Generation, purification and identification of phosphospecific rabbit anti human CXCR4 polyclonal antibodY

CROWNBIO ADVANCING ONCOLOGY | CHANGING MEDICINE

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ABSTRACT

Phosphorylation on the Serine, Threonine and/or Tyrosine residue is an important event of post-translational modification of proteins essential to correct functioning within cells. In this study, a simple method was developed to produce and to affinity purify the specific rabbit anti-phosphopeptides polyclonal antibody. Phosphopeptides crosslinked with hemocyanin of keyhole limpets (KLH) and bovine serum albumin (BSA) were used as immunogens to raise the antiserum. Phosphospecific polyclonal IgGs were obtained by “two-step” peptide-affinity column purification. The specificity of isolated rabbit IgGs was tested by indirect ELISA and Western blot, respectively. This immune method also can be used to produce anti-methylspecific and anti-acetylspecific protein polyclonal or monoclonal antibody.

Keywords: Phosphorylation; synthetic peptide; antibody; ELISA; Western blot

INTRODUCTION

Reversible phosphorylation of proteins is an important regulatory mechanism that occurs in both prokaryotic and eukaryotic organisms. Enzymes called kinases (phosphorylation) and phosphatases (dephosphorylation) are involved in this process. Many enzymes and receptors are switched "on" or "off" by phosphorylation and dephosphorylation. This is the mechanism in many forms of signaltransduction. In the late 1990s, evidence began accumulating to suggest that some GPCRs are able to signal without G proteins. In mammalian cells, the much-studied β2-adrenoceptor has been demonstrated to activate the ERK2 pathway after arrestin-mediated uncoupling of G-protein-mediated signaling. It therefore seems likely that some mechanisms previously believed to be purely related to receptor desensitisation are actually examples of receptors switching their signaling pathway rather than simply being switched off. It’s well known, phosphospecifc antibody is a very useful, but not easy to make, tool in this field. In this poster, we describe a simple and robust method developed for the generation purification of rabbit polyclonal antibody specific for phosphorylated Serine339-CXCR4.

MATERIALS & METHODS

Chemicals and reagents

Hemocyanin of keyhole limpets (KLH), bovine serum albumin (BSA), Freund’s adjuvant (Complete and incomplete), 4-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from Sigma (St. Louis, MO, USA) and Ameresco (Solon, OH, USA)), respectively. HiTrap NHS-activated HP columns is the product of GE Healthcare Life sciences (Piscataway, NJ, USA).

Peptide synthesis:

Phosphorylated P339-CXCR4 peptide and the non-phosphorylated form of it, CXCR4 peptide (Human CXCR4 C-terminal intracellular region, sequence: CGKRGGHSSpVSTESES, position 332-346, phosphorylated 339 Serine is shown in bold and Fig.1), were synthesized by Scilight Inc. (Beijing, P. R. China) with a N- terminal Cysteine to facilitate conjugation to KLH, BSA and OVA. P339-CXCR4 or CXCR4 peptide was crosslinked to KLH, BSA and OVA according to our internal procedures with GMBS as crosslinker, respectively.

Animal immunization:

Approximately 1mg of P339-CXCR4-KLH conjugates was used to immunize 2 rabbits (Japanese white species, 2kg body weight, male) with complete Freund’s adjuvant through subcutaneous injections. After 2 weeks of the first immunization, the rabbit was boosted once again with P339-CXCR4-KLH (300ug/rabbit) conjugates using incomplete adjuvant. Then, they were boosted twice with P339-CXCR4-BSA conjugates (300ug/rabbit) using incomplete adjuvant every 2 weeks. Serum was collected after 8 weeks of immunization (after 3 boosts). Rabbit serum antibody titers were screened by indirect ELISA for reactivity against P339-CXCR4 peptide and CXCR4 peptide simultaneously (Fig.2). Serum with a strong reactivity against P339-CXCR4 peptide was subjected to affinity purification by sequential column chromatography.

Indirect ELISA

Antibody titers in immune and pre-immune sera were monitored by indirect ELISA. Briefly, 96-well microtiter plates were coated overnight at 4•f1 with P339-CXCR4 and CXCR4 peptide at a concentration of 10ug/ml(100ul/well), respectively. Plates were blocked with 10% goat serum/PBS (150ul/well) for 2 hours at room temperature. Then, these plates were washed 4 times with PBS, dried in air and kept at -30•f1 . Serial dilutions of antisera or purified antibody were added (100ul/well) into each well when those plates are used. After incubation for 2 hours at 37•f1 , plates were washed 4 times with PBS/0.1% Tween 20. The same volume of HRP-goat anti rabbit IgG conjugate at a dilution of 1:5,000 were added and incubated for another 1 hour. After 4 washes, TMB working solution was added, incubated for 5 minutes at room temperature and stopped by the addition of 50ul/well 2mol/L H_2SO_4. The absorbance was read at 450nm on a SpectraMAX Plus microplate spectrophotometer (Molecular Devices Cooperation, Sunnyvale, CA).

Serum purification:

Antisera of rabbit A with the best reaction against P339-CXCR4 were picked up according to indirect ELISA result, and subjected to “two-step” peptide-affinity purification by sequential pass-through P339-CXCR4 and CXCR4 peptide-affinity column chromatography, respectively. In brief, binding buffer (20mmol/L sodium phosphate, 0.15mol/L NaCl, pH 7.4) diluted sera were firstly applied to HiTrap NHS-activated HP column conjugated with P339-CXCR4 according to the instructions of the manufacturer, washed with binding buffer and eluted with elution buffer (100mmol/L glycine, 0.5mol/L NaCl, pH 3.0). The elution fraction from this column was immediately neutralized by neutralization buffer (1mol/L Tris-HCl, pH 9.6), diluted as above and next applied to another HiTrap NHS-activated HP column conjugated with CXCR4 peptide. The flow-through fraction of the second column was collected and concentrated to 1mg/mL. It’s composed of rabbit IgG specific to phosphorylated P339-CXCR4 peptide. The reactivity and specificity of purified antibody were evaluated by indirect ELISA as described before (Fig.3)

Western blot:

2ug/lane P339-CXCR4-OVA conjugates were loaded and separated by 12% SDS-PAGE. Then, the proteins were transferred onto the PVDF membrane according to in-house standard protocols. Purified P339-CXCR4-specific rabbit IgG was used as the primary antibody at the dilution of 1:1,000, 1:5,000 and 1:10,000 overnight at 4•f1 , respectively. This was followed by incubation with AP conjugated goat anti rabbit IgG secondary antibody. The membrane was developed with BCIP/NBT as substrate. 2ug/lane CXCR4-OVA conjugates were served as negative control (Fig.4).

RESULTS

Figure 1.

Scheme of human CXCR4 molecule and 2 postulated phosphorylation sites

Figure 2.

Determination of serum antibody titers against P339-CXCR4 and CXCR4 peptides before and after immunization by indirect ELISA

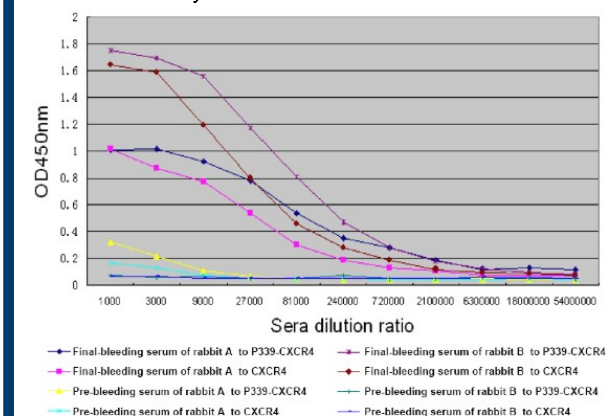


Figure 3.

Determination of “two-step” peptide-affinity purified antibody titers of rabbit A and its specificity by indirect

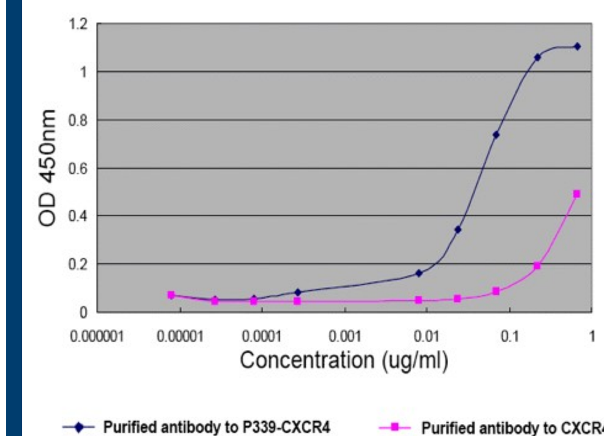
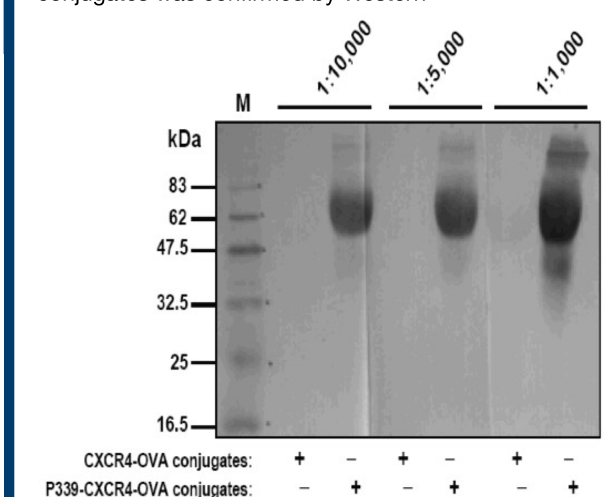


Figure 4.

Specificity of purified antibody against P339-CXCR4-OVA conjugates was confirmed by Western



SUMMARY

- An effective platform for generation of phosphospecific antibodies has been established in-house at Crown Biosience.
- Phosphorylated synthetic peptide was designed and used to generate polyclonal antisera in rabbit for generation of anti-pSer339-CXCR4 antibody.
- By KLH conjugates priming and BSA conjugates boosting, antisera with high titer and specificity against pSer339-CXCR4 was produced successfully.
- Anti-pSer339-CXCR4 antibody was purified by “two-step” peptide-affinity chromatography.
- The purified anti-pSer339-CXCR4 antibody can be used in assays, such as ELISA and Western blot.