Combinatorial proteomic analysis of intercellular signaling applied to the CD28 T-cell costimulatory receptor

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PNAS Early Edition | 1 of 10

Study Systematic characterization of intercellular signaling approximating the physiological conditions of stimulation that involve direct cell–cell contact is challenging. We describe a proteomic strategy to analyze physiological signaling mediated by the T-cell costimulatory receptor CD28. We identified signaling pathways activated by CD28 during direct cell–cell contact by global analysis of protein phosphorylation. To define immediate CD28 targets, we used phosphorylated forms of the CD28 cytoplasmic region to obtain the CD28 interactome. The interaction profiles of selected CD28-interacting proteins were further characterized in vivo for amplifying the CD28 interactome. The combination of the global phosphorylation and interactome analyses revealed broad regulation of CD28 and its interactome by phosphorylation. Among the cellular phosphoproteins influenced by CD28 signaling, CapZ-interacting protein (CapZIP), a regulator of the actin cytoskeleton, was implicated by functional studies. The combinatorial approach applied herein is widely applicable for characterizing signaling networks associated with membrane receptors with short cytoplasmic tails.

Significance

Intracellular signaling during complex cell–cell interactions, such as between immune cells, provides essential cues leading to cell responses. Global characterization of these signaling events is critical for systematically exploring and understanding how they eventually control cell fate. However, proteome-wide characterization of intercellular signaling under physiologically relevant conditions involving multiple interacting receptors during cell–cell interactions remains challenging. We developed an integrated proteomic strategy for quantitatively profiling intercellular-signaling events mediated by protein phosphorylation and protein–protein interaction. We applied this approach to determine the influence of a single receptor-ligand pair during T-cell stimulation by blocking the interaction of the CD28 costimulatory receptor with its ligand. This approach is generally applicable to other transmembrane receptors involved in signaling during complex cell interactions.

Author contributions: R.T., H.W., A.W., and T.P. designed research; R.T., H.W., G.D.G., E.P., A.P., Y.S., M.M., and R.D.B. performed research; N.Y., T.H., and A.-C.G. contributed new analytical tools; R.T., H.W., A.W., and T.P. analyzed data; and R.T., H.W., A.W., and T.P. wrote the paper. The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/suppl/doi:10.1073/pnas.1503286112/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1503286112
its corresponding receptor. However, exploring the resulting signal transduction pathways is challenging because the identity of the cell from which a particular protein is derived is lost after cell lysis. The T.P. laboratory developed a proteomic approach [termed quantitative analysis of bidirectional signaling (qBidS)] for profiling cell-specific tyrosine phosphorylation events resulting from contact between cells expressing either an Eph receptor tyrosine kinase or its ephrin ligand, respectively (10). In these experiments, the EphB2 receptor or its ephrin-B1 ligand, a transmembrane protein that itself has intrinsic signaling activity, was stably expressed in human embryonic kidney (HEK) 293 cells so that the two cell types segregated in culture due to repulsive EphB2/ephrin-B1 signaling. The two distinct cell populations were then differentially labeled with stable isotopes [stable isotope labeling by amino acid in cell culture (SILAC)] and cocultured. This strategy not only can identify tyrosine phosphorylation events modulated after the interaction between EphB2 and ephrin-B1 but also can indicate from which cell type a specific phosphopeptide originates, and thus can infer pathways activated in either cell type involved in EphB2/ephrin-B1 bidirectional signaling. However, these experiments have limitations. One is that they depend on overexpression of receptors and ligands in a model-cell system. Another is that, once two cells adhere to one another, they may interact through multiple different ligands and receptors, making it difficult to confidently define the exclusive signaling output of a single class of receptor (9, 11).

Here, we overcome these issues by using a combination of proteomic and cell-biological approaches to systematically identify specific signaling events downstream of the endogenous CD28 costimulatory receptor on T cells. CD28 is a type I transmembrane protein that binds through its extracellular region to B7 proteins (CD80 and CD86), which are transmembrane proteins expressed on the surface of antigen-presenting cells (APCs) and are up-regulated by inflammatory signals. Upon the interaction of T cells and APCs, the T-cell receptor (TCR) engages a peptide antigen-bound major histocompatibility complex molecule on the APCs, resulting in a primary signal. Simultaneously, costimulatory signaling is established predominantly, but not exclusively, through the association of CD28 on T cells with B7 ligands on the surface of the APCs. Both TCR and CD28 signals are required for a full T-cell response, resulting in, for example, production of the IL-2 cytokine. Although it lacks an intrinsic catalytic domain, the short 41 amino acid cytoplasmic tail of CD28 has highly conserved tyrosine-based motifs that are phosphorylated by cytoplasmic tyrosine kinases upon T-cell stimulation and that bind targets with SH2 domains in a pTyr-dependent manner, as well as proline-rich sequences that potentially bind SH3 domains (12). It therefore can help orchestrate the response to the signal from an APC, such as a B cell.

Because the complex cellular interactions of T cells and APCs stimulate multiple receptors (e.g., the integrin receptor) in addition to CD28, we have developed techniques to distinguish specific events uniquely dependent on CD28 signaling to undertake a comprehensive screen of tyrosine and serine/threonine phosphorylation sites selectively regulated by CD28. We have used a complementary approach using the full cytoplasmic tail of CD28 to identify the cytoplasmic targets with which it interacts. This approach involved immunoprecipitation coupled to mass spectrometry (IP-MS) to identify a network of interacting proteins immediately proximal to CD28. We then integrated these data to dissect CD28-based signaling networks activated in T cells after coculture with APCs. We targeted one newly identified CD28-regulated phosphoprotein, CapZIP, interacting protein (CapZIP), to demonstrate its importance in the CD28-dependent functional response. Our results demonstrate the value of a combinatorial and broad-based strategy to identify signaling pathways regulated by a specific endogenous receptor activated by direct cell–cell contact.

**Results**

**Global Phosphoproteomic Analysis of Endogenous CD28 Signaling.** We sought to develop a general proteomics approach to quantitatively measure global signaling events mediated by an endogenous receptor activated by direct cell–cell contact. As a test biological system, we systematically explored how CD28-mediated costimulatory signaling coordinates with TCR signaling for full activation of T cells by antigen-presenting B cells, using a combination of MS-based interaction-proteomics and phosphoproteomics. To specifically explore physiological CD28-dependent protein phosphorylation events, we used a coculture system involving Jurkat leukemic T cells and Raji lymphoblastoid B cells (Fig. L4) (13, 14). Distinct combinations of SILAC labeling were used to differentially label T cells with either “medium” (M) or “heavy” (H) SILAC isotopes, and with B cells in an unlabeled “light” (L) state. After coculture of T cells and B cells for 5 min, this differential labeling allowed us to distinguish peptides and phosphorylation sites derived from B cells (L) or T cells (M or H), as well as those derived from T cells that were fully stimulated by coculture with B cells (M) or T cells in which the TCR (and other interacting receptor/ligand pairs that are not defined here) is engaged but CD28 is not (H) (Fig. L4). Specifically, Jurkat leukemic T cells were stimulated by coculturing with Raji lymphoblastoid B cells presenting the superantigen staphylococcal enterotoxin E (SEE), which binds to the TCR and to MHC class II molecules. Under these conditions, the majority of the T-cell population (up to 88.2%) was efficiently activated, as indicated by potent ERK phosphorylation, reflecting TCR signaling (Fig. 1B and SI Appendix, Fig. 1A); consistent with this result, we observed T and B cells forming a typical immunological synapse, with strong induction of tyrosine phosphorylation at the site of synaptic contact in T cells (SI Appendix, Fig. S1B).

To selectively block CD28 signaling, we made use of cytotoxic T-lymphocyte antigen-4 (CTLA4), a coreceptor expressed on activated T cells, which binds the same family of B7 ligands on B cells as does CD28, but with more than 20-fold higher affinity (15). CTLA4-Ig, a CTLA4-Ig recombinant fusion protein that is used in the treatment of rheumatoid arthritis, was added to specifically block the interaction of CD28 on Jurkat leukemic T cells with B7 ligands on the surface of Raji lymphoblastoid B cells. Incubation with CTLA4-Ig completely inhibited CD28 signaling in the coculture assay, even after 22 h, as suggested by an efficient block in the production of the cytokine IL-2, a signature target of CD28-mediated costimulation (Fig. 1C). To focus on proximal CD28 signaling events and minimize potential secondary effects, a relative early time point after T-cell activation was chosen. We were guided by the kinetics of ERK phosphorylation after Raji-SEE stimulation was systematically investigated. Maximal ERK phosphorylation was observed at 5 min after T-cell stimulation (SI Appendix, Fig. 1A). Therefore, 5 min was chosen to activate the Jurkat T cells in the phosphoproteomic analysis. Signals that are present in M-labeled T cells cocultured with SEE-presenting B cells, but are specifically blocked by treatment with CTLA4-Ig in H-labeled T cells, are therefore dependent on B7-stimulated CD28.

We sought to develop a sensitive MS assay to identify tyrosine and serine/threonine phosphorylation events regulated by endogenous CD28 on a global scale. To this end, we optimized a two-step phosphoproteomic approach involving tryptic digestion followed by either anti-pTyr IP or strong cation exchange (SCX) fractionation and subsequent titanium dioxide (TiO₂) enrichment (Fig. L1). By combining these enrichment steps with the use of a high-resolution Orbitrap Elite instrument with fast sequencing speed (16), we identified 936 phosphorylation sites by...
pTyr IP, of which 778 were pTyr sites, and 15,861 phosphorylation sites using the SCX/TiO₂ workflow, of which 13,516 were phospho-serines (pSers), and 2,184 were phospho-threonines (pThrS) (Dataset S1). Two biological replicates gave an overlap of about 75% for sites identified by pTyr IP and 90% for the SCX/TiO₂ workflow, demonstrating reasonable reproducibility of both procedures (SI Appendix, Fig. S2A). The results obtained demonstrated high identification confidence and MS1 mass accuracy (SI Appendix, Fig. S2B and C).

To delineate T cell-specific phosphorylation sites dependent on CD28 costimulation, we applied stringent quantification filters and show the results derived from every single MS spectrum. This effort is mainly due to the fact that Jurkat leukemic T cells and Raji lymphoblastoid B cells are completely different cell types and that their proteome profile and expression level of the same protein could be significantly different. Consequently, accurate measurement of the SILAC ratios between coeluted SILAC-labeled peptides from distinct cell types could be challenging. We strictly selected phosphopeptides that were reliably identified (Andromeda score > 42) and quantified in at least three MS/MS spectra from two biological replicates, and that had negligible peak overlap, defined by the SILAC light peak overlapping into the medium isotope peak by less than 20%. Furthermore, we used the H/L and M/L ratios (T cells/B cells) to define and remove B cell-specific phosphopeptides. The cutoffs were selected based on removal of phosphopeptides from PYK2, which was predominantly phosphorylated in B cells as judged by manual inspection, and from six well-characterized B cell-specific proteins, including CD19, LYN, BTK, CD22, BLNK, and PIK3AP1, which had significantly low H/L and M/L ratios. In total, 8,208 T cell-specific phosphorylation sites were quantified, with a probability of being assigned to the correct residue of ≥0.33 and where 6,267 sites have site probability of ≥0.75 (Dataset S2). The majority of phosphorylation sites exhibited a tight distribution around zero, indicating no significant change when CD28 signaling was blocked, and demonstrating the high accuracy of the SILAC quantification (Fig. 1D). A total of 598 sites showed significant changes upon CTLA4-Ig treatment, with a P value (Wilcoxon test) less than 0.01 and log ratio H/M cutoff at ≥0.95 (98.5% and 1.5% quartiles, respectively).

Previous studies indicate that multiple signaling pathways are downstream of CD28. Our pathway analysis revealed that many proteins involved in known CD28-related signaling pathways were identified by at least one phosphorylation site, and more than 20 of those phosphorylation sites were significantly reduced upon CD28 inhibition (Fig. 2A) (12). For example, we observed a remarkable reduction of phosphorylation of Y191 in the CD28 cytoplasmic tail, which is known to recruit phosphatidylinositol 3-kinase (PI3K) through its SH2 domain, leading to PKB activation. Consistent with this observation, phosphorylation of several proteins involved in the PI3K pathway, including PI3K, cAMP-binding protein 1 (CREB1), and mammalian target of rapamycin (mTOR), changed after inhibiting CD28 signaling. We also observed increased phosphorylation of filamin A (FNLA), which interacts with CD28 and is involved in regulating cytoskeleton remodeling (17). Protein kinase C theta (PKCδ) was recently reported to associate with CD28 and control NFκB activity (18). Although we didn’t confidently detect any phosphorylation changes of PKCδ, the phosphorylation of its downstream signaling targets, CARMA1, as well as the transcription factor NFκB, was reduced after CD28 blockade. We observed dynamic CD28-dependent regulation of phosphorylation-signaling events in fully activated T cells. For example, phosphorylation of CD28-proximal signaling complexes [e.g., VAV1, phospholipase Cγ1 (PLCγ1), and SHC1] tended to be consistently decreased whereas nuclear proteins such as NFAT transcription factors mostly exhibited increased serine/threonine phosphorylation. Aside from known CD28-related signaling pathways, the global phosphorylation analysis quantitatively identified phosphoproteins belonging to a broad spectrum of canonical signaling pathways with up to 66.7% coverage of all pathway components (SI Appendix, Fig. S2D and Dataset S3). Interestingly, most of the enriched signaling pathways within the 598 CD28-regulated phosphorylation sites were down-regulated (Fig. 2B and Dataset S3). TCR signaling and a number of other immune signaling pathways were down-regulated by CD28 blockade. It is noteworthy, however, that events associated with the TCR signaling pathway did not dominate the down-regulated events, suggesting that CD28 may influence events independently of the TCR. These data provide a broad map of signaling pathways controlled by CD28.
events specifically regulated by endogenous CD28 activated by contact between Jurkat T and Raji B cells.

Identification of a Protein-Interaction Network Involving CD28. The CD28-dependent pathways identified in the preceding phosphoproteomic analysis are most likely controlled by proteins that are recruited to the evolutionarily conserved pTyr-containing or proline-rich motifs on CD28 (SI Appendix, Fig. S3A). Indeed, in the preceding analysis, we confidently identified three pTyr sites on the CD28 cytoplasmic tail that were significantly down-regulated upon CTLA4-Ig-mediated inhibition (Fig. 1E). Interestingly, two of these pTyr sites, Y191 (located in the motif YMNM) and Y209 (located in the motif PYAPP) were relatively abundant and strongly inhibited by CTLA4-Ig treatment whereas phosphorylation on Y218 was less abundant and more weakly inhibited. By IP and Western blot, we observed reduced recruitment of the p85α subunit of phosphatidylinositol 3-kinase (PI3K) to CD28 after CTLA4-Ig treatment, which corroborates the observation that phosphorylation of Y191 is down-regulated upon CD28 inhibition because this residue is in a site that favors binding to the PI3K p85α SH2 domains (SI Appendix, Fig. S3B). We also identified two phosphoserine and phosphothreonine sites on the CD28 cytoplasmic tail but with low site localization probability scores that don’t meet the data cutoff. Other function-related posttranslational modifications including ubiquitination would be possibly identified with a more dedicated workflow.

To explore the targets immediately proximal to CD28, and thus to investigate how CD28 is connected to the spectrum of proteins whose phosphorylation is regulated by its signaling, we sought to systematically profile proteins that bind the major pTyr and Pro-rich sites on CD28 identified in the phosphoproteomics screen. The comprehensive identification of proteins associated with transmembrane receptors is technically problematic due to the harsh protein extraction conditions needed to solubilize the receptors for affinity purification, to which the relevant complexes are relatively labile (19, 20). Compared with alternative strategies of using short peptide motifs corresponding to receptor docking sites, we expected that the “full-length” cytoplasmic domain with physiologically relevant modifications could recruit more critical downstream proteins associated with an activated receptor (7, 8).

To attempt to capture a relevant view of the receptor’s immediate targets and the role of the regulated pTyr sites in mediating interactions, we devised a proteomic approach to define the CD28 interactome in which a chemically synthesized CD28 cytoplasmic polypeptide was used to affinity purify CD28-binding proteins from T-cell lysates (Fig. 3A). The CD28 cytoplasmic tail contains only 41 amino acids, of which we synthesized a 33 amino acid peptide that contains all of the highly conserved motifs, including two of the identified sites of tyrosine phosphorylation, as well as proline-rich sequences (SI Appendix, Fig. S3A) (CD28Y85). We anticipated that, using longer synthetic peptides corresponding to almost the entire CD28 cytoplasmic domain, together with appropriate modifications, would provide a physiologically relevant and comprehensive view of the receptor’s immediate targets. To ensure that we identified phosphorylation-dependent interactions, we synthesized several forms of CD28 that were variously unmodified (YY), phosphorylated on either Y191 or Y209 (pYY or YpY), doubly phosphorylated on both Y191/Y209 (pYpY) or had alanine substitutions for Pro208 and Pro211 to destroy a potential PXXP SH3-binding motif (pYAA).

To examine phosphorylation-dependent protein interactions, the various forms of biotin-tagged CD28 that were immobilized on streptavidin agarose beads and incubated with SILAC-labeled T-cell lysates, and associated proteins were identified by MS. For quantitative purposes, we applied a three-channel SILAC approach that allowed us to perform two biological replicates in the same experiment with reversed SILAC labeling (21). For the purpose of identifying pTyr-dependent interactions on both Y191 and Y209, we did pulldowns using pYpY peptide with both SILAC light- and heavy-labeled T-cell lysates, while using YY peptide with SILAC medium-labeled T-cell lysates, and associated proteins were identified by MS. We thus obtained two replicate ratios of pYpY:YY peptide pulldowns as represented by H/M and L/M ratios (Fig. 3B and Dataset S4). This approach generated highly reproducible data between two biological replicates and nicely differentiated pTyr-dependent interacting proteins from the majority of other proteins that bound nonspecifically or to the nonphosphorylated YY peptide. Twenty-eight CD28-binding proteins were confidently identified, including 8 proteins previously identified as associated with the CD28 cytoplasmic domain (Fig. 3C). Based on the accurate quantification afforded by SILAC, use of the various phosphorylated and mutant forms of CD28 that identified three families of proteins that bound selectively to either the pYMNM site (group I), the PXXP motif (group II), or the PpYAPP sequence (group III) of CD28 (Fig. 3B and C, and SI Appendix, Fig. S4 A–D). Western blots for 8 CD28-associated proteins that contained either pTyr-binding SH2 domains or PXXP-binding SH3 domains also gave results that were consistent with the MS data (SI Appendix, Fig. S4E).
Pathway analysis revealed that more than 50% of the CD28 interactors are involved in immune signaling pathways, including CD28 signaling in T-helper cells, demonstrating the biological relevance of the CD28 interactome obtained by the in vitro CD28\(^{\text{vivo}}\) pulldown (Fig. 3D). Our data recapitulated previous observations demonstrating that the YMMN and PYAPP motifs are the two dominant motifs on the CD28 cytoplasmic tail (12), including the binding of PI3K family proteins as well as the GRB2 and GADS adaptors to the CD28 pYMMN motif and GRB2 SH3 domain-mediated interaction with the PYAPP motif. In addition, we identified seven previously unidentified interactions to the PYAPP motif that are dependent on pTyr, the proline-rich sequence, or both. Because these newly identified proteins contain SH2 or SH3 domains as GRB2 and GADS adaptors, they likely bind to the CD28 cytoplasmic tail in an indirect manner. However, a more dedicated experimental workflow is needed to confirm their binding modes. The PYAPP motif has been suggested to be dispensable for mediating T-cell functions such as proliferation and IL-2 secretion, in contrast to the YMMN motif (12). Therefore, our approach and findings may be the basis for further experiments to study the mechanisms underlying the importance of these motifs.

Like CD28, most immune receptors and ligands contain rather short cytoplasmic tails (Dataset S5). Peptides of this relatively short length, together with their posttranslational modifications, can be readily synthesized using current technology, indicating that the overall approach described above could have widespread applications.

**A High-Resolution in Vivo CD28 Interactome Reveals Phosphorylation-Dependent Interaction Hubs.** To validate and extend the signaling network out from these CD28-binding partners identified by in vitro CD28\(^{\text{vivo}}\) pulldown experiments, we performed IP-MS from Jurkat leukemic T cells (Fig. 4A). For this purpose, we used a piggyb ac transposon system to efficiently generate Jurkat leukemic T cells stably expressing specific 3xFlag-tagged CD28-binding proteins, such as GRB2 (22). These cells were then either left unstimulated or were stimulated by the anti-CD28 antibody and lysed, and proteins associated with the Flag-tagged polypeptide were identified by IP-MS. A three-channel SILAC approach was used to quantitatively distinguish proteins that bound constitutively and selectively to the target, proteins whose binding was induced by CD28 activation, and proteins that bound nonspecifically. GRB2 was investigated because it is known to interact with CD28 on both the YMMN and the PYAPP motifs and to function as an adaptor to link receptors to multiple downstream signaling pathways (Fig. 4B). A group of proteins that selectively, but constitutively, interacted with GRB2 in T cells was reliably identified with high confidence; in contrast, the association between GRB2 and CD28 required CD28 stimulation, consistent with an SH2–pTyr interaction. Among the constitutively GRB2-interacting proteins were CBL, SOS1, and THEMIS (23). Suppressor of T-cell receptor signaling 1 (STS1; gene name UBASH3B), a negative regulator of T-cell activation, is a potential atypical tyrosine phosphatase that contains an SH3 domain that recognizes a proline-rich motif and a UBA domain that binds ubiquitin (24, 25). The CD28\(^{\text{vivo}}\) pulldown experiments have shown that STS1 binding required both the PXXP and pTyr elements within the CD28 PYAPP motif. Interestingly, our IP-MS data showed that STS1 also interacted with GRB2 and that both of them bound to the E3 ubiquitin ligases CBL and CBL-B (Fig. 4C). The association of STS1 with CD28 was validated by immunoprecipitating endogenous CD28 (SI Appendix, Fig. S5A). We also found that the interaction between STS1 and GRB2 is enhanced upon the activation of both TCR and CD28 signaling, demonstrating its potential recruitment to CD28-associated signaling complexes through GRB2 (SI Appendix, Fig. S5B).

We further followed up experimentally an interaction hub that focused on CD28, GRB2, and CBL/CBL-B, which contains 14 proteins, including 8 identified by CD28\(^{\text{vivo}}\) pulldown experiments (Fig. 4D). By using IP-Western blot (IP-WB) and IP-MS approaches, we validated 12 pairs of protein–protein interactions within this interaction hub and found that 6 of these interactions could be enhanced upon stimulation of both TCR and CD28 signaling (Fig. 4D and SI Appendix, Fig. S5B). Interestingly, we found that the phosphorylation level of 14 phosphorylation sites on nine proteins, as identified by our initial unbiased phospho-proteomic screen in cell lysates (Fig. 1 and Dataset S3), all consistently decreased upon CD28 inhibition. Of these 14 phosphorylation sites, 10 are pTyr sites, which is consistent with the down-regulation of three pTyr sites on the CD28 cytoplasmic...
Docking protein 1 (DOK1) has been previously indicated to be tyrosine-phosphorylated upon CD28 activation by antigen-presenting cells (26). We characterized three phosphorylation sites with a decreased phosphorylation level on DOK1 (Y409, Y449, and T406) and identified a stimulation-dependent interaction of DOK1 with Crk-like protein (CRKL), which potentially links it to the CD28-mediated interaction network (SI Appendix, Fig. S5B). ADAP is a well-characterized adaptor protein that positively regulates TCR signaling (27). We identified a decreased phosphosite at Y813 of ADAP (Dataset S2) and showed that ADAP constitutively associated with CRKL (SI Appendix, Fig. S5B). ODIN is a newly characterized protein containing ankyrin repeats, a SAM domain, and a PTB domain (28). Odin constitutively bound both GRB2 and CD2-associated protein (CD2AP) (SI Appendix, Fig. S5B). The phosphorylation level of ODIN Y445 strongly decreased upon CD28 inhibition, with log₂ ratio H/M of −2.2 (Dataset S3). These results validate tight and consistent phosphorylation-dependent regulation of the CD28 interactome, demonstrating the usefulness of the combinatorial analysis of the phosphoproteome and interactome for dissecting the CD28-mediated signaling network.

To obtain a comprehensive view of the phosphorylation-dependent CD28 interaction network, we queried our 28 CD28-interacting proteins for their interactors in the STRING and BioGRID databases (Fig. 5A). Ninety-three proteins were obtained that have regulated phosphorylation sites as identified by the phosphoproteomic screen (Dataset S3). These proteins are highly enriched for protein functions such as intracellular-signaling cascades and actin-cytoskeleton organization (Fig. 5B and SI Appendix, Fig. S6A). Pathway analysis of the extended CD28 interaction network showed that immune signaling, including TCR signaling, is overrepresented and additively down-regulated by CD28 inhibition (SI Appendix, Fig. S6B and Dataset S3). The extended CD28 interaction network forms clear phosphorylation-dependent interaction hubs around proteins such as GRB2, the PI3K family, the STAT family, CD2AP and CIN85, and CBL. Interestingly, two well-characterized CD28-interacting proteins are most notable; GRB2 has extensive connections to 37 newly recruited phosphoproteins whereas PI3K p85α (PIK3R1) has broad associations with multiple components in the CD28 interactome. These observations might explain a functional importance of GRB2 as a key adaptor for regulating critical CD28-associated downstream signaling.

Costimulation-Regulated Phosphorylation of CapZIP and Its Function in Regulation of IL-2 Production. Our analysis of the phosphorylation-dependent CD28 interaction network suggested that one of the main functions of CD28 signaling is to regulate actin dynamics (Fig. 5B). Consistent with this observation, it was recently reported that RltpAr, an actin-uncapping protein, plays an essential role in CD28-mediated signaling (29). RltpAr contains a capping protein (CP) interaction (CPI) motif that interacts with the CapZ protein, thereby inhibiting actin filament capping and promoting actin polymerization. In our global phosphoproteomics study, no significant change of RLTPR phosphorylation was observed. However, the phosphorylation pattern of CapZIP, another member of the CPI motif-containing protein family, was significantly changed upon blockage of CD28 costimulation (Dataset S2). CapZIP, highly expressed in immune cells and skeletal muscle, can be phosphorylated by multiple
stress-activated protein kinases (SAPks) when cells are exposed to cellular stresses (30). A previous study suggested that phosphorylation of CapZIP may cause the dissociation of CapZIP from CapZ and thereby regulate actin-filament assembly (30). Among 18 CapZIP phosphorylation sites identified by our phosphoproteomic screening, three phosphoserine sites (S132, S135, and S333) were significantly changed upon CD28 inhibition, with S132 and S135 showing significant down-regulation and S333 showing significant up-regulation, suggesting that CapZIP might be involved in CD28-mediated costimulation (Dataset S2). To examine the role of CapZIP in the CD28-signaling pathway, we generated CapZIP-deficient cells from the Jurkat T-cell lines by using CRISPR/Cas9 technology. The double-nicking strategy was used to minimize off-target mutagenesis (Fig. 6A) (31). We succeeded in generating four single-cell clones of CapZIP-deficient (CapZIP−/−) Jurkat cells (Fig. 6B). The deficiency of CapZIP did not affect surface expression of CD3 and CD45 (Fig. 6C). Although two of four CapZIP−/− single-cell clones had reduced surface CD28 expression by up to 60%, the other CapZIP−/− clones had CD28 expression that was indistinguishable from the parental Jurkat line. The reduction of CD28 expression was not statistically significant (Fig. 6D). After stimulation with SEE presented by Raji B cells, WT and CapZIP−/− Jurkat cells up-regulated comparable amounts of CD69, indicative of intact TCR signaling in CapZIP−/− cells (Fig. 6E). CD69, a major activation marker downstream of the Ras/MAPK/ERK signaling pathway, can be induced by TCR stimulation alone. However, the IL-2 production in SEE-stimulated CapZIP−/− Jurkat cells was nearly abolished (Fig. 6F). Notably, CapZIP−/− Jurkat cells were able to produce similar levels of IL-2 compared with WT Jurkat cells, when cells were stimulated by PMA plus ionomycin, which bypass the TCR/CD28 proximal signaling (SI Appendix, Fig. S7). Collectively, these data suggest that CapZIP is required for CD28 costimulation-dependent IL-2 production.

Discussion

Although the importance of CD28-mediated costimulation for T-cell activation has been explored for more than 29 y, the downstream signaling pathways that are regulated by CD28 are still poorly understood. Current models derived from targeted experimental studies have yielded controversial views, especially for the functional role of the PI3K pathway in CD28 signaling (12). Our combinatorial proteomic analysis provides a window into the physiologically relevant CD28-regulated protein phosphorylation and its interaction networks. The extent of protein phosphorylation changes that were affected by blocking CD28 engagement was more extensive than anticipated. The global phosphorylation analysis using cell-based stimulation showed some intersection of CD28-mediated signaling with TCR signaling but also involved a number of other signaling pathways, some of which have been associated with other immune receptors.

Studying signal transduction events mediated by endogenous receptors under physiologically relevant conditions is crucial for understanding fine-tuned signaling outputs and their cellular effects. Toward this end, we have designed our SILAC approach with the following considerations. We studied endogenously expressed CD28 receptors during a physiologically relevant intercellular interaction. We used CTLA4-Ig to specifically interrupt CD28 receptor activation, leaving signaling by other receptors, including the TCR, intact. This physiologically relevant activation condition maintains the complexity of the natural cell–cell communication. It has been well-characterized that a broad spectrum of different costimulatory and coinhibitory receptors and their ligands are engaged and activated during TCR-dependent T-cell activation (9). The assumption of our study is that, in terms of T-cell activation, CD28 has some key, unique functions that either work independently or are integrated with other signals generated by TCR or other molecules involved in cell–cell interaction. In our experimental setting, the only difference between two activation conditions is CD28 blockade. We acknowledge that other receptor-mediated signals may rely on or influence the CD28 signals. Our experiments indeed aim to determine which of these pathways are CD28-dependent.

To explore the multidimensional nature of receptor signaling, a combination of different unbiased screening approaches on a global scale is required. Our combinatorial proteomic approach allowed the identification of signaling events associated with CD28 costimulation through both a global phosphorylation analysis and a protein-interactome screen. The phosphoproteome analysis provides a global view of CD28 receptor activation across a broad spectrum of different downstream signaling pathways. The synthetic cytoplasmic-domain pulldown approach captured CD28-associated protein complexes without protein solubilization issues. The combination of these phosphoproteomic approaches facilitated the study and recognition of phosphorylation-dependent recruitment of protein complexes.

The CD28 interactome analysis was characterized by a spectrum of associated signaling proteins that are shared with other important immune signaling pathways. Prominent among these...
pathways are those that involve remodeling the actin cytoskeleton and the PI3K pathway. As a preliminary effort to explore this dataset, we focused on the actin cytoskeleton because our recent studies (32) and those of others (29) suggested an important role for CD28 in regulating the actin cytoskeleton. The genetic study of Liang et al. (29), which identified an important role of Rlptr, led us to focus on CapZIP. Ablation of CapZIP expression led to impaired IL-2 production in our Jurkat-SEE/Raji system, which mimicked the effects of CD28 blockade but had no effect on TCR signaling, based on CD69 expression. Although the precise role of CapZIP is not clear and further work is required to understand its role and that of its CD28-regulated phosphorylation, these studies do support an important role for CD28 signaling in actin-cytoskeleton regulation and have identified a previously unappreciated role for CapZIP downstream of CD28. Our further systems-wide interactome analysis and experimental validation revealed a prominent GRB2-proximal interaction hub that is widely regulated by CD28-dependent phosphorylation and merits further exploration. Clearly, our CD28-regulated proteome will provide an important starting point for many other studies aimed at understanding CD28 costimulatory signaling.

Importantly, this SILAC-based phosphoproteomic approach is generally applicable for the study of other endogenous receptors activated by direct cell–cell contact because blocking reagents are available for most of the well-characterized receptors. With advances in synthetic biology, the synthetic cytoplasmic-domain pulldown approach could, in principle, be applied to study a broad spectrum of transmembrane receptors. As such, this combinatorial approach provides a widely applicable workflow to systematically understand signaling events mediated by membrane receptors with short cytoplasmic tails in more physiologically relevant contexts.

Materials and Methods

Cell–Cell Stimulation and Sample Preparation for Phosphoproteomics. Medium and heavy SILAC-labeled Jurkat leukemic T cells were starved in SILAC media without dialyzed FBS for 12 h whereas two sets of equal amounts of unlabeled Raji lymphoblastoid cells were kept in normal culture. After that, 1 × 10^6 medium and heavy SILAC-labeled Jurkat leukemic T cells were used

Fig. 6. Generation and characterization of CapZIP-deficient cells. (A) Schematic of targeting human CapZIP exon 2 using Cas9 double-nicking strategy. The target regions of each sgRNA are labeled in blue, and PAM sequences are highlighted in red. (B) Immunoblot analysis with a CapZIP-specific or GAPDH-specific antibody in total cell lysates. Single-cell clones (clones 1–7) were generated from cells transfected with Cas9-nickase and sgRNAs targeting CapZIP. CapZIP protein level remained largely unchanged in clones 1–3 and was completely deleted in clones 4–7. (C) Representative FACS analysis of surface expression of CD3, CD45, and CD28 on resting CapZIP-sufficient cells (blue, clone 2) and CapZIP-deficient cells (red, clone 3). Gray-filled histogram, unstained control. (D) Bar chart showing CD28 surface expression level on resting CapZIP-sufficient cells (blue, WT Jurkat and clones 1–3) and CapZIP-deficient cells (red, clone 4–7). (E) Jurkat cells were stimulated by SEE (30 ng/mL) presented by Raji B cells. CD69 up-regulation on CapZIP-sufficient cells (blue, clone 2) and CapZIP-deficient cells (red, clone 5) was measured by FACS 22 h after stimulation. Gray-filled histogram, untreated control. (F) The ELISA analysis of cytokine IL-2 production by Jurkat cells 22 h after stimulation with Raji B cells and SEE at indicated concentrations. **P < 0.01, ***P < 0.001, n = 4, unpaired t test. The CapZIP-sufficient samples (WT Jurkat and clone 1–3) are labeled in blue, and the CapZIP−/− samples (clone 4–7) are labeled in red.
for the following steps. Two sets of 1 x 10^6 Raji lymphoblastoid B cells were washed and resuspended into 1 mL of SILAC RPMI 1640. After adding 20 nM SEE or 20 ng/mL SEE, cells were incubated at 10 μg/mL CTLA-4a (final concentration). The Raji lymphoblastoid B cells were incubated at 37 °C for 30 min. Both Jurkat leukemic T cells and Raji lymphoblastoid B cells were then transferred to ice for 10 min, mixed together with equal volumes, quickly centrifuged at 500 x g for 5 min at 4 °C to promote cell–cell contact, and stimulated at 37 °C for 5 min without resuspending the cell pellet, to promote cell–cell contact. After that, the cells were lysed in lysis buffer (50 mM Tris- HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM sodium orthovanadate, protease inhibitors mixture (Complete mini; Roche), phosphatase inhibitor mixture (PhosSTOP; Roche)), and the two sets of cell lysates were then mixed together. The soluble proteins were centrifuged at 4 °C and were precipitated with four volumes of acetone at –20 °C overnight. The protein precipitate was dissolved in 100 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.3% Nonidet P-40 and enriched by anti-phosphotyrosine antibody 4G10-conjugated beads (Millipore). The rest of the sample was fractionated into 24 fractions by SCX chromatography as described (33) and enriched by a TiO2 kit (GL Sciences).

Flow-Cytometry Analysis and ELISA. Flow-cytometry analysis of phosphorylated ERK was performed as described (34). Briefly, after stimulation, cells were fixed in 4% (wt/vol) formaldehyde (Polysciences Inc.) and permeabilized in 95% (vol/vol) ice-cold methanol. Permeabilized cells were stained with a pERK antibody (Cell Signaling) followed by a PE-conjugated antibody against rabbit IgG and APC-conjugated anti-CD3. The level of pERK in gated CD3+ populations was measured by an LSRFortessa cell analyzer. For the ELISA, the supernatants of the coculture system were collected 22 h after stimulation, and IL-2 production was measured using a Human IL-2 ELISA Kit (BD Biosciences) according to the manufacturer's instructions.

CD28*-YPE Pulldown and Immunoprecipitation Assays. The CD28 cytoplasmic domain with bioitin tag and various modifications was synthesized in-house, HPLC-purified, and conjugated on streptavidin agrose beads. Jurkat leukemic T cells were SILAC-labeled with light, medium, or heavy lysine and arginine. Then, 50 x 10^6 SILAC-labeled cells were lysed in Nonidet P-40 lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, protease inhibitors mixture (Complete mini; Roche), phosphatase inhibitor mixture (PhosSTOP; Roche)) and incubated with different concentrations of antibody at 4 °C overnight. The beads were washed four times with washing buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40) and two times with 20 mM ammonium bicarbonate at 4 °C. The associated proteins were digested on the beads by trypsin as described (35) and combined for MS analysis. For IP-MS analysis of GRB2 and STS1, WT and triple Flag-tagged Jurkat leukemic T cells were SILAC-labeled as indicated in Fig. 2A. Then, 1 x 10^6 cells were starved in PBS for 20 min at 37 °C, and the heavy SILAC-labeled cells were stimulated with an anti-CD28 antibody for 5 min at 37 °C. The cells were lysed in Nonidet P-40 lysis buffer, precleared with mouse IgG beads (Sigma), and incubated with anti-Flag M2 beads (Sigma) overnight. The beads were washed and digested as described above.

MS Data Analysis and Definition. Samples were analyzed on LTQ-Orbitrap Elite (for phosphoproteomics) or Classic (for part of CD28*-YPE pulldown) mass spectrometers (Thermo). The obtained raw data were processed by MaxQuant (version 1.2.2.5) coupled with Andromeda for database searching against the International Protein Index (IPI) human database (version 3.7.9; 91464 entries). The assignment of phosphorylation sites to tryptic peptides was performed automatically in the MaxQuant software environment with proper evaluation of the site localization probabilities (36). Evidence tables containing all of the quantified peptide information were used to generate the final identification information as shown in Dataset S1. The R language package was used to perform quantitative analysis of the global phosphorylation dataset with data obtained from the MaxQuant evidence tables. The minimum Andromeda score was set as 42. In addition, the minimum site localization probability was 0.33. The pathway analysis was performed using gene annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (37) (P value cutoff 0.1; Fisher’s exact test) and Ingenuity Pathways Analysis (P value cutoff 0.05; Fisher’s exact test) (Fig. 3D). Further interaction analysis related to Fig. 4D was performed based on STRING v9.1 (38) (score cutoff 0.5) and BIOGRID (39).

For CD28 cytoplasmic domain pulldowns and IP-MS experiments, only proteins identified and quantified with at least two “Unique + Razor Pep- tides” were considered. Only proteins identified and quantified in at least two out of three experiments as shown in Fig. 3B and SI Appendix, Fig. S4 A and B were considered as positive hits. The log, ratio cutoff was set as 1. PtyMM motif- and pYpAPP motif-dependent interactions were defined by SILAC quantification with three-peptide pulldown combinations (SI Appendix, Fig. S4 A and B), which were cross-validated by YpP peptide pulldown (SI Appendix, Fig. S4C). The result, as shown in SI Appendix, Fig. S4D, was used to define pXpMM motif-interacting dependents, and two selected proteins (i.e., CIN85 and STS1) were further validated by Western blot (SI Appendix, Fig. S4E).

Western Blots. For IP-WB analysis of GRB2, CRKL, DOK1, CD2AP, CIN85, ODIN, and ADAP, 50 x 10^6 cells were starved in PBS for 20 min at 37 °C, and cells were stimulated with anti-TCR or anti-TCR plus anti-CD28 antibody for 5 min at 37 °C. The cells were lysed in Nonidet P-40 lysis buffer and incubated with anti-Flag M2 beads (Sigma) or anti-mouse IgG beads (Sigma) overnight. The beads were washed and boiled in 2x SDS/PAGE loading buffer for Western blot analysis. Western blot quantitation was performed with Phos-tag® SDS/PAGE gels, transferred to nitrocellulose, and analyzed.

Generation of CapZIP-Deficient Jurkat Cell Lines. Two single-guide RNAs (sgRNAs) targeting the CapZIP gene were cloned into pX335 (Addgene). Jurkat cells were cotransfected with these two sgRNA plasmids and a GFP reporter plasmid. At 24 h posttransfection, GFP+ live Jurkat cells were single cell sorted into a 96-well plate. Three weeks later, single cell clones were expanded upon cryopreservation.

MS Data Storage. The raw MS data files associated with this study can be downloaded from the Mass Spectrometry Interactive Virtual Environment (MassIVE) (massive.ucsd.edu/ProteoSAFe) via the following ftp access: address, massive.ucsd.edu/ProteoSAFe; user name, CD28; password, CD28AJII.

ACKNOWLEDGMENTS. We acknowledge K. Colwill, L. Taylor, B. Larsen, C. Zhang, and M. Tucholska for technical help and J. Jin, Y. Zheng, C. Chen, G. M. Findlay, and M. Kofler for helpful discussions. We thank A. Salomon and N. Krogan for constructive suggestions after their reading of this manuscript. We thank J. Bluestone, who provided the CTLA4a-Ig. This work was supported by grants from Ontario Research Fund GL2 (to T.P. and A.-C.G.), the Canadian Institutes of Health Research (CIHR) (Grant MOP- 848148 to T.H., A.-C.G., and H. Li; the National Cancer Institute (Grant CA82683) (to T.H.), and a Shenzhen grant (ZDSYS20160509142721429) (to R.T.). R.T. is the recipient of a fellowship from the CIHR. H.W. is the recipient of a postdoctoral fellowship from the US Arthritis Foundation.


