3. Methods

Destination vectors and LR Recombination Reactions:

Transfer reactions were done using Gateway system according to the manufacturer's protocol with the following changes: the final reaction volume was 10µl and all components were used at half the recommended volume except that 1µl LR Clonase Enzyme and 3 units Topoisomerase I were used... The mixture was either frozen or immediately used for transformation of DH5α cells.

PDEST-17 was used as a His_6-expression vector. For the other tags, pCAL-n-Flag (Stratagene), pGEX-2tk (Pharmacia) and pMal-2c (New England Biolabs) respectively were adapted to recombinational cloning by insertion of the appropriate recombination cassette using a blunt site in the multiple cloning sites and subsequent determination of the correct orientation.

Transformations into DH5α cells and DNA Minipreps:

Transformations were done in 20µl or 100µl final volume as described in (Zhao et al., 1998). Up to 384 colonies were plated robotically on 25cm x 25cm LB-Agar plates, which contained 125µg/ml Ampicillin using a TECAN Genesis RSP. Minipreps were done robotically using the Qiagen 96-well Turbo prep.

Protein Expression and Purification in 96-well format:

A streak of freshly transformed BL21pLys^s was inoculated in 1ml TB media containing 125µg/ml Ampicillin, 34µg/ml Chloramphenicol and 2% Glucose and grown for 14-16hrs. The OD_{600} was measured and the cultures diluted to a final OD_{600} = 0.1 into fresh 1.5ml TB containing the same antibiotics. The cultures were then grown for approximately 3.5hrs at 25°C and simultaneously induced with 1mM IPTG when the average OD_{600} of all cultures was 0.7-0.9. After 1.5hrs growth at 25°C, a 75µl aliquot for western blot analysis was removed, the OD_{600} measured and the remainder of the liquid culture harvested. The pellets were frozen to minus 20°C.

Frozen cell pellets were thawed for 5 minutes at room temperature and resuspended at 4°C in 100µl Lysis Buffer. Resuspension was achieved by agitating the 96-deep well block on a Beckman Shaker for 5 minutes at 600 rpm while mixing the cells and the buffer with an inverted 96-pin device. 10µl Lysozyme/Tx-100 mix were added and mixing continued for 30 minutes at 300 rpm. Subsequently 10µl DNase mix were added and mixing continued at 300 rpm for another 15 minutes. During the incubations, two Whatman GF/C plates were prepared the following way: The filtration was wetted with lysis buffer. The purification matrix was aliquotted into the second plate, and equilibrated by addition of 200µl lysis buffer followed by a centrifugation. The purification plate was sealed at the bottom with an aluminum foil and placed on top of a rubber cushion. The filtration plate was placed on top of the purification plate and the lysates transferred into this filtration plate by centrifugation for 2 min at 2000 x g. Then the purification plate was sealed on top and rotated for 45 min at 4°C. After binding, the seals were removed and the lysates separated from the beads by a centrifugation at 16 x g for 1 min 30 sec at 4°C. The matrix was washed by repeated addition of 260µl wash buffer and centrifugation. Finally, the proteins were eluted by addition of elution buffer followed by 5-minute incubation and a centrifugation. After the last elution, the plates were centrifuged once more at 2000 x g for 5 minutes to remove the remainder of liquid.

Purification Buffers and Affinity Matrices:

His_6–tag denaturing conditions: Lysis buffer: 100mM NaH_2PO_4, 10mM Tris/HCl, 6M guanidine hydrochloride, 10mM 2-Mercaptoethanol, pH 8.0, Wash buffer: 100mM NaH_2PO_4, 10mM Tris/HCl, 8M Urea, pH 8.0; Elution buffer: wash buffer containing .5M Imidazole, pH 8.0. Ni-NTA matrix from Qiagen was used for purifications under denaturing conditions.

His_6 no denaturing conditions: Lysis and wash buffer: 50mM NaH_2PO_4, 500mM NaCl, 10% Glycerol pH 8.0; elution buffer: wash buffer containing 150 – 500mM Imidazole, pH 8.0. Ni-NTA manufactured by Qiagen and Talon matrix manufactured by Clontech was used.

CBP: For CBP purifications the Buffers A, B, and D were used as described in (Vaillancourt et al., 2000). CBP-agarose was purchased from Stratagene or Pharmacia and washed thoroughly.

GST: Lysis and wash buffer I: 140mM NaCl 10mM, Na_2HPO_4, 2.7mM KCl, 1.8mM KH_2PO_4, 2mM EDTA, 10% Glycerol, pH 7.3; wash buffer II: wash buffer I, but 500mM NaCl and 0.1% Triton X-100. Elution Buffer: wash buffer II containing 20mM reduced GSH. GSH-Agarose was purchased from Pharmacia and equilibrated in wash buffer I.

MBP: Lysis and wash buffer: 20mM Tris/Cl, 500mM NaCl 10% Glycerol, 2mM EDTA, pH 7.4. Elution buffer: wash buffer containing 20mM Maltose. Amylose resin was purchased from NEB and washed thoroughly with wash buffer before use.

SDS-PAGE and Western Blot analysis:

For SDS-PAGE analysis, 4-20% Criterion precast gradient gels with 26 wells were used. GelCode® Coomassie Blue reagent from Pierce was used to visualize protein bands on the gels. Western Blot was done as described (LaBaer et al., 1997). Antibodies and dilutions: α-Tetra-His antibody from Qiagen at 0.1µl/ml in BSA, M2 α-FLAG monoclonal from Pierce in 1:1000 in Blotto, Z-5 polyclonal α-GST from Santa Cruz 1:1000 in Blotto; α-MBP antibody from NEB, 1:1000 in Blotto. The signal visualized using Pierce Femto- or Pico-West Luminol reagent and detected the signals on a Chemidoc from Biorad or film.

Purification of kinases from insect cells and kinase reactions:

The baculoviruses for Cyclin E/ GST-cdk2 and Cyclin D1/GST-cdk4 were kind gifts of Dr Chou and Dr Zhao, respectively. Cells were lysed in Cyclin D buffer as described (LaBaer et al., 1997) and purified according to the recommendations of the manufacturer. Kinase assays were performed as described (LaBaer et al., 1997).

Database and Informatics:

The FLEXProt database was created by Dr. Hu using Microsoft Access. External data for the FLEXProt database were parsed from the Swissprot, LocusLink (Maglott et al., 2000) and Gene Ontology (2001) databases. Protein hydrophobicity was calculated using the TopPred algorithm (Claros and von Heijne, 1994). Query results were visualized using Spotfire software.