Supplemental Data

Experimental Procedures

Activity Assays

Assays for wild-type and mutant human E3 activity were conducted as described previously (Liu et al., 1995), except that 3 mM lipoamide was used in the place of the lipoic acid-bound lipoyl domain. PDC was reconstituted with E1p, E2p-E3BP according to the molar ratio described previously (Hiromasa et al., 2004) and a saturating concentration (0.9 μM) of wild-type or mutant E3 carrying a disease-causing mutation. Spectrophotometric assays for the overall activity of reconstituted PDC were carried out as described (Patel, 2001). The above assays were performed at 30° C.

Binding Measurements by Isothermal Titration Calorimetry (ITC)

ITC measurements were performed in a VP-ITC microcalorimeter from MicroCal (Northampton, MA). Titrations were carried out in 50 mM potassium phosphate buffer (pH 7.5), 100 mM KCl, and 5 mM β-mercaptoethanol at 20 °C. In a typical measurement for LBD-SBDp binding, 30 injections (at 3 min intervals) with 8 μl each of the ligand (67 μM) were made into 1.8 ml of the E3 protein (15 μM, monomer) in the cell. The binding isotherms derived from heat changes was used to calculate the standard free energy of binding (ΔG^o) according to the equation: $\Delta G^o = -RT\ln K_a$, where R is the gas constant, T the absolute temperature and K_a the association constant. From the binding isotherm, the number of binding sites (n) was obtained, and changes in enthalpy (ΔH^o) and entropy

(ΔS^o) were calculated according to the equation: $\Delta G^o = \Delta H^o - T\Delta S^o$. Curve fitting and the derivation of thermodynamic parameters were carried out with the ORIGIN v. 7.0 software package provided by MicroCal. The concentrations of E3 and LBD-SBDp were determined using extinction coefficients ε_{278nm} of 0.455 $mM^{-1}cm^{-1}$ (in 6 M guadinium-HCl) and 0.704 $mM^{-1}cm^{-1}$, respectively.

To obtain the accurate high-affinity binding constant for LBD-E3BD, competitive binding experiments were performed as described previously (Sigurskjold, 2000). The weaker-binding competitor ligand, LBD-SBDb protein (0.52 mM, monomer) of human BCKDC, was mixed with the E3 protein (15 μM, monomer) in the cell. The stronger-binding ligand, LBD-E3BD (0.41 mM, monomer) was titrated into the E3/LBD- SBDb mixture. The system generated two equilibria K_A = [PA] / [P][A] and K_B = [PB] / [P][B], which were displaced with each injection, where K_A and K_B were binding constants; P was the E3 protein; A and B were ligands LBD-E3BD and LBD-SBDb, respectively; PA and PB were E3-LBD-E3BD and E3-LBD-SBDb complexes, respectively. The K_B and ΔH_B for the competitor ligand (LBD-SBDb) were obtained in the above non-competitive bimolecular binding experiments. These parameters were entered as known parameters into the equation for the apparent binding constant: $K_{app} = K_A / 1 + K_B [B]$ from the competitive binding experiment to calculate binding constant K_A for the stronger ligand LBD-E3BD. The concentrations of LBD-E3BD and LBD-SBDb were determined using extinction coefficients ε_{278nm} of 0.77 $mM^{-1}cm^{-1}$ and 0.85 $mM^{-1}cm^{-1}$, respectively.

Analytical Ultracentrifugation

Purified wild-type and mutant human E3 proteins were dialyzed extensively against 50 mM potassium phosphate buffer, pH 7.5, 100 mM KCl, and 0.5 mM β-mercaptoethanol. Sedimentation equilibrium experiments were performed in an An60-Ti rotor in a Beckman Optima XL-I analytical ultracentrifuge at 4ºC using interference optics. Samples (110 µl) were loaded into a six-channel centerpiece at 1.1 µM final concentration of wild-type and mutant E3 proteins. The data were analyzed globally at 12,000 and 17,000 rpm by nonlinear least-squares analysis using the ORIGIN and SigmaPlot software packages. Experimental data were analyzed with theoretical curves for oligomers, dimers, monomers and monomer-dimer equilibrium. The best curve fit was evaluated on the basis of the magnitude and randomness of the residuals, expressed as the difference between the experimental data and the theoretically fit curves.

Other Methods

Ultraviolet circular dichroism spectra (190 to 250 nm) for wild type and mutant E3BD (1 µM) were determined using an AVIV (Lakewood, NJ) Model 62 DS spectropolarimeter.