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Cutting Edge: Negative Regulation of Human T Cell Activation by the Receptor-Type Protein Tyrosine Phosphatase CD148[1]

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T cell activation represents a balance between positive and negative signals delivered via distinct cell surface molecules. Many cytoplasmic protein tyrosine phosphatases are involved in regulating cellular responses by antagonizing the action of protein tyrosine kinases. CD148 is a receptor-type protein tyrosine phosphatase expressed by all human mononuclear cells. We have investigated the effect of CD148 on TCR-mediated activation of human T cells. Overexpression of wild-type, but not a phosphatase-deficient, CD148 in Jurkat T cells inhibited TCR-mediated activation, evidenced by reduced expression of the early activation Ag CD69, inhibition of tyrosine phosphorylation of many intracellular proteins including the critical protein tyrosine kinase ZAP-70, and impairment of mitogen-activated protein kinase activation. Taken together, these results suggest that CD148 is an important phosphatase involved in negatively regulating the proximal signaling events during activation of Ag-specific T cells. ***The Journal of Immunology,*** **1998, 161: 3803–3807.**

T cells undergo activation in response to signals delivered via the TCR in combination with accessory molecules including CD28 (1), LFA-1 (2), CD2 (3), CD27 (4), and CD40 ligand (5). To maintain immune equilibrium, these stimulatory signals must be regulated by inhibitory signals delivered via molecules such as CTLA-4 (6) and killer cell inhibitory receptors (7). The interplay between positive and negative signals is regulated by protein tyrosine kinases (PTK[4]) and protein tyrosine phosphatases (PTP) (8–10). PTP involved in lymphocyte activation include CD45 and SHP-1 (SH2-containing PTP-1). CD45 is a critical positive regulator for initiation of Ag receptor-mediated signal transduction (9). In contrast, SHP-1 can inhibit such responses (11–13). Thus, PTP can either positively or negatively regulate lymphocyte activation. CD148 is a receptor-type PTP (R-PTP) (14–16) expressed by activated human T cells (17). Ligating CD148 with a specific mAb augmented proliferation of anti-CD3 mAb-activated T cells (17). We have now investigated the mechanism by which CD148 regulates T cell activation.

Materials and Methods

Antibodies

The following Abs were used: anti-CD148 (A3; Ref. 17); anti-CD69 (Becton Dickinson, Mountain View, CA); goat anti-mouse Ig (Jackson ImmunoResearch Laboratories, West Grove, PA); anti-CD3 (Spv-T3b; Ref. 18); anti-Jurkat TCRβ-chain (C305; Ref. 19); control IgG1 (MOPC-31; PharMingen, San Diego, CA); horseradish peroxidase-conjugated antiphosphotyrosine (4G10), anti-*myc* (9E10; Upstate Biotechnology, Lake Placid, NY); rabbit antiphosphorylated mitogen-activated protein kinase (pMAPK) antiserum (Promega, Madison, WI); rabbit anti-extracellular signal-regulated kinase (ERK) antiserum (Santa Cruz Biotechnology, Santa Cruz, CA); and horseradish peroxidase anti-rabbit and anti-mouse IgG antiserum (Amersham, Arlington Heights, IL).

Expression constructs

The full-length CD148 gene (14) was released from pSSRα by *Not*I-*Sal*I digestion, producing a 4267-bp fragment that was ligated into the *Not*I-*Sal*I sites of pSport1 (Life Technologies, Grand Island, NY). The pSport/CD148 construct was digested with *Xba*I and *Sal*I, yielding a fragment that could be ligated into the *Xba*I-*Sal*I sites of pEF-BOS (20), producing the pEF-BOS/CD148 construct. To generate a phosphatase-deficient construct, pSport/CD148 was digested with *Stu*I to release the fragment (from nucleotide 3424 to 4176) encoding the catalytic site of the phosphatase domain. Following digestion, pSport/CD148 was religated, yielding pSport/CD148$_{mutant}$. pSport/CD148$_{mutant}$ was then digested with *Xba*I and *Sal*I, and the resulting fragment cloned into the *Xba*I-*Sal*I sites of pEF-BOS, producing pEF-BOS/CD148$_{mutant}$. The C-terminal *myc*-tagged pEF-BOS/ZAP-70 construct was provided by Dr. A. Weiss (University of California, San Francisco).

Transfection, analysis, and activation of human T cells

Jurkat cells (2×10^7) were transfected by electroporation with no DNA, empty pEF-BOS, pEF-BOS/CD148, pEF-BOS/CD148$_{mutant}$ (40 μg), or a combination of pEF-BOS/ZAP-70 (10 μg) with CD148 or CD148$_{mutant}$

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[4] Abbreviations used in this paper: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SHP-1, SH2-containing protein tyrosine phosphatase-1; R-PTP, receptor-type PTP; MAPK, mitogen-activated protein kinase; pMAPK, phosphorylated MAPK; ERK, extracellular signal-regulated kinase; PE, phycoerythrin; MFI, mean fluorescence intensity.

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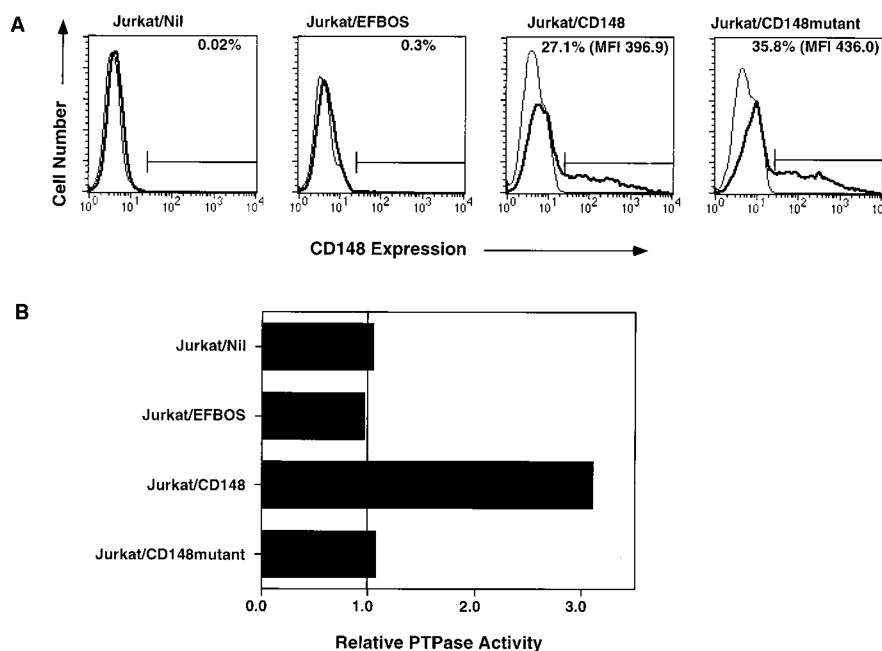

FIGURE 1. Transient expression of CD148 in Jurkat cells. *A*, Jurkat cells (2×10^7) were transfected by electroporation with either no DNA (Jurkat/Nil), pEF-BOS vector containing no insert (Jurkat/EFBOS), pEF-BOS/CD148 vector (Jurkat/CD148), or pEF-BOS/CD148$_{mutant}$ vector (Jurkat/CD148 mutant). Expression of CD148 was assessed 15 h later. The bold histogram shows the fluorescence of cells incubated with FITC-anti-CD148 mAb, while the thin histogram shows the fluorescence of cells incubated with a FITC-conjugated isotype control mAb. Fluorescence was measured on a 4-decade logarithmic scale. The values represent the percentage of CD148$^+$ cells, and the value in parentheses is the MFI of the positive cells. *B*, Cell lysates were prepared from the transfected Jurkat cells, and $\sim 10^6$ cell equivalents were incubated in the wells of a 96-well microtiter plate precoated with either a control IgG1 or anti-CD148 mAb (10 μg/ml). Phosphatase activity of each lysate was assessed by the ability to generate free phosphates following the addition of *p*-nitrophenyl phosphate substrate and quantitated by determining the absorbance of the soluble product at a wavelength of 405 nm. Results are expressed as a ratio of OD_{405} in the presence of anti-CD148 mAb/OD_{405} in the presence of control IgG1. The solid line represents a ratio of 1, indicative of negligible PTP activity.

(20). Intrinsic PTP activity of cell lysates was demonstrated as described (21). Transfected Jurkat cells (10^6/ml) were cultured with immobilized anti-CD3 mAb (1 μg/ml) or PMA (1 ng/ml) plus ionomycin (500 ng/ml). In some experiments, culture plates were precoated with anti-CD148 mAb (17), and transfected Jurkat cells or normal purified human T cells (10^6/ml; Ref. 22) were added to the wells and activated with 1 μg/ml soluble anti-CD3 mAb.

Surface immunofluorescence

Cells were incubated with FITC- or phycoerythrin (PE)-conjugated mAb and incubated on ice for 30 min. Expression of CD3 and CD69 on transfected Jurkat cells was determined by gating on CD148$^+$ and CD148$^-$ cells. Dead cells were excluded by the addition of propidium iodide (2 μg/ml).

Immunoblot analysis of activated Jurkat cells

Following transfection, the cells were activated with anti-Jurkat TCRβ-chain mAb (C305) for 3 min at 37°C, then lysed in 10 mM Tris-HCl (pH 7.8) containing 1% Nonidet P-40, 150 mM NaCl, and enzyme inhibitors (20). In some experiments, the cells were stained with FITC-anti-CD148 mAb and then sorted into CD148$^+$ and CD148$^-$ cell populations before activation. ZAP-70 was immunoprecipitated from lysates using anti-*myc* mAb absorbed onto protein G beads. Cell lysates were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) which were probed with Abs specific for phosphotyrosines, *myc*, pMAPK or total ERK. The membranes were developed using enhanced chemiluminescence (Pierce, Rockford, IL) and autoradiography.

Results and Discussion

Transient expression of CD148 in Jurkat cells

To investigate the mechanism of action of CD148, the Jurkat T cell line, which does not express endogenous CD148 (17), was transiently transfected with wild-type CD148 (CD148) or PTP-deficient CD148 (CD148$_{mutant}$). After 15 to 24 h, comparable levels of gene expression were observed in both transfectants (Fig. 1*A*). Analysis of lysates indicated that CD148-dependent PTP activity was detected in CD148-transfected Jurkat cells (Fig. 1*B*), whereas Jurkat cells transfected with CD148$_{mutant}$ exhibited no PTP activity, indicating that this mutant construct indeed encoded a PTP-deficient protein (Fig. 1*B*).

CD148 negatively regulates TCR-mediated activation

Induction of proliferation of anti-CD3 mAb-activated T cells in the presence of anti-CD148 mAb could reflect either activation or inhibition of the PTP (17). To determine whether CD148 is a positive or negative regulator of T cell activation, the effect of CD148 on TCR-mediated activation was investigated. As described above, after transfection, up to 40% of Jurkat cells expressed CD148, while the remainder did not. This allowed for flow cytometric analysis of TCR-mediated up-regulation of CD69 expression on activated transfected Jurkat cells that were either CD148$^+$ or CD148$^-$. The existence of CD148$^+$ and CD148$^-$ Jurkat cells within the one population served as an internal control for the activation procedure. In the presence of anti-CD3 mAb, 90.1% ($n = 3$) of CD148$^-$ Jurkat cells transfected with CD148 up-regulated CD69 expression (Fig. 2*A*). In contrast, CD69 expression was up-regulated on only 65.9% ($n = 3$) of activated CD148$^+$ Jurkat cells (Fig. 2*a*). Not only did fewer CD148$^+$ cells up-regulate CD69, but the mean fluorescence intensity (MFI) of CD69 expression was ~40% less than on CD148$^-$ Jurkat cells (365.0 vs 590.2; $n = 3$). The inhibitory effect of transiently expressed

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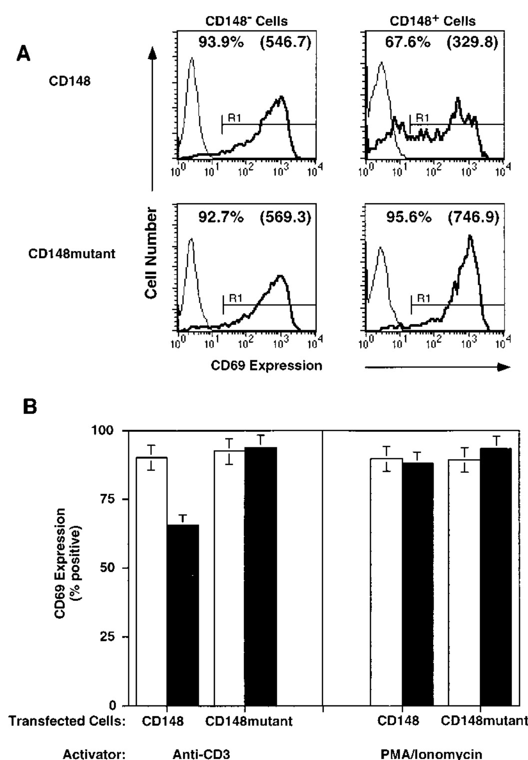

FIGURE 2. Over-expression of CD148 inhibits TCR-mediated activation of Jurkat cells. Jurkat cells were transfected by electroporation with the pEF-BOS/CD148 (CD148) vector or the pEF-BOS/CD148$_{mutant}$ vector (CD148 mutant). Fifteen hours later the cells were cultured with immobilized anti-CD3 mAb (1 μg/ml) or PMA (500 ng/ml) plus ionomycin (1 μg/ml). Expression of CD69 on the CD148$^-$ and CD148$^+$ Jurkat cells was assessed 24 h after activation by immunofluorescent staining. The bold histogram shows the fluorescence of cells incubated with PE-anti-CD69 mAb, while the thin histogram shows the fluorescence of cells incubated with a PE-conjugated isotype control mAb. Surface staining was measured on a 4-decade logarithmic scale. Dead cells were excluded by addition of propidium iodide. *A*, Flow cytometric analysis of CD69 expression on transfected Jurkat cells in response to anti-CD3 mAb. These results are representative of three independent experiments. The values represent the percentage of CD69$^+$ cells, and the value in parentheses is the MFI of CD69 expression. *B*, Percentage of transfected CD148$^-$ and CD148$^+$ Jurkat cells expressing CD69 in response to activation with anti-CD3 mAb or PMA plus ionomycin. These results represent mean ± SD of three independent experiments. The MFI of CD69 expression on anti-CD3 activated Jurkat cells was similar to that illustrated in *A*, whereas that of PMA/ionomycin-activated cells ranged from 1000 to 2000, depending on the individual experiment.

CD148 was not dose-dependent but rather threshold-dependent because activation of only Jurkat cells that expressed the highest levels of CD148 was affected. The inhibitory effect of CD148 was not due to a difference in the level of TCR expressed by the transfected cells because the MFI of CD3 expression was equivalent on CD148$^+$ and CD148$^-$ Jurkat cells (data not shown). Activation with PMA and ionomycin, which bypasses TCR-dependent signaling, overcame the inhibitory effect of CD148 as evidenced by up-regulation of CD69 on 90% of CD148$^-$ and 88.1% of CD148$^+$ Jurkat cells ($n = 3$; Fig. 2*B*). This finding demonstrated that over-expressing an exogenous protein did not grossly affect the signaling capacity of Jurkat cells. Importantly, when Jurkat cells were transfected with the CD148$_{mutant}$ cDNA CD148$^-_{mutant}$ and CD148$^+_{mutant}$ cells up-regulated CD69 expression to a comparable extent (Fig. 2). Thus, CD148 can inhibit TCR-mediated activation

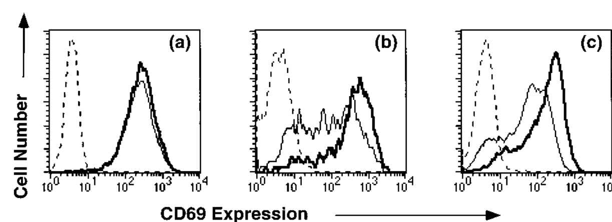

FIGURE 3. Cross-linking CD148 restores TCR-mediated activation. Transfected Jurkat cells or purified peripheral blood T cells were cultured with anti-CD3 mAb (1 μg/ml) in the presence of immobilized control IgG1 (thin histogram) or anti-CD148 mAb (10 μg/ml; thick histogram). Expression of CD69 on (*a*) CD148$^-$ Jurkat cells, (*b*) CD148$^+$ Jurkat cells, and (*c*) peripheral blood T cells was assessed after 24 h (*a* and *b*) or 48 h (*c*) by incubating the cells with PE-anti-CD69 mAb followed by flow cytometric analysis. The fluorescence of activated cells incubated with a PE-conjugated isotype control mAb is shown in the dashed line. Surface staining was measured on a 4-decade logarithmic scale. These results represent the mean ± SD of three (*a* and *b*) or five (*c*) independent experiments. The expression of CD69 induced by anti-CD3 mAb alone was no different to that induced by anti-CD3 mAb plus the control IgG1 (not shown).

in Jurkat cells, and this effect is specifically regulated by the PTP activity of CD148. This demonstration that CD148 negatively regulates T cell activation is consistent with previous observations implicating this molecule in the negative regulation of cell growth. These include the findings that the *CD148* gene was deleted in various human carcinomas (14) and that in vitro induction of CD148 expression caused a reduction in growth of some cell lines (15, 23).

The inhibitory effect of CD148 on T cell activation is neutralized by anti-CD148 mAb

If immobilized anti-CD148 mAb enhanced T cell proliferation by neutralizing the negative effect of CD148 (17), then the inhibitory effect of overexpressed CD148 on Jurkat cell activation should also be abrogated by anti-CD148 mAb. When transfected Jurkat cells were cultured with anti-CD3 mAb in the presence of immobilized anti-CD148 mAb, up-regulation of CD69 by CD148$^+$ cells was restored to the level observed for CD148$^-$ cells. (Fig. 3, *a* and *b*). To extend these observations, peripheral blood T cells were similarly cultured. Activation with anti-CD3 mAb plus a control IgG1 up-regulated CD69 expression on the majority of T cells (68.5 ± 5.6%; mean ± SEM, $n = 5$). Activation in the presence of anti-CD148 mAb moderately increased the percentage of CD69$^+$ T cells (81.7 ± 4.0%; Fig. 3*c*). Strikingly, the MFI of CD69 expression on T cells activated with anti-CD3 plus anti-CD148 mAb was 2-fold greater than T cells activated with anti-CD3 mAb plus a control IgG1 (168.3 vs 88.7, $n = 5$; Fig. 3*c*). This result suggests that cross-linking CD148 impairs its ability to negatively regulate T cell activation, causing an exaggerated T cell response as evidenced by increased proliferation and increased expression of activation Ags. A similar mechanism was proposed to account for the ability of mAb specific for the inhibitory cell surface receptors CD22 and CD5 (13, 24) to enhance proliferation of Ag receptor-activated lymphocytes (25, 26). It was proposed that the mAb increased lymphocyte proliferation by disrupting the association of the cytoplasmic domains of CD5 and CD22 with the inhibitory PTP SHP-1 (13). In further support of our data are recent studies suggesting that the activity of R-PTP is regulated by dimerization. Analysis of crystal structures of R-PTPα suggested that dimerization of the PTP domain lead to blockade of the active

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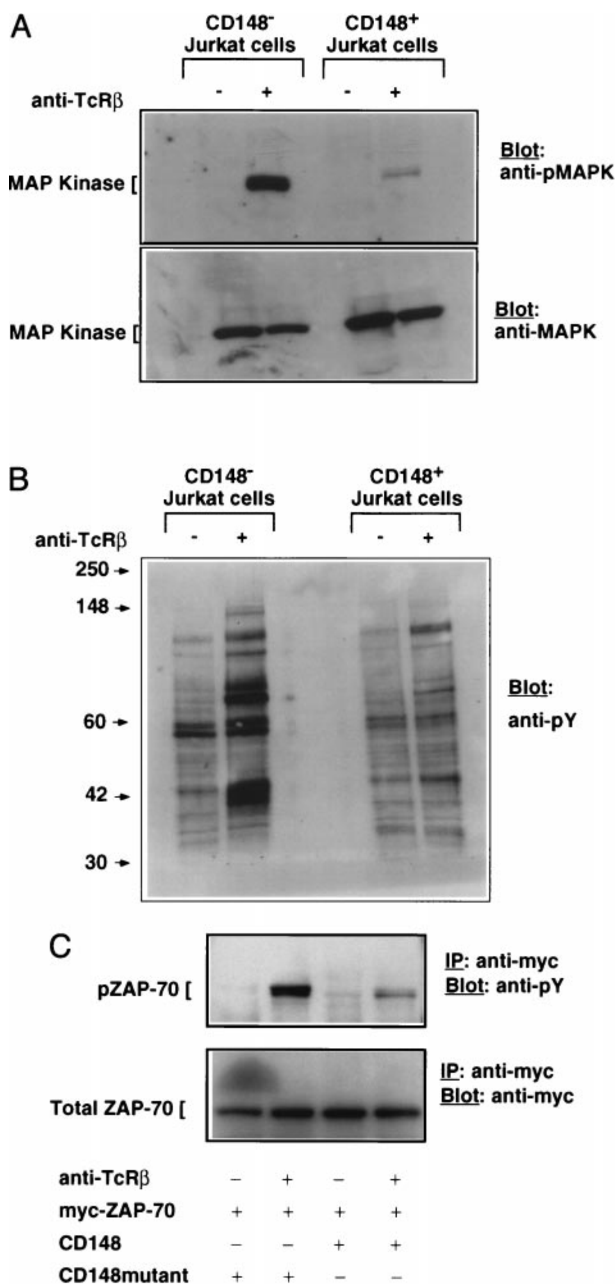

FIGURE 4. CD148 inhibits TCR-mediated signal transduction. *A* and *B*, Transiently transfected Jurkat cells were sorted into $CD148^-$ and $CD148^+$ cell populations. On re-analysis, the sorted cells were >97.5% $CD148^+$ and >99.0% $CD148^-$. The cells were either unstimulated (−) or stimulated (+) with anti-Jurkat TCRβ mAb. Cell lysates were electrophoresed through SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. *A*, Phosphorylated MAPK (*upper panel*) or total MAPK (*lower panel*) were detected using anti-pMAPK or anti-MAPK Abs, respectively. *B*, Proteins phosphorylated on tyrosine residues were detected with 4G10 phosphotyrosine-specific mAb (anti-pY). Migration of standard m.w. markers, in kilodaltons, are shown on the left-hand side. *C*, Jurkat cells were transfected with *myc*-tagged ZAP-70 and either $CD148_{mutant}$ or wild-type CD148. Fifteen hours later cells were either unstimulated (−) or stimulated (+) with anti-Jurkat TCRβ mAb for 3 min and then lysed. *myc*-tagged ZAP-70 was immunoprecipitated from the cell lysates with anti-*myc* mAb. Precipitated proteins were electrophoresed through SDS-polyacrylamide gels and transferred to PVDF membranes. Phosphorylated ZAP-70 was detected with 4G10 phosphotyrosine-specific mAb (anti-pY; *upper panel*); total myc-ZAP-70 present in cell lysates was detected with anti-*myc* mAb (*lower panel*). The *lower panels* of *A* and *C* illustrate that equal amounts of ERK-1/2 and ZAP-70 were present in the cell lysates.

site and subsequently to inhibition of PTP function (27). In addition, the restoration of TCR-mediated signal transduction in CD45-deficient Jurkat cells by a chimeric receptor could be abrogated following ligand-induced dimerization of the PTP domain of CD45 (28). Interestingly, mutation of critical residues in the cytoplasmic domain of CD45 that are believed to be involved in dimerization of CD45 abrogated the ligand-induced reduction in TCR-mediated signaling (29). Of note, the intracellular domain of CD148 contains a consensus sequence present in both R-PTPα and CD45 (27) that is important for inhibition of PTP function following dimerization (29). Thus, a generalized consequence of ligating the extracellular domain of R-PTP may be an inhibition of PTP activity induced by dimerization of the PTP domain (9).

CD148 inhibits anti-CD3-mediated signal transduction in Jurkat cells

One of the key regulators of TCR-mediated activation events, such as up-regulation of CD69 expression, is the MAPK pathway (8, 10, 30). The MAPK ERK-1 and ERK-2 were rapidly phosphorylated in TCR-activated sort-purified $CD148^-$ Jurkat cells (Fig. 4*A*, *upper panel*). In contrast, ERK-1/ERK-2 were only minimally phosphorylated in activated $CD148^+$ Jurkat cells (Fig. 4*B*, *upper panel*). This demonstrated that over-expression of CD148 inhibited TCR-mediated MAPK activation and therefore is likely to function upstream of MAPK. An early biochemical event initiated by TCR ligation is activation of PTK resulting in tyrosine phosphorylation of many cellular substrates (11). Consistent with previous studies (31), activation of $CD148^-$ Jurkat cells via the TCR induced phosphorylation of major cellular proteins of ~40 to 100 kDa (Fig. 4*B*). Strikingly, there was minimal induction of phosphorylated proteins in $CD148^+$ cells following TCR-mediated activation (Fig. 4*B*). In fact, the pattern of phosphorylated proteins in lysates of stimulated $CD148^+$ cells was similar to that of unstimulated cells, suggesting that the proximal TCR signaling machinery may be inhibited by CD148. To examine whether activation of ZAP-70, a critical PTK implicated in initiating TCR signal transduction (8), was affected Jurkat cells were transfected with *myc*-tagged ZAP-70 and either $CD148_{mutant}$ or wild-type CD148. Ectopically expressed ZAP-70 was then immunoprecipitated and assessed for its tyrosine phosphorylation status. When cotransfected with $CD148_{mutant}$, ZAP-70 was rapidly phosphorylated following TCR-stimulation (Fig. 4*C*, *upper panel*). In contrast, only a low amount of phosphorylated ZAP-70 was detected in activated Jurkat cells expressing wild-type CD148 (Fig. 4*C*, *upper panel*). This finding confirms that the inhibition observed in cells over-expressing CD148 required the PTP domain of this molecule. Taken together, over-expression of CD148 in Jurkat cells potently inhibits proximal TCR-mediated signaling events including protein tyrosine phosphorylation, ZAP-70 activation, and subsequent downstream events such as MAPK activation.

Conclusions

T cell activation can be down-regulated following recruitment of SHP-1 to cell surface receptors and intracellular molecules (12, 13, 31). TCR-mediated signaling can also be attenuated by MAPK phosphatases (10). Because it is an inducible molecule whose activity can be modulated by engagement of its extracellular domain suggest, CD148 may represent an important PTP involved in down-regulating T cell activation.

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