Supplementary Figure S1  Multiple sequence alignment of the G domain of the Rho protein family.

(A) Rho protein sequences were aligned using ClustalW program and the alignment was edited by GeneDoc. The conserved signatures of the Rho proteins are designated as G1 (P loop binds), G2 (switch I), G3 (switch II), G4 (guanine base binding) and G5 (guanine base binding). Conserved residues in G1-G5 motifs are highlighted in white and red background (X). Residues in white and black background (X) may be responsible for the fast nucleotide dissociation from RhoD and Rif. Residues in white and green background (X) are most likely responsible for the slow nucleotide association with RhoB, Rac2 and TC10. Catalytic residues that are not conserved in Rnd1, Rnd2 and Rnd3 proteins are highlighted in white and blue background (X). The alternative names of the proteins are summarized in the Supplementary Table S1.

(B) Phylogenetic tree of the G domain of the Rho protein family. The phylogenetic diagram was generated using program MEGA (http://www.megasoftware.net/) and shows the exceptional status of RhoD, Rif, TFF and the Rnd proteins. The number below the line represents the difference between sequences. Accordingly, 0.1 value is equivalent to 10% deviation in sequence.
Supplementary Figure S2  Multiple sequence alignment of the nucleotide binding residues of Rho proteins family.

(A) Nucleotide binding residues of Rho proteins sequences were aligned using ClustalW program and the alignment later was edited by GeneDoc. Invariable residues are highlighted in white and black background (X), highly conserved residues (80%) in white and grey background (X) and conserved residues (60%) in black and grey background (X). (B) Phylogenetic tree of the nucleotide binding residues of the Rho protein family. The phylogenetic diagram was generated using the program MEGA (http://www.megasoftware.net/) and shows the distinct status of Rif, RhoD and the Rnd proteins regarding in this case only active site of Rho GTPases. The number below the line represents the difference between sequences. Accordingly, 0.1 value is equivalent to 10% deviation in sequence.
Supplementary Figure S3  Real-time monitoring of nucleotide association kinetics. (A) Kinetics of the association between fluorescent labeled mantGDP (0.1 μM) and different concentrations (0.05–2 μM) of nucleotide-free RhoA were measured. Data were collected using a stopped-flow instrument with mant fluorescence as a signal of binding. Observed rate constants (k_{obs}) of associations were obtained by single exponential fitting of individual curves with increasing protein concentrations. (B) The association rate constants (k_{ass}) of 12 Rho proteins for mantGDP binding were calculated from the linear regression of the k_{obs} values plotted against the concentration of the nucleotide-free Rho proteins. The association rate (k_{ass}) is represented by the slope of regression lines. All k_{ass} values are summarized in Table 1. Association of mantGDP to nucleotide-free GTPases were carried out at 25°C in 30 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10 mM KH₂PO₄/K₂HPO₄, and 3 mM DTT.
**Supplementary Figure S4**  Intrinsic nucleotide dissociation of Rho GTPases.

The intrinsic nucleotide exchange of Rho GTPases were measured at 25°C in 30 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10 mM KH₂PO₄/K₂HPO₄, and 3 mM DTT by the monitoring of mantGDP dissociation from Rho mantGDP (0.1 μM) after the addition of 20 μM unlabeled GDP. The intrinsic nucleotide exchange reaction was completed after addition of 20 mM EDTA (arrow). Observed dissociation rate constants of intrinsic (k_{diss}) were obtained by single exponential fitting of the data. All k_{diss} values are summarized in Table 1.
Supplementary Figure S5  Intrinsic GTP hydrolysis of Rho GTPases.

Intrinsic GTPase reaction was measured by mixing 70 μM nucleotide free Rho protein with 50 μM GTP and incubating the reaction mixture at 25°C in 30 mM Tris-HCl pH 7.5, 10 mM KH₂PO₄/K₂HPO₄, 10 mM MgCl₂, 3 mM DTT. Samples have been taken at different time intervals, immediately frozen in liquid nitrogen and later analyzed for the amount of guanine nucleotide by HPLC assay in a time-dependent manner. Relative GTP contents were calculated from the ratio of (GTP)/(GDP+GTP). Intrinsic catalytic rate constants (kcat) of different proteins were obtained by single exponential fitting of the data (see also Table 1).
### Supplementary Table S1  Rho family proteins in the human genome.

Rho proteins are presented with their alias names, accession number and number of amino acids. Rho proteins highlighted in bold are investigated in this study.

### References


of Rac1 generates Rac1b, a self-activating GTPase. J. Biol. Chem. 279, 4743–4749.