frac{k_B}{D}}+1}\right) \qquad [9]$$

k_B is the *in vivo* rate of dissociation of export complexes, which is assumed to have similar value to k ($k \approx k_B$); k_n is a derived first-order constant for the association rate of nuclear export signal (NES) cargoes with CRM1 and RanGTP and includes the concentration of NES cargoes (n). k in Eq. **1** was substituted by $k_B + k_n$. l denotes the length of the diffusion barrier formed by the NPC. The boundary condition at $x = 0$ describes the situation where the export complex is trapped within the nucleus. η^{-1} denotes the efficiency by which a drift term would reduce the rate of reverse export, and η therefore describes the extent to which directionality is imposed on the diffusion of export complexes in the forward direction. The value of η is determined by different parameters depending on the mechanism, if any, by which directionality is imposed on transport complexes moving within the NPC, via e.g. ratcheting or a potential field (4). For derivation of Eq. **2** $k_B = 0$ to calculate J_C (max).

Protein Constructs. The NES-GST constructs were cloned into the pHAT2 vector between the *Bam*HI and *Hin*dIII sites for Rev and An3 NES and between the *Nco*I and *Hin*dIII sites for NS2 and Mut(NS2) NESs. GST was cloned from a pETHTG vector (5). The C terminus of GST was tagged by a heart-muscle kinase (HMK) recognition sequence RRASVN. The N-terminal NESs had the following amino acid sequences: CLPPLERLTLGI, CVLNLDQQFAGLDLNGI, MACEMTKKFGTLTI, and MACEATKKAGTATA for Rev, An3, NS2, and Mut(NS2) NESs, respectively. The zz-GST was obtained by inserting the *Nco*I-*Hin*dIII fragment of pHAT2Mut(NS2)GST construct into a pQE60zz vector digested with *Nco*I and *Hin*dIII. The dihydrofolate reductase (DHFR)-An3-GST construct was obtained by inserting the *Bam*HI-*Hin*dIII fragment of pQE16, which includes DHFR and a *Bgl*II site downstream into pQE30. The *Bam*HI-*Hin*dIII fragment of pHAT2An3GST was ligated into the pQE30-DHFR digested with *Bgl*II and *Hin*dIII. DHFR was cloned into a pETHTG vector to obtain the DHFR-GST construct.

Protein Expression and Biotinylation. The NES-GST and DHFR-NES-GST construct were purified on TALON resin by using PBS/8.7 % glycerol for extraction and wash. After elution by 250 mM imidazole, the buffer was exchanged to 100 mM NaCl/20 mM HEPES, pH 7.5/2 mM $MgCl_2$/8.7% glycerol. The zz-GST was purified on glutathione-sepharose. PBS/8.7 % glycerol was used for extraction and wash, and the extraction buffer was supplemented by 0.25% Triton X-100 and 1 mM DTT. After elution with 20 mM glutathione, the buffer was exchanged to 100 mM NaCl/20 mM HEPES, pH 7.5/2 mM $MgCl_2$/8.7% glycerol. RanBP1 and RanGAP (Rna1p) were made as described (6). Strepavidin was purchased from Serva.

For biotinylation of the NES-GST and DHFR-NES-GST constructs N-hydroxysuccinimido-biotin (NHS-B) was dissolved in DMSO and was added to the protein in a 1:20 molar ratio. The reaction was supplemented with Na-bicarbonate pH 8.3 at 100 mM final concentration. After an incubation of 60 minutes at room temperature the reaction was quenched by 5 mM ethanolamine·HCl. The unbound biotin was removed by a 10 K Nanosep concentrator. zz-GST was labeled with biotin-maleimide in a 1:20 molar ratio. The reaction was quenched by 5 mM DTT.

Oocyte injections. Microinjection of proteins, incubations and extraction were performed as described (7), with the following modifications: the nuclear and cytoplasmic pellets were dissolved in the SDS-PAGE sample buffer, and were vigorously shaken for 15 minutes. No ultrasonication or heat treatment was applied. Contents of two oocytes (nuclear or cytoplasmic fractions) were loaded per lane for PAGE.

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