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ORIGINAL ARTICLE

Preparation and Application of an Antibody Specifically Targeting Tyrosine-Phosphorylated PI3K p85 at Position 452

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ABSTRACT

Background: **The current invention pertains to the development and utilization of an antibody that specifically recognizes tyrosine-phosphorylated PI3K p85 at position 452. The process encompasses antigen preparation, immunization, and the construction of affinity chromatography columns.**
Methods: **To facilitate efficient peptide conjugation to a carrier protein and subsequent peptide-based affinity purification, a cysteine residue (C) was incorporated at the C-terminus of the peptide as a linker. The final peptide sequence was identified as KLHEY(p)NTQFQE. The antibody was purified through a two-step affinity purification protocol: initially, the antiserum was passed through a phosphorylated peptide column to enrich phospho-specific antibodies, followed by passage through a non-phosphorylated peptide column to eliminate non-specific binders.**
Results: **This methodology enables the scalable production of the anti-phospho-PI3K p85 (Tyr452) antibody, which demonstrates high specificity for phosphorylation and strong affinity.**
Conclusions: **In comparison to traditional protein A purification, this approach is markedly more efficient.**
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KEYWORDS

PI3K p85, tyrosine phosphorylation, site-specific antibody, affinity chromatography, high-affinity antibody

INTRODUCTION

Phosphoinositide 3-kinase (PI3K) serves as a pivotal component within cellular signal transduction networks, and its dysregulation is closely associated with oncogenesis, metabolic disorders, and immunodeficiency [1-3]. The p85 regulatory subunit of PI3K not only regulates the activity and subcellular localization of the catalytic p110 subunit but also influences downstream pathway selectivity through its own phosphorylation [4-6]. Recent mass spectrometry studies have identified phosphorylation at tyrosine 452 (Tyr452) on p85 as a critical switch for the activation of the PI3K-AKT pathway [7-9]. The presence of this modification is strongly correlated with tumor invasiveness, drug resistance, and poor clinical prognosis [10-12]. However, the lack of highly specific detection tools for the Tyr452-phosphor-

ylated state has significantly impeded mechanistic studies and diagnostic applications.

Conventional antibodies, typically purified via Protein A/G chromatography, are easy to obtain but fail to reliably distinguish between phosphorylated and non-phosphorylated epitopes, leading to high background noise and reduced specificity [13,14]. Consequently, there is an urgent need to develop a high-affinity antibody that selectively recognizes PI3K p85 phosphorylated at Tyr-452 and can be produced at scale.

To overcome this bottleneck, we developed and validated a comprehensive workflow that includes antigen optimization, immunization, and a two-step affinity purification process [15]. A cysteine residue was added to the C-terminus of the epitope sequence KLHEY(p) NTQ FQE, which encompasses Tyr452, to facilitate directional conjugation to carrier proteins such as KLH or BSA, while avoiding interference from disulfide bonds with internal cysteines. Target antibodies were subsequently enriched using a phosphopeptide affinity column, followed by the removal of cross-reactive species using a non-phosphopeptide column. This approach significantly improved both the specificity for phosphorylation and the binding affinity. Moreover, the entire procedure is easily scalable under standard laboratory conditions. The antibody produced through this method serves as a robust molecular tool for studies on PI3K signaling, tumor biomarker detection, and drug development applications.

MATERIALS AND METHODS

Materials

Animal serum; peptide-coupled affinity column; 1 M Tris·HCl, pH 7.5; 100 mM glycine buffer, pH 3.0; 10 mM PBS, pH 7.4; 10 mM PBST, pH 7.4 (containing 0.05% Tween-20); storage buffer.

Methods

Antigen preparation: The antigen design and structure-guided screening processes identified the phosphotyrosine epitope KLHEY(p)NTQFQE, to which a C-terminal cysteine was appended for conjugation purposes, resulting in KLHEY(p)NTQFQE-C.

Conjugation process: Keyhole limpet hemocyanin (KLH) (100 mg) was activated using sulfo-SMCC, desalted, and subsequently coupled to the peptide at a 1:1 mass ratio, with the reaction proceeding overnight at 4℃. Residual maleimide groups were quenched with cysteine, and the resultant conjugate was dialyzed into 0.01 M PBS, achieving an approximate yield of 30%. The conjugate was then aliquoted and stored at -70℃.

Immunization protocol: Rabbits were initially primed subcutaneously with 300 μg of peptide-KLH in complete Freund's adjuvant, followed by three booster injections at two-week intervals using 200 μg in incomplete Freund's adjuvant.

Serum collection: Blood samples (0.3 - 0.4 mL) were collected from the marginal ear vein seven days post-boost, and serum was separated by centrifugation and stored at -20℃.

Affinity column preparation: Sulfolink gel (2 mL) was coupled with 2 mg of peptide overnight at 4℃, blocked with 50 mM cysteine, and washed sequentially with PBS, 1 M NaCl, and Gly-HCl, before being stored in 0.02% NaN at 4℃ [16-20]. For further specific details, please refer to the supplementary materials.

Procedure

Peptide conjugation protocol

1) Desalting: To prepare KLH-SMCC, sulfo-SMCC is added to the KLH solution. The mixture is then subjected to desalting using a column pre-equilibrated with three column volumes of coupling buffer to remove unreacted sulfo-SMCC. The KLH-SMCC solution is loaded onto the column, and once it enters the gel bed, it is eluted with coupling buffer. The protein peak of KLH-SMCC is monitored at A_{280}, and the relevant fractions are collected and pooled.

2) Quantification and aliquoting: The concentration of KLH-SMCC is determined using the Lowry method. The solution is then aliquoted into tubes, each containing 3 mg.

3) Reaction: A reaction mixture is prepared by combining 3 mg of KLH-SMCC with a peptide solution at a concentration of 20 mg mL^{-1}. This mixture is incubated overnight at 4℃ on a silent rotator. Subsequently, a cysteine-C solution is added, and the mixture is gently shaken for 2 hours at a temperature range of 2 - 6℃.

4) Dialysis: The reaction mixture is transferred into a freshly prepared dialysis bag and dialyzed against 0.01 M PBS. Following dialysis, the entire contents are collected, and the dialysis bag is rinsed with PBS. All rinse fluids are pooled together.

5) Storage: The peptide-KLH conjugate is diluted to a concentration of 1 mg mL^{-1} in 0.01 M PBS. The solution is then aliquoted into vials and stored at a temperature of ≤ -50℃.

Antigen emulsification protocol

1) Preparation of antigen solution: Dilute the peptide-KLH antigen to a concentration of 1 mL per rabbit using 0.01 M PBS.

2) Syringe preparation: Draw the entire antigen solution into one syringe and an equivalent volume of Freund's adjuvant into a separate syringe.

3) Connection of syringes: Utilize a double-ended needle to connect the two syringes.

4) Emulsification process: Rapidly inject the antigen solution into the adjuvant and perform repeated push-pull movements until the mixture is fully emulsified.

5) Emulsion testing: To assess emulsification, deposit a droplet of the mixture onto cold water. If the droplet maintains its integrity, emulsification is deemed complete; otherwise, continue the emulsification process.

6) Transfer of emulsion: Once qualified, transfer the emulsion through the double-ended needle into a glass injection syringe and attach the appropriate needle.

Immunization schedule

1) Primary immunization: Restrain the rabbit and administer 0.3 - 0.5 mL of the emulsion subcutaneously at 8 - 10 different sites.
2) Booster immunization: Administer 0.1 - 0.3 mL of the emulsion subcutaneously at five dorsal sites and two intramuscular sites on the thighs.
3) Serum collection: Perform blood collection 7 days following the first booster to obtain serum A, and again 7 days after the second booster to obtain serum B. Collect positive serum 7 days subsequent to the third booster.
4) Serum separation: Separate the serum by centrifuging the collected blood samples.

Affinity-column preparation

1) Peptide coupling to sulfolink gel: Allow the sulfolink gel to reach room temperature. Wash the column with one bed volume of coupling buffer, ensuring a small volume remains at the bottom. Insert the bottom disc.
2) Column packing: Pack 2 mL of sulfolink gel slurry into the column and equilibrate it with at least 20 mL of coupling buffer. Secure the bottom with a cap.
3) Peptide loading: Dissolve 2 mg of peptide in 2 mL of coupling buffer and load the solution onto the column. Incubate the column at 4℃ overnight with gentle agitation.
4) Effluent collection: On the following day, allow the column to reach room temperature for approximately 30 minutes. Remove the caps from top to bottom, drain the column, and collect the effluent.
5) Blocking: Add 2 mL of 50 mM cysteine-C to the column, cap it, and mix for 2 hours at room temperature. Allow it to rest for 30 minutes, then drain any excess solution.
6) Washing: Sequentially wash the column with 20 mL of PBS, 1 mol L^{-1}NaCl, PBS, and Gly-HCl buffer.
7) Storage: Cap the bottom of the column, add 10 mL of storage buffer until the meniscus is 0.8 ± 0.2 cm above the disc, cap the top, and store the column at 4℃.

Antibody purification

Serum handling: Pool the batches of serum and adjust the pH to 7.5 by adding 1/10 volume of 1 mol L^{-1} Tris·HCl. Incubate briefly at room temperature and filter the serum through a sterile filter.

Phospho-peptide (P-peptide) column chromatography protocol

1) Equilibrate the phospho-peptide column to room temperature. Remove the top cap followed by the bottom cap, allowing the storage buffer to drain. Subsequently, wash the column with 20 mL of 10 mM PBS at pH 7.4.
2) Introduce 2 mL of P-peptide-coupled sulfolink gel into the treated serum and incubate the mixture overnight at 4℃ or for 2 hours at room temperature with rotation.
3). Reassemble the column, expel any air using 10 mL of PBS, and load the serum-gel mixture. Allow the gel to settle, then wash with 5 - 7 mL of PBS and collect the flow-through.
4) Perform a wash with 20 mL of 10 mM PBS at pH 7.4. Cap the column when the liquid level is 1 - 2 cm above the gel, insert the top disc, and wash again with 10 mL of PBS, followed by 20 mL of 10 mM PBS-T (0.05% Tween-20).
5) Prepare two 1.5 mL tubes, each containing 100 µL of 1 M Tris·HCl at pH 7.5, and one 30 mL bottle.
6) Conduct a pre-wash with 1 mL of 100 mM glycine at pH 3.0, discarding the wash. Elute twice with 1 mL of glycine at pH 3.0 into the tubes, neutralize the eluate, and continue collection until no protein is detected.
7) Measure the absorbance at 280 nm (A_{280}) and pool fractions exhibiting an absorbance greater than 0.1.
8) Re-absorb the P-peptide flow-through by repeating steps 1 through 8 until the antibody is completely depleted.
9) Clarify any precipitates by centrifugation and record the final absorbance.
10) Retain the purified serum samples for subsequent ELISA testing.

RESULTS

ELISA evaluation of phosphorylation specificity

The phosphorylation specificity of the anti-pTyr452 PI3K p85 antibody was evaluated using a direct ELISA. Microtiter plates were coated with either phosphorylated or non-phosphorylated PI3K p85 peptides and incubated with four-fold serial dilutions of the purified antibody (ranging from 1:1,000 to 1:64,000). As depicted in Table 1 and Figure 1, the antibody exhibited strong, titratable binding to the phosphorylated peptide (P-peptide), with OD450 values of 1.308, 0.984, 0.693, and 0.441 at dilutions of 1:1,000, 1:4,000, 1:16,000, and 1:64,000, respectively. In contrast, the signals for the non-phosphorylated peptide (N-peptide) remained at background levels across all dilutions (OD450 ≤ 0.303). Consequently, the ratio of phosphorylated to non-phosphorylated signals ranged from 4.3-fold at the lowest dilution to greater than 140-fold at the highest dilution, thereby confirming that the antibody specifically recognizes the tyrosine-phosphorylated PI3K p85 epitope at position 452.

Western blotting evaluation of phosphorylation sensitivity

Western blotting revealed that total PI3K protein levels were comparable between mouse lung adenocarcinoma and matched peritumoral tissues, whereas phosphorylated PI3K was markedly elevated in the lung tumor (LUAD) samples (Figure 2). The unchanged total signal indicates that the anti-phospho-PI3K antibody recognizes only the activated form, underscoring its high specificity.

Table 1. ELISA evaluation of phosphorylation specificity.

Antibody dilution ratio	1:1,000	1:4,000	1:16,000	1:64,000
Phosphorylated PI3K p85 peptide	1.308	0.984	0.693	0.441
Non-phosphorylated PI3K p85 peptide	0.303	0.107	0.085	0.003

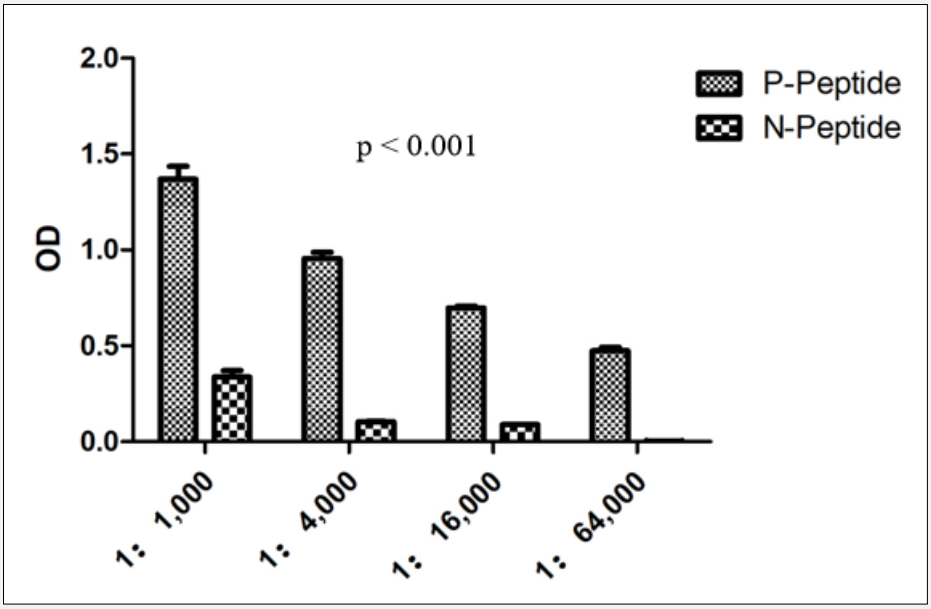

Figure 1. ELISA evaluation of phosphorylation specificity.

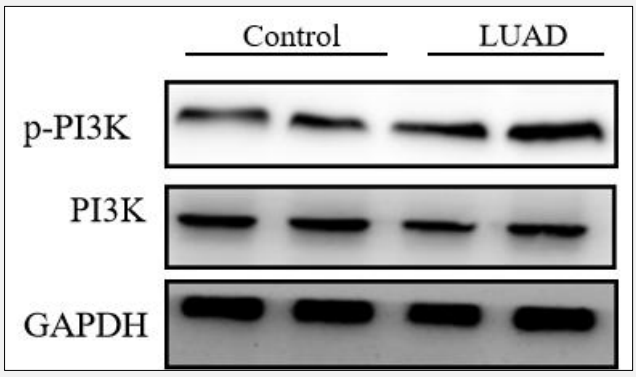

Figure 2. Western blotting evaluation of phosphorylation sensitivity.

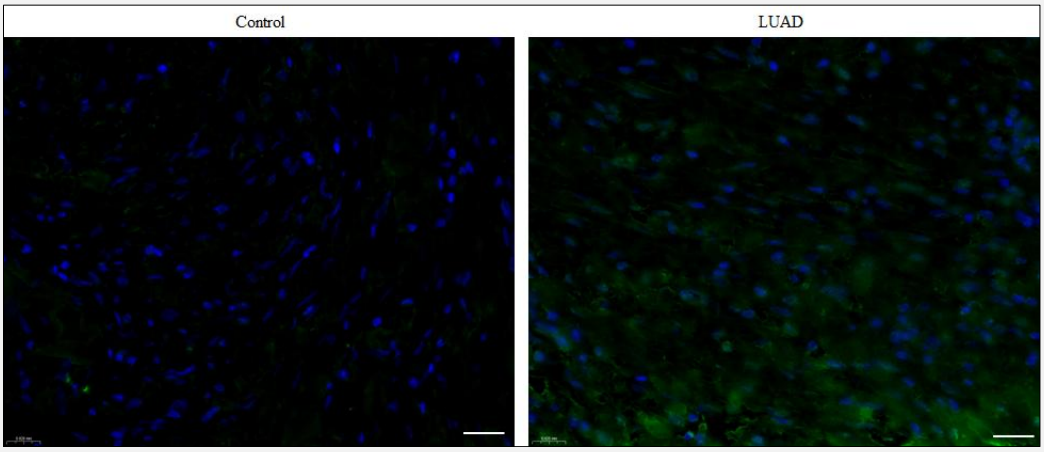

Figure 3. Immunofluorescence validation results.

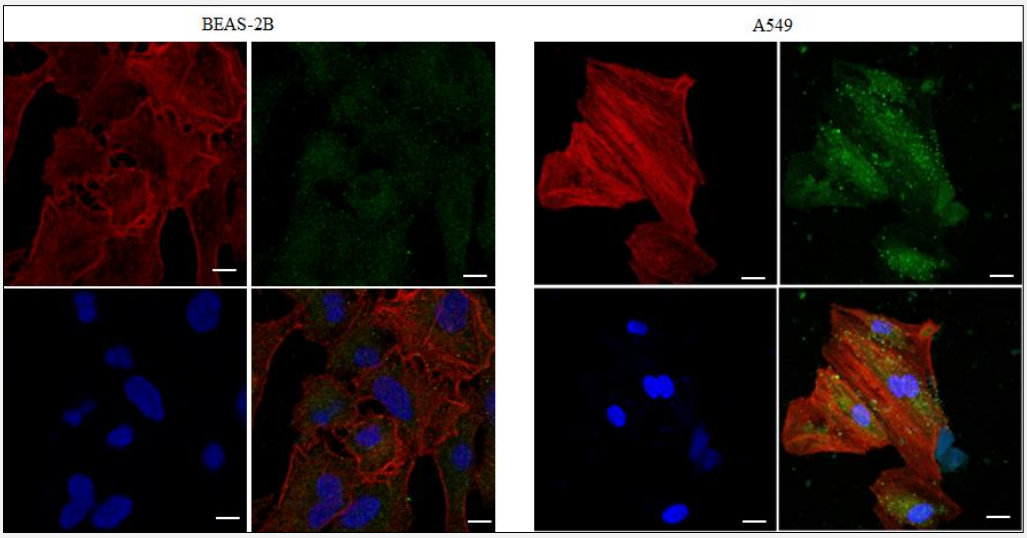

Figure 4. Immunofluorescence validation results.

Immunofluorescence evaluation tissue of phosphorylation specificity

Immunofluorescence staining with the anti-p-PI3K antibody revealed a striking increase in phospho-PI3K signal in murine lung adenocarcinoma tissues compared with matched adjacent non-tumor lung tissues. The tumor areas exhibited intense, membrane-proximal cytoplasmic staining (Figure 3).

Immunofluorescence evaluation cells of phosphorylation specificity

When BEAS-2B bronchial epithelial cells were stained with the anti-phospho-PI3K antibody, only faint green cytoplasmic signal was detected. In contrast, A549 lung adenocarcinoma cells exhibited a markedly stronger P-PI3K signal that appeared as bright, punctuate aggregates in the perinuclear region and along cellular exten-

sions. In both cell lines, red phalloidin labeling of F-actin revealed intact filament architecture and served as an internal reference for cell morphology. The striking difference in P-PI3K immunoreactivity between the weakly responsive BEAS-2B and the highly activated A549 cells, visualized at 63 x oil-immersion magnification, confirms the high specificity of the antibody for its phosphorylated target (Figure 4).

Data Availability Statement:
The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

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Declaration of Interest:
The authors declare no competing interests.

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