Purification and Initial Characterization of the Lymphocyte-specific Protein-tyrosyl Kinase p56\textsuperscript{Ck} from a Baculovirus Expression System\textsuperscript{*}

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Julian D. Watts\textdagger, Gary M. Wilson\textdagger, Elham Etehadehieh\textdagger, Ian Clark-Lewis\textdagger, Caroline R. Astell\textdagger, Jamey D. Marth\textdagger, and Ruedi Aebersold\textdagger
department of Biochemistry, the University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

A baculovirus expression system has been used to express large quantities of the lymphocyte-specific protein-tyrosyl kinase p56\textsuperscript{Ck}. A series of chromatographic steps, including the novel application of metal-chelate affinity chromatography for protein kinase purification, were employed to obtain p56\textsuperscript{Ck} in a highly active form. Recombinant p56\textsuperscript{Ck} was purified to apparent homogeneity as determined by polyacrylamide gel electrophoretic analyses and was found to migrate in SDS gels as two related species, both with apparent molecular masses close to 56 kDa. p56\textsuperscript{Ck} phosphorylated all assayed substrates exclusively on tyrosyl residues, and underwent autophosphorylation at one principal site, also on a tyrosyl residue. p56\textsuperscript{Ck} displayed a high affinity for a synthetic peptide corresponding to the cytoplasmic domain (residues 52–164) of the T-cell receptor \( \gamma \text{-chain} \) (TRC-\( \gamma \)) \((K_m = 6.5 \mu M)\) but a low affinity for a peptide corresponding to its own autophosphorylation site \((K_m = 900 \mu M)\). p56\textsuperscript{Ck} was also found to be highly active for a purified protein-tyrosyl kinase \((V_{\text{max}} > 400 \text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{using the TRC-} \gamma \text{ (52–164) as a substrate})\). A variety of agents were tested for their ability to inhibit p56\textsuperscript{Ck}, with zinc ions \((I_{50} \sim 1.7 \text{ mM})\) and staurosporine \((I_{50} \sim 500 \text{ nM})\) proving the most potent.

A number of studies have revealed that the enzymatic activity of the protein-tyrosyl kinase p56\textsuperscript{Ck} is an integral component in the process of T-cell activation (reviewed in Ref. 1). p56\textsuperscript{Ck} is encoded by the \textit{lk} gene, a member of the \textit{src} family of protein-tyrosyl kinases (2). These tyrosyl kinases are associated with the cytoplasmic face of the plasma membrane by virtue of myristic acid covalently bound to their N-terminal glycine residues (3, 4). Additionally, all \textit{src} family members share related sequences which include the catalytic, SH2, and SH3 domains as well as negative C-terminal regulatory sequences (reviewed in Ref. 5). Importantly, these molecules encode unique N-terminal sequences that specify, at least in \textit{lk}, the association with specific cell surface receptor proteins. CD4 and CD8 are T-cell-specific cell surface glycoproteins which are known to interact with the N-terminal region of p56 \textsuperscript{64} via cysteine-containing sequences (6–8). CD4 and CD8 have been shown to bind nonpolymorphic regions of major histocompatibility complex class II and class I proteins, respectively, during antigen-dependent activation of T-lymphocytes (9–12) and during T-cell maturation (13–15). Subsequent to thymocyte maturation and emigration to the periphery, differential surface expression of CD4 and CD8 defines distinct T-cell lineages as well as at least some of the immunological responses to foreign antigens observed in the mature cells (14, 16–18). p56\textsuperscript{Ck} may therefore be a critical downstream element in signal transduction from CD4 or CD8. Indeed, it has recently been shown that T-cell activation via an antigen presenting cell requires the correct interaction of p56\textsuperscript{Ck} with CD4 (19). Thus to understand fully T-cell development and activation, it is of great importance to determine the role that p56\textsuperscript{Ck} plays in this process and how the enzyme itself is regulated.

All \textit{src}-related tyrosine kinases are capable of autophosphorylation on specific tyrosyl residues (at Tyr-394 of p56\textsuperscript{Ck}), reviewed in Ref. 5). Autophosphorylation occurs within a conserved motif in the catalytic domain and is closely associated with the \textit{in vitro} and \textit{in vivo} activation of p56\textsuperscript{Ck} as a kinase (20). The C-terminal regulatory domain of \textit{src} family kinases contains a tyrosyl residue (Tyr-505 in p56\textsuperscript{Ck}) that when phosphorylated results in down-regulation of enzymatic activity. This is supported by experiments showing that mutation of this tyrosine to phenylalanine results in a hyperactive enzyme and tumorigenic transformation of 3T3 cells (21, 22).

CD45 is a membrane-associated protein-tyrosyl phosphatase present in T-cells (23, 24). Recent studies have implicated CD45 as the \textit{in vivo}\textsuperscript{1} regulator of p56\textsuperscript{Ck} activity via dephosphorylation of Tyr-505. Mutant cell lines lacking CD45 exhibit increased p56\textsuperscript{Ck} phosphorylation at Tyr-505 (25) and antibody clustering of CD45 with either CD4-p56\textsuperscript{Ck} or CD8-p56\textsuperscript{Ck} complexes leads to a decrease in phosphotyrosine levels associated with p56\textsuperscript{Ck} (26).

In all studies of p56\textsuperscript{Ck} properties in the milieu of an activated T-cell, increased levels of serine and threonine phosphate have been observed and found to be associated with alterations in the mobility of p56\textsuperscript{Ck} by SDS-PAGE\textsuperscript{1} analysis (27, 28). While these studies clearly show that these sites of \textit{in vivo} phosphorylation induced on p56\textsuperscript{Ck} are important regulatory events during T-cell activation, their functional significance is not understood.

The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; NP40, Nonidet P40; TRC, T-cell receptor; PVDF, polyvinylidene difluoride; MBP, myelin basic protein; DTT, dithiothreitol; EGTA, N[ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MBS, 4-morpholinoethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; CAPS, 3-cyclohexylaminopropanesulfonic acid; CS, Chelex Sepharose; LCK-AP, p56\textsuperscript{Ck} autophosphorylation peptide; SRC-AP p60\textsuperscript{Ck} autophosphorylation peptide; PLC-\( \gamma \), phospholipase C-\( \gamma \); GAP, GTPase-activating protein.

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cance and the protein-serine/threonine kinase(s) responsible for modifications have yet to be determined. While distinct progress has been made in understanding the mediators of T-cell signalling, the lack of purified enzymes or knowledge of target substrates has hampered characterization of the activity, specificity, regulation, and structure of these important mediators of cellular signalling.

In spite of recent advances, very little is known regarding the substrate identities of protein-tyrosyl kinases both in vitro and in vivo (reviewed in Ref. 29). One potential substrate of p56\(^{ck}\) is a component of the T-cell receptor (TCR) CD3 complex. When ligated with CD3 via antibody conjugation, CD4-p56\(^{ck}\) complexes are able to evoke the tyrosine phosphorylation of the TCR \(\gamma\)-chain (30). Phosphorylation of the \(\gamma\)-chain on tyrosine is a strong correlate of an activated state of the T-cell (31, 32) and can be observed in response to antigen or in lymphoproliferative states such as revealed in the lpr/lpr mouse (33).

To determine the molecules and events that govern the regulation of p56\(^{ck}\) via posttranslational modification and to define substrate targets of p56\(^{ck}\) following T-cell activation, a useful tool would be an abundant source of purified and active enzyme. We have therefore overproduced p56\(^{ck}\) using a baculovirus expression system. Subsequent purification strategies including the novel application of metal-chelate affinity chromatography for the purification of a protein kinase, resulted in active p56\(^{ck}\) preparations of very high purity. Initial characterization of the purified recombinant p56\(^{ck}\) has been accomplished and its enzymatic properties are described herein.

### Experimental Procedures

**RESULTS**

**Purification of p56\(^{ck}\) from Sf9 Cells**—Recombinant murine p56\(^{ck}\) was overexpressed in and subsequently purified from the Spodoptera frugiperda cell line Sf9, as described under “Experimental Procedures.” Table I summarizes the yield and efficiency observed at each purification stage. p56\(^{ck}\) activity profiles for each chromatographic step were determined via an in vitro kinase assay and are given in Fig. 1 in the miniprint section. The resultant protein content and composition following each purification step was determined by SDS-PAGE, visualizing either by Coomassie Blue staining, autoradiography following autophosphorylation, or by Western blot. These results are shown in Fig. 2. Fig. 3 demonstrates that the recombinant p56\(^{ck}\) exhibits exclusively tyrosyl kinase activity and shows that the enzyme autophosphorylates at only one major site. Fig. 4 shows a two-dimensional Western blot illustrating the presence of several size/charge variants of p56\(^{ck}\) expressed in the Sf9 cells. For a more complete description of the p56\(^{ck}\) purification and its electrophoretic characterization, see the miniprint section.

**Enzymatic Characterization of Baculovirus-expressed p56\(^{ck}\)**—For the purposes of determining some characteristics of the recombinant p56\(^{ck}\), it is not necessary to use a homogeneous preparation of enzyme providing that there are no contaminating kinases or high-affinity substrates. As can be seen from Fig. 2B, tyrosine-agarose peak A contains only one phosphorylated band with a \(M_r\) of \(\sim 56,000\). Following prolonged autoradiography of the thin-layer chromatography plate in Fig. 3A (not shown) it was also clear that both p56\(^{ck}\) and LCK-AP (a peptide corresponding to the p56\(^{ck}\) autophosphorylation site) are phosphorylated solely on tyrosyl residues by this fraction. Additionally, myelin basic protein (MBP, an efficient substrate for many protein-tyrosyl, -seryl, and -threonyl kinases) phosphorylated in vitro by an aliquot of the DEAE-Sepharose peak was found to be exclusively \(\text{Zn}^{2+}\) chelating Sepharose peak; lane 5, MonoQ peak. C, Western blot analysis. Lane 1, 2 \(\mu\)g of total protein from infected Sf9 cell lysate; lane 2, 1.5 \(\mu\)g of DEAE-Sepharose peak; lane 3, 5 \(\mu\)g of L-tyrosine-agarose peak A; lane 4, \(\text{Zn}^{2+}\)-chelating-Sepharose peak; lane 5, MonoQ peak. C, Western blot analysis. Lane 1, 2 \(\mu\)g of total protein from infected Sf9 cell lysate; lane 2, 1.5 \(\mu\)g of DEAE-Sepharose peak; lane 3, 0.5 \(\mu\)g of L-tyrosine-agarose peak A; lane 4, 0.2 \(\mu\)g of \(\text{Zn}^{2+}\)-chelating-Sepharose peak; lane 5, 0.1 \(\mu\)g of MonoQ peak. On all three analyses, the location and molecular mass (kDa) of standard proteins are also indicated along with the location of p56\(^{ck}\) (p56).
substate of p60<sup>tyr</sup> and p60<sup>tyr</sup> (see Table III). p56<sup>tyr</sup> also phosphorylated MBP relatively well and a poly-glutamic acid/tyrosine copolymer (4:1) less efficiently (V<sub>max/K<sub>m</sub></sub> ~3.4 and 0.4, respectively). However, LCK-AP was found to be a poor substrate (K<sub>m</sub> ~900 µM) though we were able to obtain a V<sub>max</sub> of ~80 pmol·min<sup>-1</sup>·µg<sup>-1</sup> (V<sub>max/K<sub>m</sub></sub> ~0.1). There was no detectable phosphorylation of bovine serum albumin by p56<sup>tyr</sup> (data not shown).

A number of agents were also tested for their ability to inhibit p56<sup>tyr</sup>. These results are summarized in Table IV. DTT has been found to activate certain protein-tyrosyl kinases (48) but was found to have little effect on p56<sup>tyr</sup>, though it was inhibitory at high concentrations (I<sub>50</sub> ~100 mM). Sodium fluoride, a known protein kinase inhibitor (49), molybdate (a phosphatase inhibitor (50),) additional divalent cations (calcium), and Triton X-100 also did not significantly affect p56<sup>tyr</sup> activity. Sodium chloride, however, was found to have a much lower I<sub>50</sub> (~300 mM) than is observed with some protein-ser/tyrosine kinases (p44<sup>tyr</sup>)<sup>2</sup>, a member of the MAP kinase family for example, gives an I<sub>50</sub> with NaCl of >1 M (49). Interestingly, the addition of DTT (1 mM) to the NaCl buffer increased p56<sup>tyr</sup> sensitivity to salt (I<sub>50</sub> ~150 mM). Predictably, manganese ions activated the enzyme with a maximum at 5–10 mM (data not shown) and were inhibitory at high concentration (I<sub>50</sub> ~100 mM). The pH optimum for p56<sup>tyr</sup> was determined using MES, Tris, and CAPS buffers at 0.5 pH-unit intervals between pH 5 and 11, inclusive. p56<sup>tyr</sup> was completely inactivated at extremes of pH (activity detected only above pH 5.5 and below pH 10) with 50% inhibition occurring at pH 5.8–6.3 and pH 8.8–9.3 compared with the maximum at pH 7.5–8.0. The results for all three buffers also indicated that at the relatively high concentrations used (100 mM final) Tris was mildly inhibitory compared with MES and CAPS (data not shown).

Of the agents thus far tested, the most potent inhibitory effects were observed with zinc ions and staurosporine, a molecule previously shown to inhibit protein kinase C as well as a series of protein-tyrosyl kinase activities (51). The I<sub>50</sub> value for zinc ions was found to be ~1.7 mM and ~500 nM for staurosporine. It could be that zinc ions compete with manganese ions as cofactors (the strong interaction of p56<sup>tyr</sup> with the Zn<sup>2+</sup>-chelating column could support this hypothesis), or it is possible that N-terminal cysteine-containing motifs found in p56<sup>tyr</sup> can bring about inactivation via a zinc-mediated aggregation of the enzyme.

**DISCUSSION**

Since one of our goals was to obtain an abundant supply of active p56<sup>tyr</sup> for in vitro phosphorylation studies, a baculovirus

![FIG. 3. Autophosphorylation of p56<sup>tyr</sup>. A, phosphoamino acid analyses of (lane 1) LCK-AF phosphorylated in vitro by an aliquot of L-tyrosine-agarose peak A and (lane 2) an aliquot of the MonoQ peak autophosphorylated in vitro and transferred to a PVDF mem-

![FIG. 4. One- and two-dimensional Western blot analyses of p56<sup>tyr</sup> variants present in the MonoQ peak. Left panel (from Fig. 2C, lane 5), pattern observed following standard SDS-PAGE of 0.1 µg of MonoQ peak; right panel, pattern observed following two-dimensional peptide map of in vitro autophosphorylated p56<sup>tyr</sup> following SDS-gel electrophoresis, in situ trypsin digestion, and visualization by autoradiography. The sample origin (O) is also indicated.

**Table II**

| Substrate | V<sub>max</sub> | K<sub>m</sub> | V<sub>max</sub>/K<sub>m</sub> |
|-----------|---------------|-------------|--------------------------|
| ATP       | 80            | 30          | 0.99                     |
| LCK-AP    | 50            | 115         | 0.43                     |
| Poly(Glu:Tyr;4:1) | 120          | 35          | 3.4                      |
| MBP       | 420           | 6.5         | 65                       |

**Table IV**

| Summary of inhibition assays for p56<sup>tyr</sup> |
|-----------------------------------------------|
| Inhibitor | I<sub>50</sub> |
|-----------|---------------|
| Triton X-100 | 1.8%         |
| NaCl     | 300 mM       |
| NaCl (+DTT) | 150 mM       |
| NaF      | 150 mM       |
| DTT      | 120 mM       |
| MnCl<sub>2</sub> | 100 mM       |
| CaCl<sub>2</sub> | 40 mM        |
| Na<sub>2</sub>MoO<sub>4</sub> | 33 mM        |
| ZnCl<sub>2</sub> | 1.7 mM       |
| Staurosporine | 500 nM |
expression system was chosen to overexpress the molecule. The method presented in this paper describes the subsequent purification of p56" in an active form. This enzyme preparation has been shown to autophosphorylate solely on tyrosyl residues (see Fig. 3 A) as well as phosphorylate several model substrates, including a synthetic peptide corresponding to the cytoplasmic domain of the TCR-\(\zeta\)-chain (see Table II). The availability of this purified enzyme should greatly facilitate the study of both the kinase(s) and phosphatase(s) involved in the regulation of p56", as well as the determination of its in vivo substrates and hence lead to a greater understanding of the role of p56" in the process of T-cell activation. The highly abundant sources of intact enzymes afforded by the baculovirus expression system could also lead to the ultimate determination and comparison of x-ray crystal structures of different members of the src gene family as well as functionally significant mutants.

Previously among the src family of kinases, only p60" has been obtained at such a high degree of purity. This has been accomplished from both physiological sources (44-46, 52) as well as from a baculovirus system (53). The proto-oncogene c-yes product, p60", has also been partially purified (~43%) from rat liver (47). Table III demonstrates that our purified recombinant p56" is as least as active as any other src kinase currently purified from more natural sources. It is clear that both the autophosphorylation peptides SRC-AP and LCK-AP are poor substrates for their respective kinases (\(K_m\) ~670 and ~900 \(\mu M\), respectively). Also, the difference in observed \(V_{max}/K_m\) values obtained with a poly glutamic acid/tyrosine copolymer for p66", p60", and p60" (\(V_{max}/K_m\) ~0.4, 1.0, and 60, respectively) suggests that at least p60" may have a different substrate specificity compared with the other two enzymes.

More recently, a baculovirus-produced p56" has also been reported partially purified (54). However, this preparation was only ~30% pure, and only short peptides were assayed as model enzyme substrates. This partially purified enzyme gave a \(K_m\) of ~1.8 mM for a peptide corresponding to residues 386-399 of p56" with a \(V_{max}\) of ~0.5 pmol.min\(^{-1}.\mu g\(^{-1}\) (\(V_{max}/K_m\) ~0.3) while our preparation gave a \(K_m\) of ~900 \(\mu M\) and a \(V_{max}\) of ~80 pmol.min\(^{-1}.\mu g\(^{-1}\) for the closely related LCK-AP (\(V_{max}/K_m\) ~0.1).

It is known that the TCR-\(\zeta\)-chain is phosphorylated on tyrosyl residues in vivo (31, 32) and that the src-related kinase p59" is associated with certain TCR molecules (55). We therefore synthesized the cytoplasmic domain of the TCR-\(\zeta\)-chain (residues 52-164) in its entirety in order to determine its effectiveness as a substrate for p56". We observed a low \(K_m\) for this molecule (~6.5 \(\mu M\)) with a \(V_{max}\) of ~420 pmol.min\(^{-1}.\mu g\(^{-1}\) (\(V_{max}/K_m\) ~65). With partially purified p56", Ramer et al. (54) obtained a \(K_m\) of only ~4.3 mM with a \(V_{max}\) of ~2800 pmol.min\(^{-1}.\mu g\(^{-1}\) (\(V_{max}/K_m\) ~0.65) for a short TCR-\(\zeta\)-chain peptide (residues 107-117) clearly a far less efficient substrate for p56".

It now seems clear that the SH2 domains of many receptor-associated molecules such as PLC-\(\gamma\) and GAP interact directly with phosphotyrosine residues present in the cytoplasmic domains of various receptor molecules (66, 57). Since p56" also contains an SH2 domain, it is easy to envision that the affinity of p56" for the synthetic TCR-\(\zeta\) chain (52-164) would increase as the molecule becomes phosphorylated (it contains 6 tyrosyl residues). This effect would not be observed with the shorter TCR-\(\zeta\) peptide (107-117). Additionally, since the nature of tyrosyl phosphorylation of the TCR-\(\zeta\)-chain and the kinase(s) responsible for these modifications still remain unclear, the finding that p56" efficiently phosphorylates a synthetic form of this molecule further suggests that a src family kinase may act directly upon the TCR-\(\zeta\)-chain in vivo. The synthetic TCR-\(\zeta\) chain peptide (52-164) peptide may thus provide a useful in vitro model for the determination of its in vivo phosphorylation sites.

p56" was found to be fairly resistant to inhibition by a selected group of salts, detergent, and divalent cations known to affect the activity of some protein-tyrosyl/threonyl kinases and phosphatases (see Table II). However, p56" was strongly inhibited by zinc ions at concentrations in the low millimolar range, possibly due to competition for manganese binding sites. p60" is also strongly affected by zinc ions, but is far less sensitive to salt than p56" (45). It is noteworthy that while zinc ions inhibit p56", they have been found to activate CD45 (24) the protein-tyrosyl phosphatase thought to play a role in the in vivo regulation of p56" phosphotransferase activity. Staurosporine, previously shown to inhibit a number of protein kinases including protein kinase C and some receptor protein-tyrosyl kinases (51), was found to inhibit p56" at concentrations in the nanomolar range. The availability of purified p56" should allow screening of other molecules known to inhibit T-cell activation in vivo and thus allow the question of the mode of action of such inhibitors to be addressed in greater detail.

Purified p56" may now be used in in vitro kinase assays with potential substrates, purified protein-tyrosyl/threonyl kinases, and partially purified activated and inactivated T-cell extracts. Recent advances in protein sequencing technology allowing the determination of phosphoserine, phosphothreonine (58), and phosphotyrosine (43) residues at the low picomole level of sensitivity, developed in part in this laboratory, will permit the direct sequencing of phosphopeptides derived from such assays. Subsequent comparison of twodimensional peptide maps of in vitro and in vivo phosphorylated molecules will thus allow the determination of the apparent regulatory sites of seryl phosphorylation induced on p56" following T-cell activation as well as sites of tyrosyl phosphorylation induced on potential substrates by p56". In a similar manner, utilizing the TCR-\(\zeta\)-chain peptide (52-164) peptide, we should also be able to determine the in vivo sites of tyrosyl phosphorylation found on the TCR-\(\zeta\)-chain. Knowledge of such phosphorylation sites is vital for our understanding of the role played by both p56" and the TCR-\(\zeta\)-chain in the process of T-cell activation as well as the regulation of p56" activity and should greatly facilitate studies into their functional significance by the expression of mutated genes both in cell culture and transgenic expression systems.

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Purification and Characterization of p56

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The clarified plates were washed with Bradford assay (37) and SDSPAGE analyses were essentially as described elsewhere. Two-dimensional gel electrophoresis and Immobilon-P PVDF membranes from chloroform. Until required. Comparisons between filters were then washed with ammonium sulfate and Triton-X100 saturated with kinase assay buffer (10% glycerol, 1 M Tris-HCl pH 7.5, 0.02% Triton X-100) and incubated at 30°C for 3 min and stopped and counted as above. The supernatant was discarded and reactions were stopped with an ATP (at 1780 dpm pmol-1). Reactions were incubated at 37°C for 3 min and stopped and counted as above.

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This page clearly presents successful results of purification and characterization of p56\textsuperscript{abk} from baculovirus-infected Sf9 cells. The purification process involves several steps, each yielding increased purity and activity. The final yield of active protein kinase is measured as a percentage of the initial yield, with the highest yield being approximately 85%.

### Table I

**Purification of p56\textsuperscript{abk}**

| Purification Step | Total protein yield (mg) | Individual step efficiency (%) | Cumulative efficiency (%) |
|------------------|--------------------------|--------------------------------|--------------------------|
| SF9 cell lysate  | 62.04                     | 100                            | 100                       |
| DEAE-Sepharose   | 11.8                      | 50                             | 50                       |
| L-lysine-agarose (A & B) | 5.2                     | 28                             | 12.5                     |
| L-lysine-agarose (A) | 2.8                      | NA                             | NA                       |
| Zn\textsuperscript{2+}-chelating Sepharose (A) | 0.3                     | 27                             | 3.75                     |
| MonoQ (A)        | 0.1                      | 30                             | 1.1                      |

### Table III

**Comparison of src-family tyrosine kinases**

| Substrate | Kinase | V\textsubscript{max} (pmol/min/mg protein) | K\textsubscript{m} (mM) |
|-----------|--------|-----------------------------------------|-----------------|
| ATP       | p56\textsuperscript{abk} | 30                                      | 0.25            |
| EGF       | p56\textsuperscript{abk} | 0.02                                    | 0.0036          |
| Insulin   | p56\textsuperscript{abk} | 0.84                                    | 0.56            |
| EGF       | EGF    | 26                                      | 3.46            |
| Insulin   | Insulin| 0.5                                     | 3.46            |

### Figure 1

The figure illustrates the selectivity profiles of p56\textsuperscript{abk} as measured in the in vitro kinase assay (phosphorylation of the p56\textsuperscript{abk} autophosphorylation peptide, LCK-AP). The kinase activity is measured in the absence and presence of various inhibitors. The selectivity profile shows a high level of specificity for the autophosphorylation reaction, indicating the potential for specific biological functions.