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Focal Localization of Placental Protein 14 Toward Sites of TCR Engagement

Jacob Rachmilewitz,* Zipora Borovsky,* Galit Mishan-Eisenberg, * Einat Yaniv,* Gregory J. Riely,† and Mark L. Tykocinski‡

TCR signal transduction is amplified by the dynamic accumulation of accessory molecules at APC-T cell contact sites, along with the simultaneous exclusion from these sites of negative regulators, such as certain tyrosine phosphatases and large glycosylated proteins. However, given the general nature of the cytoskeleton-driven clustering mechanism underlying molecular segregation events at the APC-T cell interaction site, the possibility exists that negative regulators might similarly be segregated at these sites. Using fluorescence microscopy, we have demonstrated that placental protein 14 (PP14), a direct T cell inhibitor, focuses toward APC-T cell contact sites in conjunction with conjugate formation. We have further established that the function of PP14 is dependent upon its localization to the sites of TCR triggering, where it negatively regulates T cell activation. Thus, PP14 provides an example of a soluble negative T cell regulator whose inhibitory activity is linked to modulation of the APC-T cell contact site, thereby hindering early events triggered by the TCR. The Journal of Immunology, 2002, 168: 2745-2750.

Placental protein 14 (PP14)3 (progesterone-associated endometrial protein; glycodelin) is a 28-kDa glycoprotein of the lipocalin structural superfamily with documented immunoinhibitory properties (1–4). This glycoprotein is expressed by cells of the female and male reproductive tracts (5, 6), as well as by platelets (4), and is present at high levels in amniotic fluid (AF) and maternal serum. Recently, we reported that PP14 directly inhibits human T cells and accounts for the T cell inhibitory activity of AF (7). That study further suggested that PP14 targets early events during TCR signal transduction (7), and our subsequent data have established the intriguing capacity of PP14 to elevate T cell activation thresholds (8). This latter finding points to a unique immunoregulatory mechanism for PP14 that is distinct from that of other T cell suppressive factors (such as cyclosporin A).

T cells interacting with APCs undergo rearrangement of critical surface receptors, signaling molecules, and cytoskeletal elements, so that these components face the zone of contact with APC (9). This polarization process forms a specialized domain on the T cell surface, also known as an "immune synapse," which serves to increase the amplitude and duration of TCR signaling (10). Following activation, some cell surface molecules are enriched in the central zone of the immune synapse, while large, highly glycosylated molecules and certain phosphatases (such as CD43 and CD45) migrate away from this zone (11–13).

These dynamic changes in surface molecular topology are thought to be important in sustaining T cell activation after the initial TCR triggering event. However, because this clustering phenomenon is general and involves many molecules linked to cytoskeletal actin (10), it is plausible that certain negative regulators of T cell activation might also localize to contact sites. In view of our previous demonstration that PP14 functions in a unique way at an early step of T cell activation to elevate the TCR activation threshold (8), we hypothesized that PP14 might achieve this inhibitory effect by modulating TCR signaling components within activation clusters. Such an immunoregulatory mechanism, based upon localized interference with TCR signaling within immune synapses, would be distinct from the alternative mechanism that entails negative signaling through independent, dispersed inhibitory receptors.

In this study, we use immunofluorescence microscopy to show that when APC and T cells form conjugates, PP14 translocates to the sites of cell contact. Significantly, the data further indicate that the inhibitory activity of PP14 depends upon its access to the triggered TCR, and leads to decreased stability of TCR-induced phosphorylated proteins. Taken together, these results support a model wherein PP14 attenuates TCR signaling within the signaling unit. Thus, PP14 may represent a new type of soluble regulatory protein that dampens T cell responses by its physical presence in the contact site at the time of TCR triggering.

Materials and Methods

Cells

PBMC were purified from the venous blood of healthy donors by density gradient centrifugation as described (7). As a source of APC, monocytes were isolated from mononuclear cell populations by adherence to plastic. PBMC (2 × 10^6 cells/ml) were incubated in serum-free medium for 1 h at 37°C. The adherent cells were washed extensively with medium to remove any residual nonadherent cells, and fresh complete medium was added. Following a 24-h incubation period, adherent monocytes were removed by gentle scraping with a plastic cell scraper. Cell viability was >80% as determined by trypan blue exclusion. Cells were washed with medium, resuspended at 1 × 10^6 cells/ml, and then pulsed with the superantigen staphylococcal enterotoxin B (SEB) at the indicated concentrations at 4°C.
for 2 h. The SEB-pulsed cells were then washed three times with medium, diluted to $1 \times 10^6$ cells/ml, and incubated in culture medium at 37°C for 20 min before adding them to cultures.

CD4+ T cells were isolated from the PBMC pool by first depleting monocytes by adherence to tissue culture flasks, as described above, and then further purifying the nonadherent T cells with a magnetic cell isolation system (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were maintained in RPMI medium (Biological Industries, Beit-Haemek, Israel) supplemented with 10% heat-inactivated FCS (Biological Industries), 2 mM glutamine, and penicillin/streptomycin. The Jurkat cell line was obtained from the American Type Cell Culture Collection (Manassas, VA).

**AF samples and PP14 immunoabsorption**

Discarded human AF samples were obtained from the Center for Human Genetics Laboratory at University Hospitals of Cleveland (Cleveland, OH) and stored at −80°C. Samples obtained from several patients (collected at 14–16 wk of gestation) were pooled and filter-sterilized before use. Anti-PP14 polyclonal Ab (4) was coupled to protein A-Sepharose beads (Sigma Aldrich, St. Louis, MO) to generate an immunoabsorbent. Immunoabsorption was conducted by adding Ab to Ab-coupled beads and incubating the mixture overnight at 4°C with gentle rotation. The beads were pelleted by centrifugation, and the supernatant was filtered and used in assays as described. The presence of PP14 was verified by Western blotting.

**Production of PP14-Fcγ1**

The coding sequence for full-length human PP14 (4), including its signal sequence, was fused to the 5′-end of the coding sequences for the hinge, CH1 and CH2 domains of human IgG1. This chimeric sequence was inserted into the EBV episomal expression vector pglG/REP7β (14), generating pPP14-Fcγ1. For expression, 293 cell (ATCC) transfectants secreting PP14-Fcγ1, were grown in Ultraculture (Biosciences, West Grove, PA). A total of $1 \times 10^6$ cells/sample were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software.

**Conjugate formation and immunofluorescence microscopy**

CD4+ T cells were mixed at a 1:1 ratio with autologous monocytes that had been pulsed with SEB (1 μg/ml) in the presence of 2 μg/ml PP14-Fcγ1. This relatively low concentration of PP14-Fcγ1 was chosen to optimize the visualization of PP14 at the cell surface as a ring of fluorescence. Of note, significantly higher concentrations (20–40 μg/ml) of PP14 are generally needed for inhibiting T cells in standard assays, including decreasing conjugate formation (data not shown). After centrifugation for 5 min at 100 × g, the cells were washed with 1 ml PBS and then methanol fixed. Fixed cells were labeled with PE-conjugated F(ab)2 fragment goat anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). A total of $1 \times 10^6$ cells/sample was analyzed on a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software.

**Cell stimulation and measurement of cytokine production**

Jurkat cells ($5 \times 10^5$/well in 1 ml volume) were cultured in individual wells of 24-well culture plates, and were stimulated with PHA or immobilized anti-CD3 plus soluble anti-CD28 mAb for 20 h. PP14-Fcγ1 was added either as soluble protein or immobilized on A beads, or on beads previously coated with anti-CD3 mAb. Butanedione monoxide (BDM; Calbiochem) was added to a final concentration of 20 μM, because this concentration does not significantly inhibit IL-2 secretion by Jurkat cells. IL-2 levels in the conditioned media were assayed by ELISA (Genzyme, Cambridge, MA).

**Cell stimulation and assessment of tyrosine phosphorylation**

For tyrosine phosphorylation analysis, $2 \times 10^6$ Jurkat cells were stimulated with anti-CD3 mAb (2 μg/ml) for 5 min at 37°C in 0.5 ml culture medium. In other experiments, protein A-Sepharose beads were sequentially coated with anti-CD3 mAb (OKT3 at 10 μg/ml) followed by anti-CD28 mAb (clone 9.3 at 10 μg/ml). These beads were combined with Jurkat cells and centrifuged, incubated for 5 min at 37°C, and then the selective inhibitor of the src family tyrosine kinases PP2 (10 μM; Calbiochem) was added for varying incubation periods. Harvested cells were lysed for 30 min on ice in Nonidet P-40 lysis buffer (15). Western immunoblotting was performed as described (7) using the antiphosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY).

**Results**

In a previous study using FITC-conjugated, periplasmically produced (and hence nonglycosylated) PP14 as a probe, we were unable to detect a PP14 receptor on peripheral T cells (16). Consequently, we turned to PP14-Fcγ1, which was produced in a glycosylated form by a mammalian expression system. The binding of PP14-Fcγ1 to the T cell surface was detected by flow cytometry using anti-human IgG, PP14-Fcγ1, but not CTLA-4-Fcγ1 (as a negative control for nonspecific Fc-mediated interactions), bound to purified CD4+ T cells, establishing the presence of a surface receptor for PP14. This binding was specific, because it could be abrogated by preincubating the cells with AF as a rich source of native PP14 for competitive blockade (Fig. 1A, left). Similar results were obtained with Jurkat cells (Fig. 1A, right); receptor binding was also documented for these cells using radio-labeled 125I-labeled PP14-Fcγ1 as a probe (data not shown). Thus, specific binding of PP14 to T cells can be detected.

Next, we used immunofluorescence microscopy to localize PP14 during APC-T cell interactions. APC-T cell conjugates were formed by mixing purified CD4+ T cells with monocytes that had been previously pulsed with the superantigen SEB. Cells were incubated for 5–90 min at 37°C, and conjugates were stained for PP14. Talin translocation, a hallmark of Ag-specific conjugate formation (17), was used in parallel experiments to confirm APC-T cell contact site formation under these conditions. Although PP14 was distributed in a continuous ring on the cell surface of unbound T cells (Fig. 1Bb), it was markedly enriched within the interaction sites of APC-T cell conjugates (Fig. 1Bd). A high density of PP14 epitopes was observed in the APC-T cell contact sites of 67% ± 11.6% (±SD; from three separate experiments) of those T cells forming conjugates, which comprised 10–20% of the total T cells added to the assay. Interestingly, the enrichment of PP14 in contact sites was observed as early as 5 min following conjugate formation. In the absence of Ag, few cell conjugates were formed; none of them clustered talin (data not shown; Refs. 12 and 18), and PP14 staining was uniformly distributed on the T cell surface, as seen in the unbound T cells in Fig. 1Bb. These data demonstrate that in conjunction with APC-T cell conjugate formation, cell surface PP14 is polarized toward the contact site.

In parallel experiments, we tested whether PP14 can inhibit T cell activation when the T cells are stimulated under the conditions that allowed demonstration of the translocation of PP14 to contact sites. T cells were induced to secrete IL-2 by mixing them with SEB-pulsed monocytes, as in Fig. 1B, in the absence or presence of PP14-Fcγ1 or first-trimester AF, which was used as a natural source of PP14. Both PP14-Fcγ1 and AF inhibited IL-2 secretion by the T cells (Fig. 2A). The AF inhibitory activity was PP14-dependent because it could be abrogated by depleting the AF of its PP14. Similar results were obtained when T cells or Jurkats were stimulated with PHA instead of SEB (Fig. 2B).
With evidence for PP14 enrichment at contact sites, several experiments were performed to evaluate its possible functional significance. First, we asked whether PP14 function requires its direct access to the triggered TCR complex. Using a dual-chamber culture system devised by Poo et al. (19), Jurkat cells were forced into the pores of 2-μm nucleopore membranes within Swinnex filter holders. The anchored cells in this system hydrostatically seal the membranes, leaving their cell membranes regionally exposed to both upper and lower chambers of the filter holders. PHA was chosen as a stimulus for these anchored Jurkat cells, since this T cell activator can, in its soluble form and on its own, stimulate IL-2 secretion. First-trimester AF was used as a natural source of PP14,

FIGURE 1. PP14 · Fcγ binds to T cells and translocates toward APC-T cell interaction sites following conjugate formation. A, Purified T cells (left) or Jurkat cells (right) were incubated with PP14 · Fcγ at 37°C for 30 min. For purposes of competitive inhibition, cells were preincubated with AF (50% v/v) at 37°C for 20 min before the addition of PP14 · Fcγ. CTLA-4 · Fcγ was used as a negative control to measure nonspecific binding. The cells were prepared and labeled as described in Materials and Methods, and 1 × 10⁶ cells were analyzed by FACS to detect bound protein. B, Purified T cells and SEB-pulsed monocytes were allowed to form conjugates in the presence of PP14 · Fcγ, fixed, and stained for PP14. The cells were analyzed by immunofluorescence confocal microscopy. a and b, Anti-PP14 · Fcγ immunostaining of T cells demonstrating that PP14 is uniformly distributed on the cell surface. c and d, An example of an APC-T cell conjugate in which PP14 accumulation is detected at the contact site. Differential interference contrast images of T cells or a conjugate formed between a monocyte (lower cell) and a T cell (upper cell) are shown in a and c, respectively. Fluorescence images of the same cells are shown upon staining with anti-human Ig to detect PP14 · Fcγ in b and d.

FIGURE 2. PP14 inhibits IL-2 secretion by SEB- and PHA-stimulated T cells and accounts for the inhibitory activity of AF. A, Peripheral T cells were stimulated with SEB-pulsed monocytes in the presence or absence of either PP14 · Fcγ (50 μg/ml; left) or AF and PP14-depleted AF (25% v/v; right). B, T cells in the presence of monocytes (left) or Jurkats (right) were stimulated with PHA at the indicated concentrations, in the absence or presence of AF or PP14-depleted AF (25% v/v). IL-2 was assayed by ELISA in the conditioned medium. The data represent the mean of triplicate samples.
supported by the previous demonstration that PP14 accounts for AF’s T cell inhibitory activity (Fig. 2; Refs. 7 and 8). For this experiment, AF was either combined with PHA in the same chamber, or alternatively, the AF and PHA were added on opposite sides of the Jurkat-sealed membranes. AF-mediated inhibition of IL-2 secretion by Jurkat cells was evident only when the AF and PHA (at concentration of both 1 and 2 μg/ml) were added to the same chamber (Fig. 3A). Hence, under activation conditions that maximize the generation of T cell polarity, the PP14 inhibitor and the PHA activator must be in physical proximity to each other.

The requirement for physical proximity between activator and inhibitor was further addressed by attaching anti-CD3 mAb (OKT3) as activator, and a chimeric PP14·Fcγ1 fusion protein as inhibitor, to either the same or separate protein A precoated beads. Significantly, potent T cell inhibitory activity, as measured by decreases in IL-2 secretion from Jurkats, was observed when anti-CD3 mAb and PP14·Fcγ1 were colocalized on the same beads (Fig. 3B). The level of inhibition associated with the immobilized PP14·Fcγ1 was considerably greater than that for the soluble form. In contrast, no inhibition was observed when anti-CD3 mAb and PP14·Fcγ1 were anchored on separate beads (Fig. 3B). The level of inhibition associated with the immobilized PP14·Fcγ1 was considerably greater than that for the soluble form. In contrast, no inhibition was observed when anti-CD3 mAb and PP14·Fcγ1 were anchored on separate beads (Fig. 3B), conditions under which PP14·Fcγ1 cannot readily access triggered TCRs. These findings provide additional support for the dependence of PP14-mediated T cell inhibition on the proximity of PP14 to the triggered TCR.

Clustering of receptors at contact sites is actively driven by the T cell cytoskeleton and depends upon myosin motor proteins (10). Although the inhibition of the myosin motors with BDM interferes with the activation of naive T cells (Ref. 10 and data not shown), in our hands, a low concentration of BDM (20 μM) did not significantly inhibit the capacity of Jurkat cells to secrete IL-2. However, this concentration of BDM significantly interfered with AF-mediated inhibition of IL-2 secretion from Jurkats triggered with either PHA or immobilized anti-CD3 (Fig. 3C). Several additional BDM findings supported the notion that the inhibitory effect of PP14 requires cytoskeleton-dependent PP14 transit to surface sites where TCR triggering transpires. First, BDM’s interference with AF-mediated inhibition could be attenuated by adding more AF (data not shown). Second, as expected, BDM treatment had no effect on inhibitory activity when PP14·Fcγ1 and anti-CD3 mAb were coinmobilized on the same beads (Fig. 3C). In this setting, the need for myosin motor proteins to drive colocalization is obviated. Third, similar results were obtained when taxol, an antitumor agent that binds β-tubulin and locks the microtubule apparatus, was substituted for BDM (Fig. 3D). Thus, our data indicate that PP14 requires access to the triggered TCR site to implement its inhibitory effects on T cell activation.

Active accumulation of molecules at the interface of the T cell and the APC serves to increase the overall amplitude and sustain

![FIGURE 3. PP14 requires access to sites of TCR triggering for T cell inhibition. A. Jurkat cells were stimulated with PHA in a two-chamber system in which these cells serve to seal the intervening membrane (19). AF (25% v/v) was added either to the same (S), or the opposite (O), chambers containing the PHA stimulus. B. Jurkat cells were stimulated with beads coated with anti-CD3 mAb alone, in the presence of soluble PP14·Fcγ1 (80 μg/ml); with beads coated with both anti-CD3 mAb and PP14·Fcγ1 (25 μg/ml); or with a mixture of beads separately coated with anti-CD3 mAb and with PP14·Fcγ1 (25 μg/ml). C. Jurkat cells were cultured in the absence or presence of BDM (20 μM) and stimulated with either PHA or with beads coated with anti-CD3 mAb, in the absence or presence of AF (25% v/v), or with beads bearing coinmobilized PP14·Fcγ1 and anti-CD3 mAb. D. Jurkat cells were stimulated with PHA (5 μg/ml) in the absence or presence of taxol (50 and 800 nM), with or without AF (25% v/v). The level of IL-2 in 24-h conditioned media was determined by ELISA. Results in B–D are presented as a percentage of inhibition of IL-2 secretion. The data are from one experiment; similar results were obtained in two other experiments.](http://www.jimmunol.org/)

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T cell signaling (10). Given the evidence that PP14 targets proximal events during TCR signaling (7), the accumulation of PP14 in the contact site could result in reduced levels of receptor signaling. Therefore, the effect of PP14 on tyrosine phosphorylation, an essential element in early T cell activation, was evaluated. Jurkats were stimulated with soluble anti-CD3 mAb, and the impact of AF on the induction of tyrosine phosphorylation events was assessed using antiphosphotyrosine mAb. Although AF did not significantly perturb the overall pattern of tyrosine phosphorylation in these cells, some differences in band intensities were noted (Fig. 4A).

Given that tyrosine phosphorylation is rapidly lost once TCR triggering is terminated, effective T cell activation requires sustained TCR engagement or mechanisms that prolong the phosphorylated state, for example, costimulation through CD28 (20). Along these lines, it is possible that at least some T cell inhibitors might function by shortening the phosphorylated state. Because we previously demonstrated that B7-1 costimulation counters PP14 inhibition (8), we now evaluated the possibility that PP14 impedes TCR signaling by shortening the phosphorylated state, rather than by inhibiting tyrosine kinase activity. Jurkat cells were stimulated with anti-CD3 and anti-CD28 mAb coimmobilized on beads, in the absence or presence of AF, and the extent of residual tyrosine phosphorylation was measured at varying times after the addition of the tyrosine kinase inhibitor PP2 (Fig. 4B). After the addition of PP2, tyrosine phosphorylation persisted for >1 min, as previously reported (20). In contrast, in the presence of AF, accelerated substrate dephosphorylation was elicited for multiple molecular species (Fig. 4, B and C). There is some degree of specificity in this dephosphorylation effect, given that phosphorylation of the 70-kDa band is retained despite AF treatment (Fig. 4B). Interestingly, this same 70-kDa band appears to be the only one that persists when resting T cells are triggered with anti-CD3 mAb in the absence of costimulation (20). In aggregate, these results suggest that PP14 functions within sites of TCR triggering, where it reduces the half-life of TCR-induced tyrosine phosphates by augmenting their dephosphorylation.

Discussion
PP14 is one of a relatively limited set of immunoregulatory proteins known to target T cell directly. Previous evidence has suggested that PP14 acts on an early step of T cell activation to somehow desensitize TCR signaling (7). We propose here that PP14 inhibits events in the TCR signaling pathway, and to accomplish this, PP14 must be recruited to the APC-T cell contact site and be in proximity to the triggered TCR. The specific findings supporting this hypothesis include: 1) PP14 translocates and accumulates at the APC-T cell interface; 2) in a Jurkat cell-sealed two-chamber system, PP14 and TCR stimulus (PHA) must be in the same chamber for PP14-mediated inhibition to be evident; 3) in bead immobilization experiments, PP14 and TCR stimulus (anti-CD3 mAb) must be coimmobilized on the same beads to observe PP14-mediated inhibition; and 4) functional cytoskeletal myosin motor proteins are required for PP14 inhibition, presumably enabling bound PP14 to access critical sites at the cell surface.

The confocal microscopic analysis, focusing on PP14’s transit to the APC-T cell interface, as well as the dual-chamber bead coimmobilization and cytoskeletal inhibitor experiments, together raise the intriguing possibility that an exogenously added soluble inhibitor (such as PP14) may function by transiting to the immune synapse. Hence, not only proteins known to promote activation, but also inhibitory proteins may segregate into functional sites at the APC-T cell interface. This broader effect on multiple membrane proteins is a consequence of the nonspecific nature of cytoskeleton-driven molecular clustering at the T cell surface (10). It is likely that PP14’s translocation to the contact site is an early event during activation of the T cell because a significant PP14 accumulation in the contact site is already seen by 5 min following stimulation.

The explanation for the inhibitory effect of PP14 on TCR signaling is supported by our demonstration that it reduces the half-life of TCR-induced tyrosine phosphates via augmenting their dephosphorylation. In view of these findings, it is tempting to speculate that PP14 exerts its immunoregulatory effects on T cells by entering contact sites and altering the organization of the immune synapse. Activation of T cells by APC is restricted to their site of contact, where receptors on the T cells engage their counter receptors on the APCs. The formation of the immune synapse serves to facilitate and stabilize TCR signaling (10, 20), probably by excluding phosphatases from the contact site. The expected outcome of PP14’s recruitment to the contact site might be to physically disrupt this functional site and to alter the balance between protein tyrosine kinases and phosphatases within them, thereby negatively regulating proximal signaling processes. This type of contact site-directed inhibitory mechanism may be relevant to other negative T cell regulators as well, such as galecitin-3 (21).

This model unifies positive and negative signals within a single mechanistic framework, whereby the immune synapse functions to dynamically integrate positive and negative inputs into a finely tuned T cell response. Systematic confocal microscopic analysis of the effect of PP14 on the segregation of various molecules during
contact site formation, as well as its effect on the duration of TCR-evoked signaling, should serve to substantiate the proposed model for the mode of action of PP14.

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