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Characterization of the Kinase Domain of the Ephrin-B3 Receptor Tyrosine Kinase Using a Scintillation Proximity Assay

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Abstract: We have characterized a recombinantly expressed N-terminally tagged GST fusion of the tyrosine kinase domain of human EphB3. The EphB3 kinase domain was shown to phosphorylate a group of synthetic tyrosine-containing peptides derived from a proprietary biotinylated kinase-biased peptide substrate library. In addition, the enzyme activity was stimulated by the divalent cation, manganese, and inhibited by addition of magnesium. The most active tyrosine-containing peptide, a biotinylated 49-mer, displayed saturation kinetics with an apparent K_m of ~0.4 μM. The apparent K_m for ATP was determined to be ~3 μM. The kinetics of the reaction was linear from concentrations of enzyme of 0.5 to 2 nM, and at or below the K_m concentrations of the two substrates for at least 2 h at room temperature. Moreover, the tryrosine kinase inhibitor, PP2, produced an IC_{50} of roughly 0.8 μM. In addition, the enzyme tolerated the solvent DMSO and was stable to multiple freeze/thaw cycles. Stability of the enzyme at 4°C storage was seen out to 6 h with an ~50% reduction of activity by 24 h. Formatting the assay in a 384-well microtiter plate produced good uniformity of signal at 100% inhibition, 50% inhibition, and no inhibition. The coefficient of variance was at or below 10% with a signal-to-background ratio of ~24 and a z value of 0.72. Collectively, these results showed the ability to configure a robust HTS for a truncated recombinantly expressed family member of the Ephrin tyrosine kinases.

Introduction

The Ephrin family of receptors represents the largest subfamily of RTKs.[1-3] Together with their concomitant binding partners, the ephrin ligands, this class of RTKs regulates cell–cell communication by altering cell mobility, attachment, and shape. Unlike unidirectional tyrosine kinases, the Eph receptors and their liganded ephrins represent unique cell surface signaling molecules. The activation of the Eph RTK results in not only forward signaling into the receptor-bearing cell, but the generation of a reverse signal transduced into the ephrin ligand-bearing cell.[4] The Ephs and ephrins are categorized into one of two classes. Ephrin-As are tethered to the membrane via a glycosylphosphatidylinositol linkage, are cataloged within nine RTKs, EphA1–EphA9, and bind promiscuously to six A-ephrins. Ephrin-Bs, on the other hand, extend through the membrane via a transmembrane spanning domain, possess a highly conserved cytoplasmic domain, and interact with three different liganded B-ephrins (ephrinB1–ephrinB3). Crystallographic studies have aided in the understanding of the molecular basis of Eph–ephrin recognition. Thus, structural determinations of the N-terminal ligand-binding do-

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ABBREVIATIONS: BSA, bovine serum albumin; cpm, counts per minute; DMSO, dimethyl sulfoxide; DOE, design of experiment; DTT, dithiothreitol; EGFR, epidermal growth factor receptor; EphB3, Ephrin-B3 receptor; FRET, fluorescence resonance energy transfer; GST, glutathione-*S*-transferase; HTR, homogeneous time-resolved; nGST, N-terminal glutathione-*S*-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; RTK, receptor tyrosine kinase; SDS, sodium dodecyl sulfate.