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Genomics 88 (2006) 841–845
GENOMICS
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Method

A genetic library screen for signaling proteins that interact with phosphorylated T cell costimulatory receptors

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Received 14 March 2006; accepted 23 August 2006
Available online 2 October 2006

Abstract

In the past decade, the fundamental importance and therapeutic potential of costimulatory signals for lymphocyte activation have spurred a large amount of work in immunology, infection, cancer, autoimmune diseases, etc. However, the mechanisms behind T cell costimulation remain unclear, partly due to the lack of suitable techniques. There is an urgent need for functional genomic research to develop comprehensive approaches to direct identification of protein–protein interactions that are dependent on the posttranslational modification of one component of the complex, particularly in the field of T cell immunology. Using inducible costimulator (ICOS) as a model, we failed to find any proteins that associated with the cytoplasmic tail of ICOS by the yeast two-hybrid approach. Therefore, we have developed a new yeast three-hybrid system that facilitates the rapid screening of cDNA libraries to find signaling molecules that interact with phosphorylated T cell costimulatory receptors. We demonstrate the utility of this technique to detect the interaction between ICOS and the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). The p85 unit of PI3K is the only signaling molecule identified so far that interacts with ICOS. This system may be of great help in dissecting the mechanisms of T cell costimulation and could be applied to other receptors.

Keywords: Functional genomics; Genetic screening; T lymphocytes; Costimulation; Phosphorylation; Inducible costimulator; Phosphatidylinositol 3-kinase

It is now clear that T cell activation is initiated by the engagement of the antigen receptor and that the response that follows is regulated and shaped by other molecules collectively known as costimulators. The prototypical costimulatory receptor is CD28, which upon interaction with B7-1 or B7-2 provides signals that are required for optimal activation. The CD28 homolog CTLA-4, which also binds B7-1 and B7-2, attenuates T cell responses [1–3]. Recently additional molecules in the extended CD28/B7 family that orchestrate the emerging T cell response have been found [3–5]. These include inducible costimulator (ICOS) and programmed death 1 (PD-1) [6], which bind respectively B7h [7–11] and PD-L1/PD-L2 [12–15]. B7H3 [16] and B7x [17] (also known as B7H4 [18] and B7S1 [19]) are the newest members of the B7 ligand family and their receptors are currently unknown. The precise roles of these new pathways in regulating T cell responses are not well established, and their signaling pathways are poorly understood. For example, while it is clear that ICOS can regulate Th2 cytokine production and plays a critical role in T/B cooperation, the mechanism of its action is as yet unknown.

A central question in the study of T cell costimulatory receptor-mediated signaling pathways is to determine what other proteins interact with them. Many approaches have been taken to identify protein–protein interactions. One approach is the yeast two-hybrid system [20,21], which has been a very powerful tool in identifying molecules that interact with the cytoplasmic domains of cell surface receptors. However, we found that the conventional two-hybrid system did not reveal any proteins that associated with the cytoplasmic tail of ICOS. The two-hybrid system does not allow detection of components that interact with members of signaling pathways that require modification to

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doi:10.1016/j.ygeno.2006.08.012

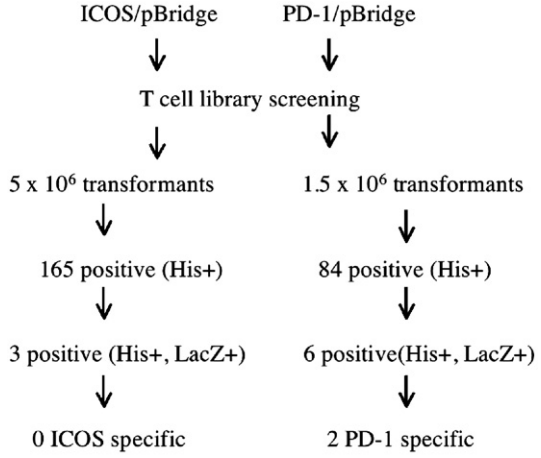

Fig. 1. Summary of yeast two-hybrid screen with ICOS and PD-1. The cytoplasmic domain of mouse ICOS was used as bait to screen a cDNA library from T cells, using the yeast two-hybrid system. It did not yield any molecules specifically binding to the cytoplasmic tail of ICOS. Analogous to the ICOS screen, the entire cytoplasmic region of PD-1 was used to screen the same mouse T cell library, and two PD-1-specific positive clones were obtained.

associate. A modified version of the two-hybrid system was used to show that phosphorylated human killer cell receptors and CD28 can bind signaling molecules [21–23]. Using ICOS as a model, we describe a yeast three-hybrid system to identify a molecule that interacts with the phosphorylated cytoplasmic tail of ICOS. This system may be a valuable tool for screening signaling molecules that interact with phosphorylated T cell costimulatory receptors, as well as with other receptors.

Results

ICOS is a type I transmembrane protein with a 35- to 44-amino-acid intracellular cytoplasmic domain that lacks intrinsic catalytic functions, suggesting that intracellular signals are mediated by other molecules that associate with ICOS. To analyze the signal transduction of ICOS, we searched for potential candidates that bind the cytoplasmic region of ICOS by first utilizing a standard yeast-two hybrid system. The entire cytoplasmic region of mouse ICOS was used as bait. A mouse T cell library constructed in the GAL4 activation domain plasmid pACT was screened using this bait. Screening of approximately 5×10^6 cotransformed clones, surprisingly, did not yield any ICOS-specific positive clones (Fig. 1). This was in contrast to similar screens using unmodified CD28 and CTLA-4 cytoplasmic tails as baits, which yielded serine/threonine phosphatase PP2A [24] and clathrin-coated pit adaptor protein AP50 [25–28]. Analogous to the ICOS screen, we used the entire cytoplasmic region of PD-1 as a bait to screen the same mouse T cell library and obtained two PD-1-specific positive clones by screening only about 1.5×10^6 cotransformed clones (Fig. 1). These results suggest that ICOS has a unique signaling pathway.

The ICOS gene has been cloned from human, mouse, rat, dog, bovine, and chicken. Protein sequence alignment analysis reveals that the cytoplasmic tail of ICOS is evolutionarily conserved (Fig. 2A). In particular, the C-terminal tyrosine is located within a highly conserved Tyr-Met-Phe-Met motif, suggesting that some signaling molecules may bind only tyrosine-phosphorylated ICOS. To determine whether the tyrosine residue of the ICOS cytoplasmic tail is phosphorylated during T cell activation, cells from the lymph nodes and spleens of DO11.10 TCR transgenic mice were activated with OVA323-339 peptide for 60 h. Following a 10-min treatment with the phosphatase inhibitor pervanadate, which increase the level of tyrosine phosphorylation, ICOS was immunoprecipitated to examine tyrosine phosphorylation using a mAb against phosphotyrosine. As expected, activated, but not naïve, T cells expressed ICOS. More importantly, at least some parts of the ICOS protein from activated T cells were tyrosine phosphorylated (Fig. 2B), suggesting that ICOS can be induced to undergo tyrosine phosphorylation during T cell activation.

To identify signaling molecules that bind phosphorylated ICOS, a new yeast three-hybrid system was developed (Fig. 3A). Because yeast cells do not contain tyrosine kinases, we cloned the mouse tyrosine kinase p56Lck into the *Not*I site of the multiple cloning site II of ICOS/pBridge, thus creating

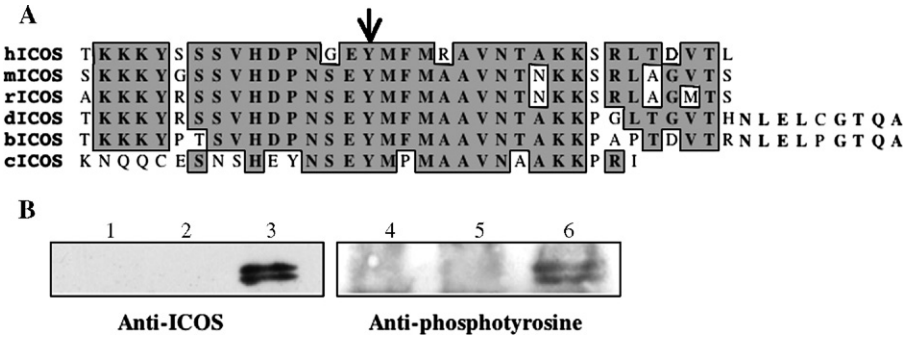

Fig. 2. The tyrosine residues of the ICOS cytoplasmic tail are conserved and could be phosphorylated during T cell activation. (A) Comparison of the cytoplasmic tail of ICOS found in human (GenBank Accession No. CAC06612), mouse (GenBank Accession No. CAB71153), rat (GenBank Accession No. BAA82128), bovine (GenBank Accession No. AAX46485), dog (GenBank Accession No. AAQ20845), and chicken (GenBank Accession No. XP–421959). Identical amino acids are highlighted in boldface. The potential tyrosine phosphorylation site is indicated with an arrow. (B) Cells from lymph nodes and spleens of DO11.10 TCR transgenic mice were activated with OVA323-339 peptide and treated with pervanadate. The lysates of activated (lanes 3 and 6) and nonactivated cells (lanes 2 and 5) were immunoprecipitated with mAb to ICOS and then detected by Western blot with mAb against ICOS (lanes 1–3) or mAb against phosphotyrosine (lanes 4, 5–6).

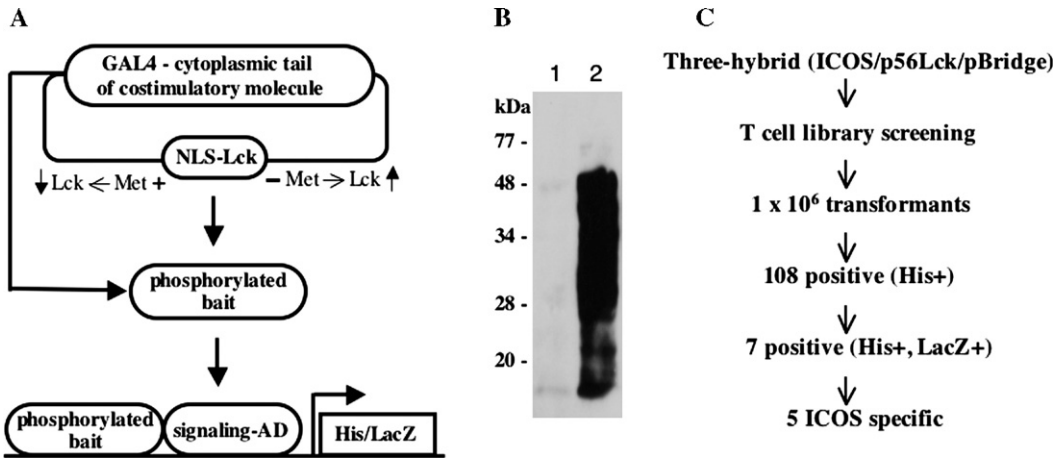

Fig. 3. A genetic system for screening signaling proteins interacting with phosphorylated ICOS. (A) Scheme for the new screening system. A single vector expresses two proteins, a bait and Lck, which is controlled by a conditional methionine promoter. Both are targeted into the nucleus, where Lck can phosphorylate the bait protein. Phosphorylated bait binds a protein from the T cell library and initiates the expression of the reporter genes. (B) Yeast cells transformed with ICOS/pBridge (lane 1) or ICOS/p56Lck/pBridge (lane 2) were grown on SD/−Trp/−Met medium. Phosphorylated proteins were detected by Western blot of total yeast protein extracts and detection using a mAb against phosphotyrosine. (C) Yeast cells containing ICOS/p56Lck/pBridge were transformed with a mouse T cell library and screening of approximately 1 million cotransformed clones yielded 5 positive clones.

a new bait plasmid, ICOS/p56Lck/pBridge. ICOS/p56Lck/pBridge expresses two fusion proteins, one containing the GAL4 DNA binding domain and the ICOS cytoplasmic tail, the other containing a nuclear localization sequence and p56Lck. The nuclear localization sequence ensures that p56Lck is in the nucleus, where it can phosphorylate target proteins. The expression of p56Lck is controlled by a conditional methionine promoter, so that it is expressed in the absence of methionine. This allows p56Lck expression to be switched on or off by a simple replica plating step. The tyrosine kinase activity of p56Lck in yeast was verified by Western blot (Fig. 3B). Expression of plasmid ICOS/p56Lck/pBridge in yeast cells enabled phosphorylation of yeast proteins, while no phosphotyrosine was detected in yeast containing ICOS/pBridge. The same mouse T cell library was screened using this new system and screening of approximately 1×10^6 cotransformed clones yielded 108 clones selected by growth on SD/−Trp/−Leu/−His/−Met plates; 7 were positive for β-galactosidase activity, and 5 were ICOS specific. All 5 positive clones encoded phosphatidylinositol 3-kinase (PI3K) p85 (Fig. 3C).

PI3K p85 protein contains one SH3 domain and two SH2 domains. To see if the whole PI3K p85 molecule or just one domain is necessary for binding to ICOS, we sequenced all inserts from the five positive clones. Protein sequence alignment revealed that all five clones corresponded to the second SH2 domain of the p85 regulatory subunit of PI3K (Fig. 4A), suggesting that PI3K p85 binds phosphorylated ICOS via the SH2 domain.

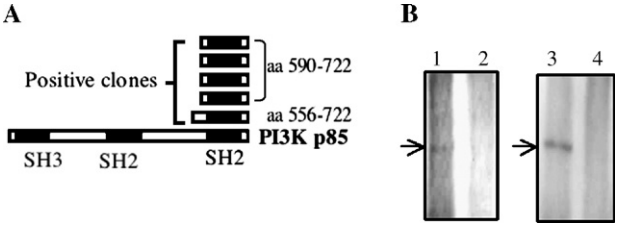

Fig. 4. PI3K p85 can be detected as a component of the ICOS signaling complex. (A) Schematic diagram of five positive cDNA clones encoding PI3K p85. (B) Cells from the lymph nodes and spleens of DO11.10 TCR transgenic mice were activated with OVA323-339 peptide and treated with (lanes 3 and 4) or without (lanes 1 and 2) pervanadate. The cellular lysates were immunoprecipitated with a mAb to ICOS (lanes 1 and 3) or control Ab (lanes 2 and 4) and then Western blotted with a mAb against phosphotyrosine.

To determine whether PI3K p85 interacts with ICOS in vivo, ICOS was immunoprecipitated from OVA323-339 peptide-activated DO11.10 TCR transgenic T cell lysates and the presence of ICOS-associated endogenous PI3K p85 was examined by Western blot. As shown in Fig. 4B, PI3K p85 was found to coimmunoprecipitate with ICOS. Thus, in vivo, PI3K p85 can be detected as a component of the ICOS signaling complex.

Discussion

Protein–protein interactions are intrinsic to virtually every cellular process, including replication, transcription, secretion, signal transduction, and metabolism, and alterations of protein–protein interactions are known to contribute to many diseases. Traditionally, the tools to detect interacting proteins include techniques such as affinity chromatography, coimmunoprecipitation, crosslinking, and glycerol gradient sedimentation [29]. These biochemical approaches are time-consuming and can be difficult undertakings. The yeast two-hybrid system is a genetic system that has provided enormous insights into the understanding of protein–protein interactions in various signal pathways and has very recently been applied to the entire protein complement of yeast [30], *Caenorhabditis elegans* [31], and *Drosophila melanogaster* [32].

ICOS is a T cell costimulatory receptor with an intracellular cytoplasmic domain that lacks intrinsic catalytic functions, suggesting that intracellular signals are mediated by other molecules that associate with ICOS. Therefore, a standard yeast two-hybrid approach was first used to search for potential candidates that

bind the cytoplasmic region of ICOS. Despite an extensive library screen, however, we failed to detect any molecules that could bind the unmodified form of ICOS. There is an urgent need to develop comprehensive approaches for direct identification of protein–protein interactions that are dependent on the posttranslational modification of one component of the complex.

ICOS contains an evolutionarily conserved tyrosine residue in the cytoplasmic region and can be induced to undergo tyrosine phosphorylation during T cell activation. The tyrosine kinases responsible for ICOS tyrosine phosphorylation in activated T cells have not yet been identified. We used p56Lck in our three-hybrid screen system since it has been reported that Lck can induce tyrosine phosphorylation of CD28 and CTLA-4 when coexpressed in cell lines [33]. Using the new yeast three-hybrid system, we rapidly identified that the p85 regulatory subunit of PI3K bound tyrosine-phosphorylated ICOS. This result is in agreement with other reports using biochemical approaches to show that the phosphorylated cytoplasmic domain of an ICOS–GST recombinant protein bound PI3K p85 [34] and that ICOS and PI3K p85 coimmunoprecipitated after anti-CD3/ICOS crosslinking [35]. We further demonstrated that the binding is mediated through the second SH2 domain of the p85 unit. PI3K is the only signaling molecule known to interact with ICOS so far; the role of this pathway in ICOS-meditated Th2 cytokine production and T/B cooperation remains to be explored.

Costimulatory molecules are essential for the communication of T cells with virtually all other host cells. Because of the fundamental biological importance and therapeutic potential of T cell costimulatory receptors, there has been great interest in identifying components of their signaling pathways. However, the mechanisms behind T cell costimulation mediated by CTLA-4, ICOS, and PD-1 remain unclear. Despite considerable research on CD28, the molecular mechanism by which CD28 crosslinking affects T cell activation is incompletely understood. In recent years, one of the emerging paradigms for T cell signal transduction is that all known costimulatory receptors undergo tyrosine phosphorylation upon interaction with the B7 ligand family and that signaling molecules interact not in isolation but through the recruitment of a complex of other proteins. To facilitate the study of posttranslational modifications that mediate interactions in T cell costimulatory signal transduction, we have demonstrated here the use of a three-hybrid system for rapid screening of cDNA libraries, to find signaling molecules that interact with phosphorylated ICOS. This strategy could be applied to the other recently discovered T cell costimulatory molecules, as well as to other receptors.

Materials and methods

Antibodies, cDNA library, peptide, and mice

Antibodies used in the study include monoclonal anti-ICOS (eBioscience), anti-phosphotyrosine (Upstate Biotechnology), and polyclonal anti-p85 of PI3K (Upstate Biotechnology). A mouse T cell cDNA library constructed in the GAL4 activation domain plasmid pACT was previously described [25]. OVA323-339 peptide was synthesized at the Cancer Research Laboratory Microchemical Facility of the University of California at Berkeley, by standard fluorenylmethoxycarbonyl synthesis. Peptides were purified by reverse-phase HPLC (>99%) and purity was verified by mass spectroscopy. DO11.10 TCR transgenic mice were bred in-house.

Plasmid construction

The ICOS/pBridge and PD-1/pBridge plasmids were generated by polymerase chain reaction (PCR) amplification of the cDNAs encoding the murine ICOS cytoplasmic domain amino acids 166–200 and the PD-1 cytoplasmic domain amino acids 196–288, respectively, and subcloning into the *Sal*I site of the yeast expression vector pBridge (Clontech). The ICOS/pBridge and PD-1/pBridge express a fusion protein containing the GAL4 DNA binding domain and ICOS cytoplasmic tail or PD-1 cytoplasmic tail, respectively. The open reading frame of murine p56Lck was PCR amplified from a plasmid (a gift from Dr. Sue Sohn, University of California at Berkeley) and inserted into the *Not*I site of pBridge and ICOS/pBridge to generate p56Lck/pBridge and p56Lck/ICOS/pBridge, respectively. Both p56Lck/pBridge and p56Lck/ICOS/pBridge express a hybrid p56Lck protein that is targeted to the yeast nucleus by N-terminal nuclear localization sequences.

Yeast two-hybrid screen

The ICOS/pBridge or PD-1/pBridge construct was transfected into yeast strain Y190 along with a mouse peripheral T cell library constructed in the GAL4 activation domain plasmid pACT, and the two-hybrid screen was performed as previously described [25]. A result was scored positive if both growth in the absence of histidine occurred and β-galactosidase activity was observed.

Yeast three-hybrid system

Yeast strain Y190 transformed with ICOS/p56Lck/pBridge was grown on SD/−Trp plates and then in SD/−Trp/−Met medium overnight. The ICOS/p56Lck/pBridge/Y190 cells were transformed with a mouse T cell library constructed in the GAL4 activation domain plasmid pACT [25] according to the high-efficiency transformation protocol [36] and grown on SD/−Trp/−Leu/−His/−Met plates containing 5 mM 3-AT. A result was scored positive if both growth on an SD/−Trp/−Leu/−His/−Met plate occurred and β-galactosidase activity was observed. The plasmids were extracted from a single positive clone and transformed into *Escherichia coli* XL1-Blue before sequencing. Yeast proteins were obtained with the Y-PER yeast protein extraction reagent (Pierce) using the manufacturer's protocol.

T cell stimulation, immunoprecipitation, and Western blot

Cells from the lymph nodes and spleens of DO11.10 TCR transgenic mice were activated with OVA323-339 peptide for 48–60 h and then lysed for 4 h in the lysis buffer (1% NP-40, 300 mM NaCl, 5 mM EDTA, 10 mM iodoacetamide, protease inhibitor cocktail, 50 mM Tris–HCl, pH 7.5). Cell lysates were clarified by centrifugation and immunoprecipitated using protein G beads coated with anti-ICOS antibody. After 6–8 h of incubation at 4°C, the immunoprecipitates were washed using 1% NP-40 lysis buffer, and bound proteins were eluted by boiling in SDS sample buffer, separated by SDS–polyacrylamide electrophoresis, and transferred onto nitrocellulose membranes. Membranes were blocked with 4% BSA in PBS and then incubated with anti-ICOS, anti-phosphotyrosine, or anti-p85 of PI3K and subsequently with horseradish peroxidase-conjugated secondary antibody before visualization using the ECL reagents (Amersham).

Pervanadate treatment

The pervanadate solution was made fresh as previously described [26]. H_2O (0.45 volume), 1 M H_2O_2 (0.5 volume), and Na_3VO_4 (0.05 volume) were mixed and left for 15 min at room temperature. Cells were mixed with 1/50 volume of the pervanadate solution and incubated for 10 min at 37°C.

Sequence analysis

Public databases were explored using BLAST searches with protein and nucleotide sequences. All the sequence alignment and homology comparison were done with MacVector 7.0.

Acknowledgments

We thank Tsvetelina Hoang, Pete Savage, and Alejandro Sepulveda for critically reading the manuscript. This work was supported by the National Institutes of Health (2R01CA040041-19) and the Howard Hughes Medical Institute. X.Z. is a Fellow of the Cancer Research Institute, P.L. is the recipient of a Wellcome International Research Fellowship, and J.P.A. is an Investigator of the Howard Hughes Medical Institute.

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