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CXCR4 Physically Associates with the T Cell Receptor to Signal in T Cells

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Summary

SDF-1α (CXCL12) signaling via its receptor, CXCR4, stimulates T cell chemotaxis and gene expression. The ZAP-70 tyrosine kinase critically mediates SDF-1α-dependent migration and prolonged ERK mitogen-activated protein (MAP) kinase activation in T cells. However, the molecular mechanism by which CXCR4 or other G protein-coupled receptors activate ZAP-70 has not been characterized. Here we show that SDF-1α stimulates the physical association of CXCR4 and the T cell receptor (TCR) and utilizes the ZAP-70 binding ITAM domains of the TCR for signal transduction. This pathway is responsible for several of the effects of SDF-1α on T cells, including prolonged ERK MAP kinase activity, increased intracellular calcium ion concentrations, robust AP-1 transcriptional activity, and SDF-1α costimulation of cytokine secretion. These results suggest new paradigms for understanding the effects of SDF-1α and other chemokines on immunity.

Introduction

Stromal cell-derived factor-1α (SDF-1α; CXCL12) is a member of the superfamily of chemokines that regulates the growth, survival, adherence, and migration of multiple cell types (Ansel and Cyster, 2001; Campbell and Butcher, 2000). CXCR4, the receptor for SDF-1α, is expressed by most T cell subsets. SDF-1α signaling via CXCR4 results in T cell adhesion, chemotaxis, and gene expression important for regulating cell-cycle progression and apoptosis (Thelen, 2001; Suzuki et al., 2001). Evidence suggests that SDF-1α signaling via CXCR4 modulates lymphocyte development (Zou et al., 1998; Hernandez-Lopez et al., 2002), homeostasis (Sawada et al., 1998), and human immunodeficiency virus type-1 (HIV-1) pathology; however, the molecular mechanisms of CXCR4 signaling in T cells are incompletely understood.

Like other chemokine receptors, CXCR4 is a member of the G protein-coupled receptor (GPCR) superfamily and consists of a single polypeptide that spans the membrane seven times. Chemokine receptors signal by activating Gi-type and other G proteins (Thelen, 2001). SDF-1α treatment activates multiple downstream signaling pathways in T cells (Cherla and Ganju, 2001; Chernock et al., 2001; Soede et al., 2001; Ganju et al., 1998; Vicente-Manzanares et al., 1999; Sotsios et al., 1999). In particular, SDF-1α signaling via CXCR4 in T cells results in prolonged activation of the extracellular signal-regulated kinases, ERK-1 and ERK-2 (ERK) (Tilton et al., 2000; Kremer et al., 2003). Prolonged ERK activation critically induces gene expression in other cell types (Murphy et al., 2002), and SDF-1α-mediated ERK pathway activation induces multiple genes that regulate T cell signal transduction, adhesion, cell shape, DNA repair, and apoptosis (Suzuki et al., 2001). We have shown that SDF-1α signaling via CXCR4 in T lymphocytes utilizes both the tyrosine kinase, ZAP-70, and the scaffold molecule, SLP-76, to stimulate prolonged ERK activation in T cells (Kremer et al., 2003). Others have shown that ZAP-70 enhances SDF-1α-induced T cell migration (Ottoson et al., 2001; Soede et al., 1998; Ticchioni et al., 2002). However, the molecular mechanisms that allow CXCR4 to signal via ZAP-70 are not clear. The two amino-terminal SH2 domains of ZAP-70 bind the dually tyrosine phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) domains of ITAM-containing receptors (Hatada et al., 1995; Cambier, 1995). In T cells, the primary ITAM-containing receptor is the T lymphocyte antigen receptor (TCR). Ligation of the TCR by a strong agonist (either TCR mAbs or cognate peptide bound to MHC class I or II proteins) induces tyrosine phosphorylation of ITAM domains of the ε, δ, γ, and ζ TCR subunits. Phosphorylated ITAM domains recruit ZAP-70 (Iwashima et al., 1994), which mediates signal transduction by phosphorylating downstream substrates including SLP-76 and by acting as a scaffold to nucleate signaling proteins (Kane et al., 2000).

We hypothesized that crosstalk between CXCR4 and the TCR might permit CXCR4 to utilize ZAP-70 for signal transduction. Here we show that in response to SDF-1α, CXCR4 closely associates with the TCR and utilizes TCR ITAM domains for signal transduction. Structural characterization of the ITAM domain elements suggests that the ITAM domain mediates CXCR4 signaling by binding ZAP-70. This signal transduction pathway contributes to the physiologically important actions of SDF-1α on T cells, substantially enhancing the stimulation of AP-1-dependent transcriptional activity and the costimulation of TCR-stimulated AP-1 activity and cytokine secretion. These results increase understanding of the molecular mechanisms responsible for CXCR4 signal transduction in T cells and suggest potential new roles for ITAM-containing molecules throughout the immune system as mediators of CXCR4 signaling.

Results

SDF-1α Treatment of T Cells Induces the Close, Physical Association of CXCR4 and the TCR

To address the possibility that the TCR participates in CXCR4 signal transduction, we first used confocal microscopy to visualize Jurkat T cells expressing fluorescent fusion proteins of CXCR4 and the TCR-CD3-ζ subunit. CXCR4 and TCR-CD3-ζ fusion proteins were

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primarily expressed on the cell surface of unstimulated cells (Figure 1A). SDF-1α treatment redistributed CXCR4 from the cell surface into intracellular compartments (Figure 1A). Another chemokine, MIP-3β (also known as CCL19, a ligand for CCR7), did not induce this response (Figure 1A). SDF-1α induced a portion of TCR-CD3-ζ fusion proteins to move and colocalize with CXCR4 fusion proteins, both on the cell surface and within small vesicles resembling endosomes (Figure 1A). Similar results were obtained with normal, human, peripheral blood T cells (PBMC T cells) (data not shown). Direct stimulation of the TCR via mAb (OKT3) did not alter the subcellular localization of CXCR4 (Figure 1A). TCR-CD3-ε fusion proteins also colocalized with CXCR4 in response to SDF-1α stimulation (data not shown). Coimmunoprecipitation further indicated that CXCR4-YFP copurifies with the TCR-CD3-ζ subunit (Figure 1B).

To obtain better resolution of the proximity of CXCR4 and the TCR in living cells, we utilized two different fluorescence resonance energy transfer (FRET) methods. FRET occurs when specific pairs of chromophores come within approximately 10 nm of each other (Stryer, 1978). First, we assayed FRET between fluorescent fusion proteins of CXCR4 and TCR-CD3-ζ (Figure 1C). Jurkat T cells expressing both fusion proteins were placed in a cuvette and excited with 433 nm light, and the emission spectrum was recorded before (Figure 1D, blue line) and after (Figure 1D, green line) stimulation with SDF-1α for 30 min. The reciprocal changes in the YFP emission peak (528 nm) versus the CFP emission peak (475 nm) indicated that SDF-1α treatment induced FRET between the fusion proteins. Confocal microscopy similarly showed that FRET developed in response to SDF-1α treatment (Figure 1E). For the second FRET method, we assayed FRET between endogenous, cell-surface CXCR4 and TCR molecules that were fluorescently labeled via mAbs (Figure 1F). PBMC T cells were labeled with receptor-specific mAbs, stimulated with SDF-1α, and assayed for FRET with a flow cytometer. SDF-1α treatment increased per cell FRET fluorescence, as shown by the increase in FL3 channel fluorescence (Figure 1G). SDF-1α also increased the per cell FRET fluorescence of Jurkat T cells assayed in a similar manner (see Figure S2 in the Supplemental Data available with this article online). The SDF-1α-induced CXCR4-TCR FRET signals gradually increased with time after SDF-1α addition and plateaued after approximately 20 min, were sensitive to either low temperature or a CXCR4 antagonist, and displayed dose-response relationships for both Jurkat and PBMC T cells, consistent with SDF-1α acting by binding to CXCR4 (Figures S1 and S2). No CXCR4-TCR FRET signals were induced when cells were stimulated with MIP-3β (data not shown). As a control, we confirmed that cells labeled with either phycoerythrin (PE) or allophycocyanin (APC) alone did not show FRET signals, either before or after SDF-1α treatment (data not shown). As another control, we confirmed that FRET signals were undetectable when a similar approach was used to assay the proximity of CXCR4 to another abundantly expressed cell-surface receptor, CD45 (Figure 1H). Similarly, no FRET signals were detected with this approach to assay the proximity of the TCR to another chemokine receptor, CCR7 (data not shown). Together, the results in this section indicate that SDF-1α treatment causes CXCR4 and the TCR to move into close, physical proximity in both normal human T cells and the Jurkat T cell line.

Characterization of the CXCR4-TCR Association Induced by SDF-1α

To address the proportion of the CXCR4-TCR complexes induced by SDF-1α that were located on the cell surface versus intracellularly (e.g., after endocytosis), we used an acid wash to remove cell-surface mAbs (and associated FRET signals) from cells treated with SDF-1α to generate FRET signals as in Figures 1F and 1G. The acid wash removed approximately half of the FRET signal associated with the SDF-1α-treated cells, whereas the rest of the FRET signal was insensitive to the acid wash (Figure 2A, bottom). Control experiments confirmed that the acid wash removed the majority of mAbs bound to CXCR4 and TCR-CD3-ε when the receptors were kept on the cell surface by low temperature (Figure 2A, top). Thus, a substantial proportion of the CXCR4-TCR FRET signal that arises in response to subsequent SDF-1α treatment derives from within the cell, presumeably from receptors that have undergone endocytosis. Endocytosis is not by itself sufficient to generate the FRET signals, however. Inhibitors of either actin polymerization (cytochalasin D) or PI3 kinase activity (LY294002) did not prevent SDF-1α-induced CXCR4 endocytosis (data not shown; Francois and Klotman, 2003). These drugs partially inhibited the ability of SDF-1α to induce CXCR4-TCR FRET signals (Figure 2B). The formation of large numbers of CXCR4-TCR complexes might therefore require one or more cellular polarization events that are sensitive to these drugs (Vicente-Manzanares et al., 1999; Sotsios et al., 1999). Finally, we tested the effects of pertussis toxin (PTX) on CXCR4-TCR association. PTX does not inhibit the endocytosis of CXCR4 (Forster et al., 1998); however, several downstream CXCR4 signals are sensitive to PTX. PTX had no detectable effect on the ability of SDF-1α to induce CXCR4-TCR FRET signals (Figure 2C), even though PTX blocked the ability of SDF-1α to stimulate downstream ERK activation (Figure 2D). The results in this section are consistent with SDF-1α treatment bringing CXCR4 into close proximity with the TCR via a cellular polarization event.

SDF-1α Signal Transduction Requires TCR ITAM Domains

We next asked whether SDF-1α signal transduction via CXCR4 was altered by CXCR4 associating with the TCR. The mutant Jurkat T cell line, JRT3, is deficient in expression of the TCR-β subunit and is consequently deficient in cell-surface TCR as compared to the parental Jurkat cell line (Figure 3A, left; Ohashi et al., 1985; Weiss and Stobo, 1984). Nevertheless, JRT3 cells express normal levels of cell-surface CXCR4 (Figure 3A, right). SDF-1α stimulation of T cells normally results in prolonged ERK activation, defined as elevated ERK activity after 8–12 min of SDF-1α stimulation (Figure 3B; Kremer et al., 2003; Tilton et al., 2000). Here we found that TCR-β-deficient cells displayed a substantial defect in SDF-1α-dependent ERK activation at 8 and 10 min (Figure 3B). ERK activation in TCR-β-deficient Jurkat T cells after 8 or 10 min of SDF-1α stimulation was significantly lower than in normal Jurkat T cells (44% ± 15%

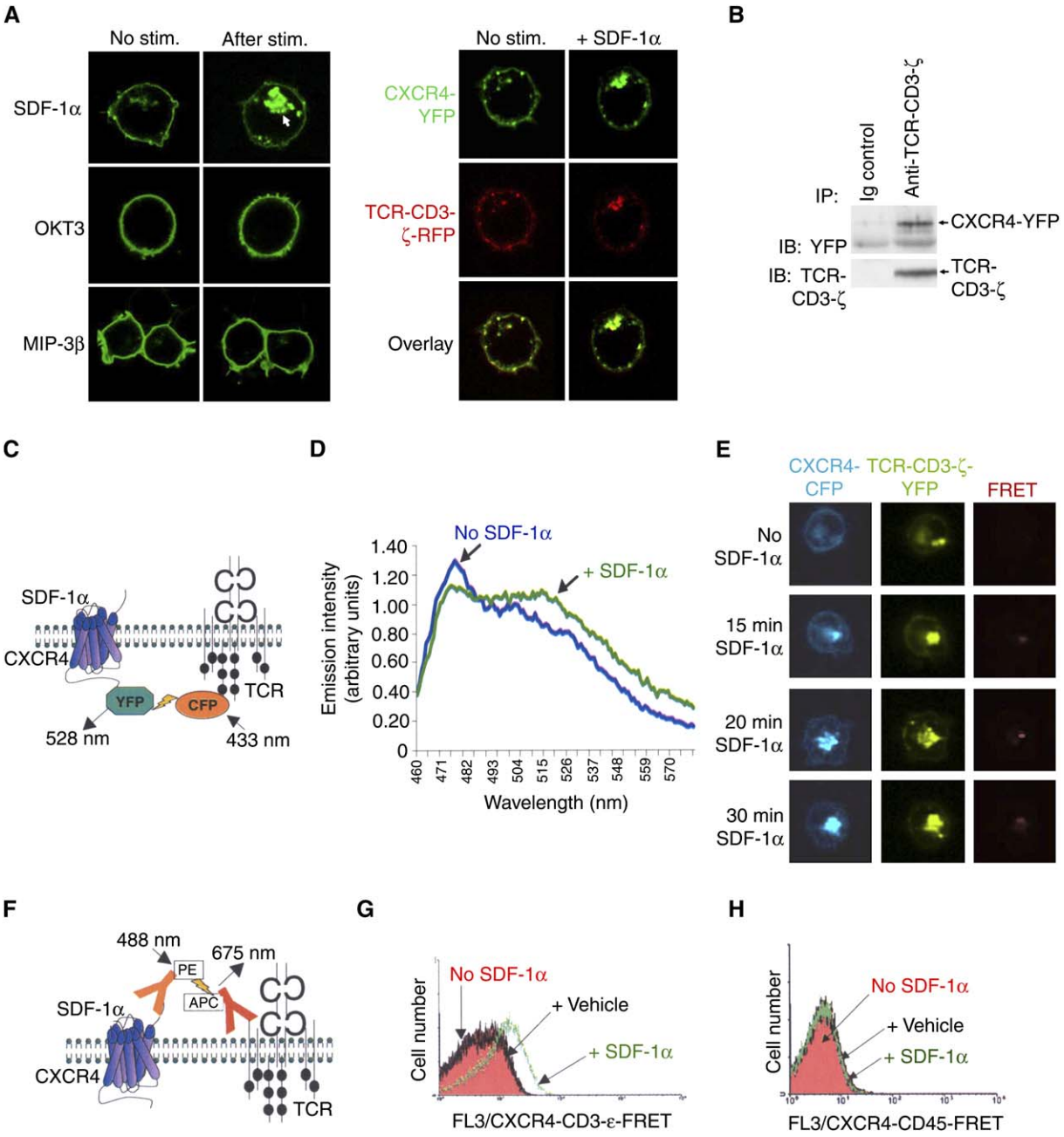

Figure 1. SDF-1α Treatment Induces the Movement and Close, Physical Association of CXCR4 and the TCR

(A) Confocal microscopy of CXCR4-YFP (green) and/or TCR-CD3-ζ-RFP (red) fusion proteins in live, Jurkat T cells. The left panels show cells expressing CXCR4-YFP before and after 30 min stimulation with SDF-1α, TCR-CD3 mAb (OKT3), or MIP-3β. The arrowhead denotes intracellular CXCR4-YFP fluorescence. The right panels show a single cell expressing both CXCR4-YFP (green) and TCR-CD3-ζ-RFP (red), before and after 30 min stimulation with SDF-1α.

(B) Jurkat T cells expressing CXCR4-YFP were stimulated with SDF-1α for 20 min. TCR-CD3-ζ was immunoprecipitated with either specific (right lane) or control (left lane) immunoglobulin. Copurifying CXCR4-YFP was detected by immunoblotting with anti-YFP (upper gel). As a control, the same blot was stripped and reprobed with anti-TCR-CD3-ζ (lower gel). Results are representative of three independent experiments.

(C) Cartoon depicting FRET between fluorescent fusion proteins of CXCR4 and the TCR.

(D) Fluorescence spectrometer assay of CXCR4-TCR FRET in Jurkat T cells via the approach in (C). The fluorescence emission spectra of the same cells in response to 433 nm excitation before (blue line) and after (green line) 30 min of SDF-1α treatment are shown.

(E) Confocal microscope assay of CXCR4-TCR FRET in Jurkat T cells via an approach similar to (C). CXCR4-CFP (blue), TCR-CD3-ζ-YFP (yellow), and FRET fluorescence (red) of a single, live Jurkat T cell before and after SDF-1α treatment are shown.

(F) Cartoon depicting FRET between endogenous CXCR4 and TCR receptors linked to fluorophores via mAbs.

(G) Flow cytometer assay of FRET of endogenous CXCR4 and TCR on normal human T cells via the approach in (F). Both CXCR4-PE and TCR-CD3-ε-APC mAb were bound to the cell-surface CXCR4 and TCR-CD3-ε, and after 20 min stimulation with either SDF-1α or water (vehicle), FRET was assayed by flow cytometry.

(H) No FRET was observed between CXCR4 and CD45 of Jurkat T cells via an approach similar to (F). Results are representative of 3–15 independent experiments.

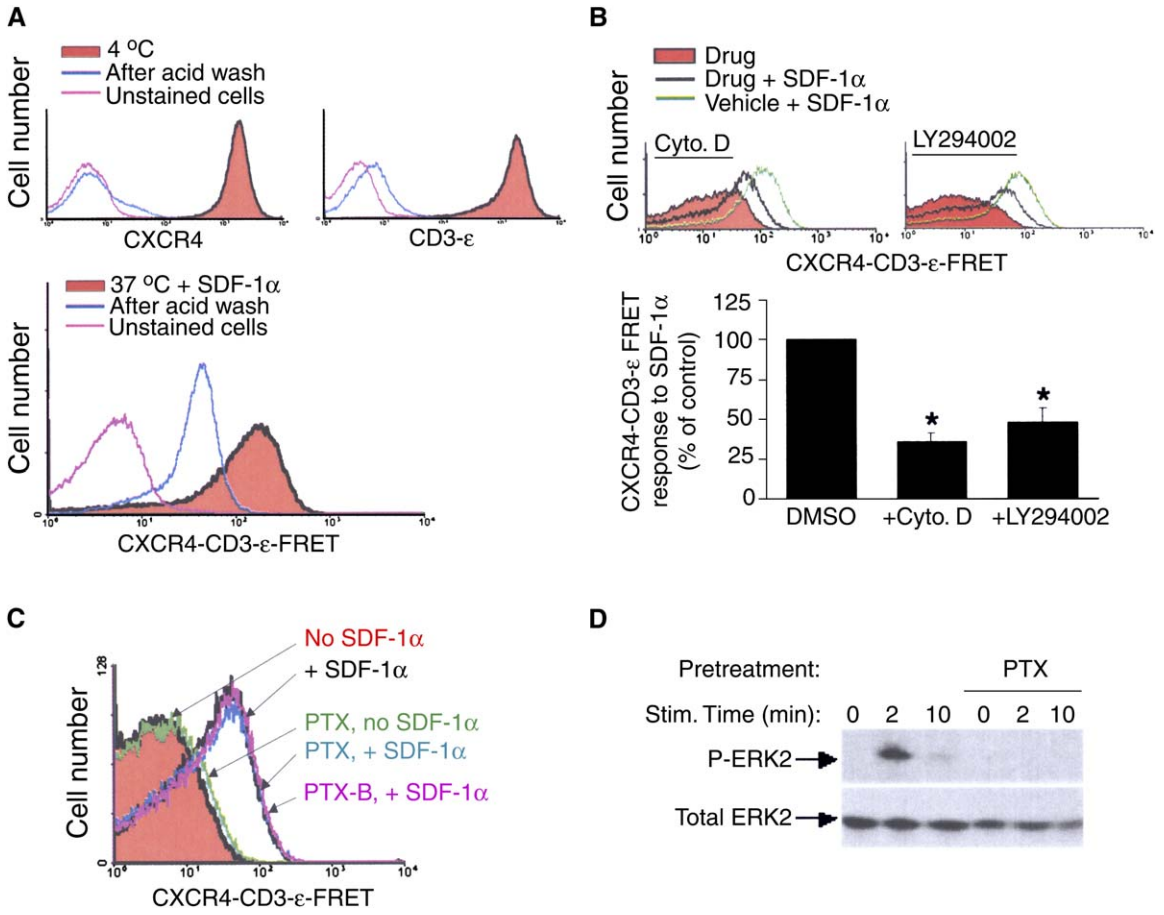

Figure 2. Characterization of the CXCR4-TCR Association Induced by SDF-1α

(A) Top: Results of control samples in which cells were stained with CXCR4-PE and TCR-CD3-ε-APC mAb as in Figure 1F, stimulated with SDF-1α for 30 min and kept at 4°C to prevent receptor endocytosis, and then analyzed for mAb bound to CXCR4 or TCR-CD3-ε before (solid red histograms) and after (blue histograms) an acid wash designed to remove cell-surface mAbs. Bottom: Results of experimental samples in which cells were stained with CXCR4-PE and TCR-CD3-ε-APC mAb as in Figure 1F, stimulated with SDF-1α for 30 min at 37°C, and analyzed for FRET before and after acid wash designed to remove cell-surface mAbs and consequently FRET signals.

(B) Jurkat T cells were pretreated with inhibitors as indicated, and then the CXCR4-TCR FRET of endogenous receptors in response to SDF-1α was assayed as in Figure 1F. Cyto D., cytochalasin D. Histograms show representative results; the bar graph shows the mean SDF-1α-dependent FRET response ± SEM for 3–4 experiments as a % of controls analyzed the same day; *significantly different from controls ($p < 0.05$).

(C) Jurkat T cells were pretreated with either nothing, pertussis toxin (PTX), or an inactive control toxin (PTX-B). The CXCR4-TCR FRET of endogenous receptors in response to SDF-1α was then assayed as in Figure 1F. A representative experiment showing histograms of FRET signals of PTX-treated cells is shown.

(D) PTX-pretreated T cells were stimulated with SDF-1α and assayed for active, phosphorylated ERK by immunoblotting (upper gel). The same blots were stripped and immunoblotted with anti-total ERK2 as a control (lower gel).

of control responses at 8 min, $p = 0.03$, $n = 4$; and 42% ± 9% of control responses at 10 min, $p = 0.01$, $n = 4$). This SDF-1α signaling defect arises from the TCR-β deficiency, because JRT3 cells stably reconstituted with TCR-β (creating the PF2.4 cell line) activated ERK normally in response to SDF-1α (Figures 3A and 3B). Indeed, SDF-1α-dependent ERK activation in the TCR-β-reconstituted PF2.4 cell line was not markedly different from that of normal Jurkat T cells ($p = 0.80$ and $p = 0.63$ for responses at 8 and 10 min, respectively; $n = 4$). Thus, SDF-1α signal transduction resulting in prolonged ERK activation requires expression of TCR-β and correlates with expression of the TCR at the cell surface.

We next asked whether SDF-1α signaling requires the ITAM domains of the TCR. For this purpose, we utilized constructs encoding CD8-CD3-ζ chimeras consisting of the extracellular and transmembrane regions of CD8α fused to the cytoplasmic region of TCR-CD3-ζ, which normally contains three ITAM domains (Figure 3C). Transient expression in TCR-β-deficient cells of CD8-CD3-ζ ("wt" or "wild-type" in Figures 3C–3E) restored SDF-1α-mediated ERK activation (Figures 3D and 3E). In contrast, the CD8-CD3-ζ construct missing most of the TCR-ζ portion including all three ITAM domains ("STOP") failed to rescue SDF-1α-mediated ERK activation (Figure 3E). The CD8-CD3-ζ construct with each ITAMs' critical tyrosines modified by point mutation ("6F") also failed to rescue SDF-1α-mediated ERK activation, a result specifically indicating the critical importance of the ITAM tyrosines in mediating SDF-1α signaling. Because ZAP-70 binds to ITAM domains only when both tyrosines are phosphorylated, we determined whether both ITAM tyrosines were required for SDF-1α signaling. SDF-1α-mediated ERK activation was

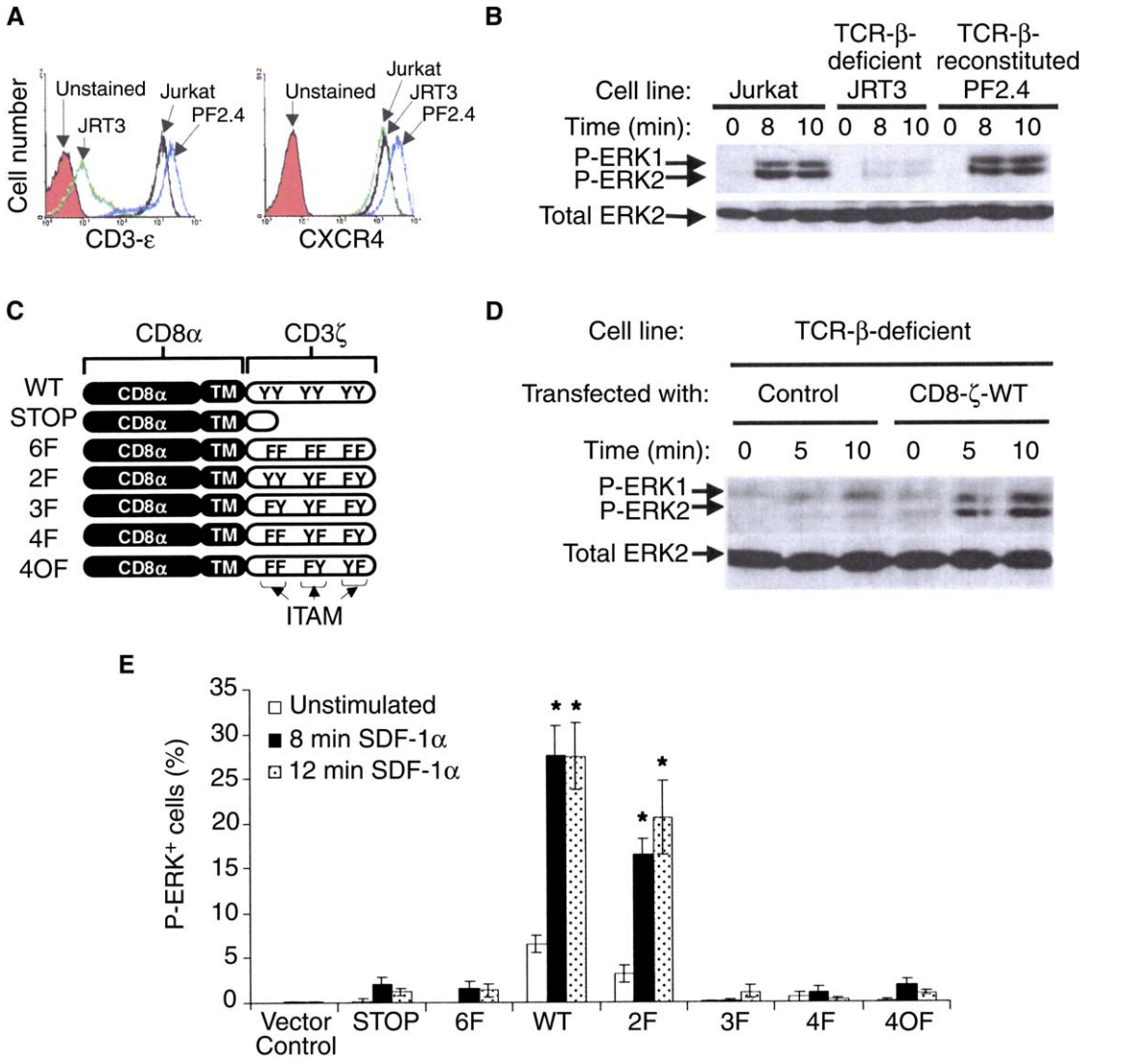

Figure 3. SDF-1α Uses TCR ITAM Domains to Stimulate Prolonged ERK Activation

(A) Flow cytometric analyses of the cell-surface expression of TCR-CD3-ε and CXCR4 on the Jurkat T cell line, the TCR-β-deficient JRT3 cell line, and JRT3 stably reconstituted with TCR-β (PF2.4).

(B) The indicated cell lines were stimulated with SDF-1α, then lysed and assayed for active ERK as in Figure 2D. Results are representative of four independent experiments.

(C) Cartoon of CD8-CD3-ζ fusion proteins showing the CD8α extracellular domain (black), the CD8α transmembrane region (TM), and TCR-ζ cytoplasmic domains that are either wild-type (wt), truncated (STOP), or with ITAM tyrosines (Y) mutated to phenylalanines (F). Each YY denotes one ITAM domain.

(D) TCR-β-deficient (JRT3) Jurkat T cells were transiently transfected with the indicated plasmids, stimulated with SDF-1α, and then assayed for active ERK as in Figure 2D.

(E) TCR-β-deficient (JRT3) Jurkat T cells were transiently transfected with the indicated plasmids, stimulated with SDF-1α, and then assayed for active ERK by flow cytometry. Each bar shows the mean % of CD8-CD3-ζ-expressing cells with elevated phosphorylated ERK in response to SDF-1α ± SEM for six independent experiments; *significantly different from vector control or STOP construct results ($p < 0.05$).

defective in cells reconstituted with "3F," "4F," or "4OF" constructs, all of which have all three of their TCR-ζ ITAM domains mutated (Figure 3E). In contrast, the "2F" construct, which has one intact ITAM domain, restored SDF-1α-mediated ERK activation in a manner indistinguishable from the wt CD8-ζ chimeric protein ($p \geq 0.5$). Together, these results indicate that SDF-1α signaling leading to prolonged ERK activation requires expression of the TCR and characterize structural features of the ITAM domain that are required for this novel signal transduction pathway.

SDF-1α Stimulation of Robust Ras Activation Requires Both the TCR and ZAP-70

We previously showed that SDF-1α signaling leading to prolonged ERK activation requires the expression of ZAP-70 (Figure 4A; Kremer et al., 2003). To gain further insight into this signaling pathway, we examined the role of Ras. SDF-1α treatment of both Jurkat and PBMC T cells stimulated similar amounts of active, GTP bound Ras (Figure 4B). SDF-1α-dependent Ras activation was deficient in TCR-β-deficient cells as compared to either wild-type Jurkat cells or TCR-β-reconstituted

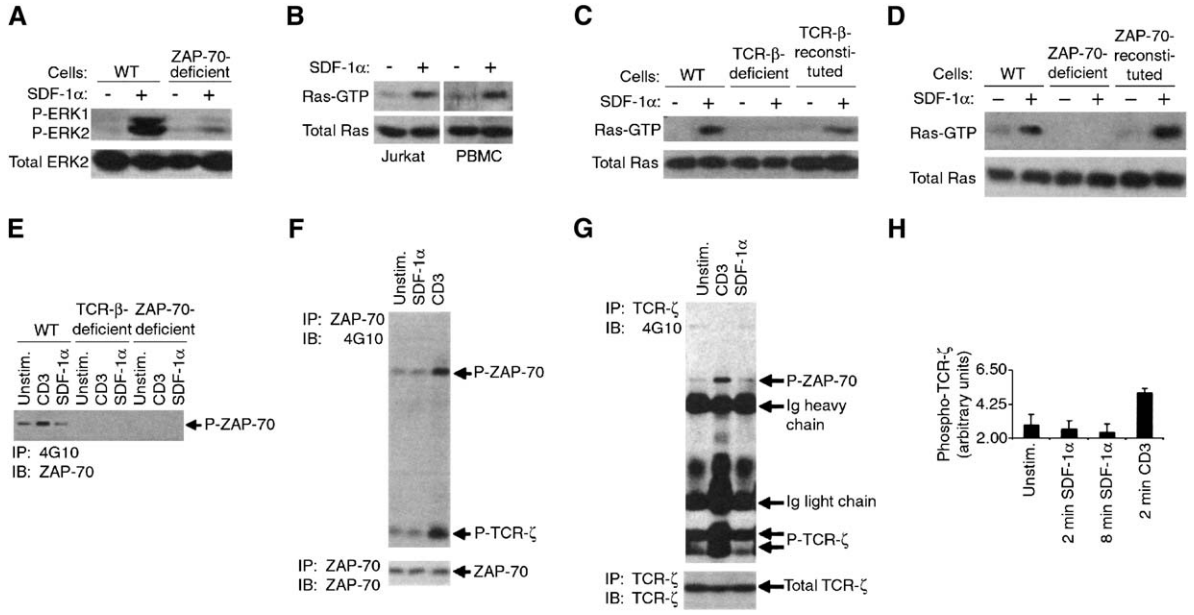

Figure 4. Constitutively Tyrosine Phosphorylated TCR and ZAP-70 Mediate Robust Ras Activation in Response to SDF-1α

(A) Wild-type (wt) Jurkat T cells or ZAP-70-deficient (P116) Jurkat T cells were stimulated for 8 min with SDF-1α, then assayed for active ERK as in Figure 2D.

(B) Wild-type Jurkat T cells or PBMC T cells were stimulated for 8–10 min with SDF-1α, and then active, GTP bound Ras (RAS-GTP) was detected by affinity purification and immunoblotting with anti-Ras (upper gel). As a control, whole-cell lysates were immunoblotted for total Ras (lower gel).

(C and D) Ras activation of the indicated cell lines, determined as in (B).

(E) The indicated cell lines were stimulated for 2 min with either crosslinked TCR mAb (CD3) or SDF-1α, and then phospho-tyrosine-containing proteins were immunoprecipitated with 4G10 mAb and immunoblotted with anti-ZAP-70.

(F) Jurkat T cells were stimulated as in (E), and then ZAP-70 was immunoprecipitated and immunoblotted with anti-phosphotyrosine (4G10; upper gel) or anti-ZAP-70 (lower gel).

(G) Jurkat T cells were stimulated as in (E), and then TCR-ζ was immunoprecipitated and immunoblotted with anti-phosphotyrosine (4G10; upper gel) or anti-TCR-ζ (lower gel).

(H) Densitometric quantitation of three independent experiments as in (G) ± SEM. Results in (A)–(H) are representative of 3–6 experiments.

cells (Figure 4C). SDF-1α-mediated Ras activation was also deficient in ZAP-70-deficient Jurkat T cells, and this defect was restored by reconstituting ZAP-70 (Figure 4D). Thus, SDF-1α signaling via the TCR and ZAP-70 enhances Ras activation in addition to prolonging ERK activation.

SDF-1α Signaling Occurs via Constitutively Tyrosine Phosphorylated TCR and ZAP-70

We next asked whether constitutively tyrosine phosphorylated ITAM domains or inducibly tyrosine phosphorylated ITAM domains participate in mediating SDF-1α signal transduction. Constitutively tyrosine phosphorylated ZAP-70 was easily detected in unstimulated, wild-type Jurkat cells (Figure 4E). Constitutively tyrosine phosphorylated ZAP-70 was not seen in unstimulated cells of the TCR-β-deficient cell lines, suggesting that it requires cell-surface TCR expression. SDF-1α treatment failed to increase the tyrosine phosphorylation of ZAP-70. In contrast, inducible tyrosine phosphorylation of ZAP-70 was easily detected after direct ligation of the TCR via specific mAb (CD3) (Figure 4E). SDF-1α also failed to elevate the amounts of ZAP-70 phosphorylated on tyrosine 493, a site that is phosphorylated when the kinase is active (Chan et al., 1995; data not shown). Finally, SDF-1α did not detectably alter tyrosine phosphorylation of either TCR-ζ (Figures 4F–4H) or TCR-ε (data not shown). These results suggest that SDF-1α signal transduction proceeds via constitutively tyrosine phosphorylated, rather than inducibly tyrosine phosphorylated, TCR and ZAP-70.

The TCR and ZAP-70 Are Required for Robust AP-1 Transcriptional Activity and $[Ca^{2+}]_i$ Mobilization

Prolonged ERK activation prolongs the half-life of AP-1 subunits (Murphy et al., 2002). SDF-1α significantly stimulated AP-1-dependent transcriptional activity in Jurkat T cells by approximately 2.5-fold as compared to unstimulated cells ($p < 0.05$) (Figure 5A). This action of SDF-1α was inhibited by PD098059, an inhibitor of MEK-1 and downstream ERK. The TCR-β-deficient Jurkat T cells displayed deficient SDF-1α-mediated activation of AP-1 transcriptional activity, but this was restored to normal amounts after reconstitution with TCR-β ($p \leq 0.01$) (Figure 5B). SDF-1α stimulation of AP-1 transcriptional activity also required ZAP-70 (Figure 5C). These results indicate that SDF-1α signaling via the TCR and ZAP-70 increases AP-1 transcriptional activity. We also asked whether the TCR might participate in SDF-1α-mediated $[Ca^{2+}]_i$ mobilization, because we previously showed that this response requires ZAP-70 (Kremer et al., 2003). The TCR-β-deficient Jurkat T cells displayed deficient SDF-1α-mediated $[Ca^{2+}]_i$ mobilization as compared to the TCR-sufficient parental cell line (Figure 5D). Together, the results in this section demonstrate that SDF-1α signaling via the TCR and ZAP-70 is required

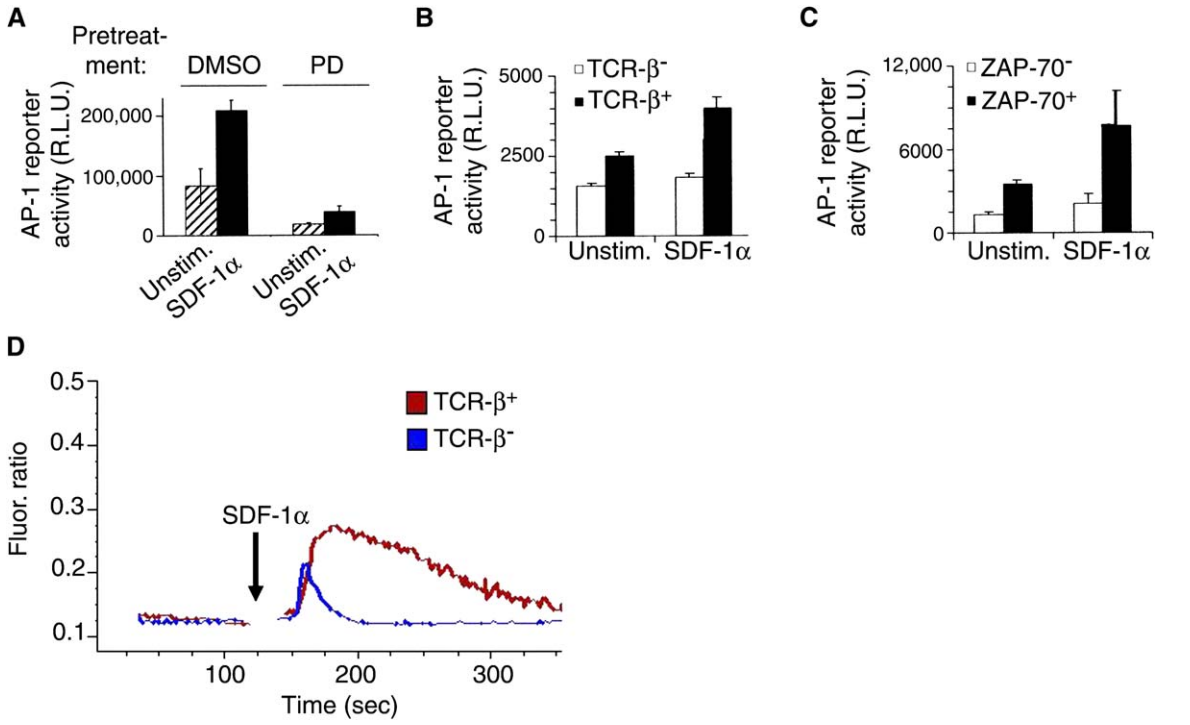

Figure 5. SDF-1α Signaling via the TCR Enhances AP-1 Transcriptional Activation and Ca^{2+} Mobilization

(A) Jurkat T cells were transiently transfected with an AP-1 luciferase reporter construct, pretreated with either vehicle (DMSO) or a MEK-1 inhibitor (PD098059; PD), and stimulated as indicated, and transcriptional activity of AP-1 was assayed by measuring luciferase activity; R.L.U., relative light units ± SD (n = 3).

(B and C) AP-1 transcriptional activities of the indicated cell lines, as determined in (A).

(D) Flow cytometric $[Ca^{2+}]_i$ assays of the indicated cell lines in response to SDF-1α (arrow). Fluor. Ratio, the 405 nm:495 nm fluorescence ratio that correlates with $[Ca^{2+}]_i$. Results are typical of three independent experiments.

both for robust AP-1 transcriptional activity and $[Ca^{2+}]_i$ mobilization.

SDF-1α Costimulation of TCR-Stimulated T Cells Selectively Enhances Expression of CD69, IL-10, and IL-2

SDF-1α is abundant within the lymph nodes (Muller et al., 2001), where the immune activation of naive T cells occurs via ligation of the TCR. We therefore addressed the effects on T cell activation of simultaneously ligating both CXCR4 and the TCR. Costimulation with SDF-1α and CD3 mAb significantly enhanced AP-1-dependent transcription by approximately 7.3-fold as compared to unstimulated cells ($p < 0.05$), a response approximately 2.7-fold higher than that induced by either SDF-1α or CD3 mAb alone (Figure 6A). In contrast to AP-1, neither NF-AT (Figure 6B) nor NF-κB (Figure 6C) -dependent transcriptional activity was affected by either SDF-1α stimulation or costimulation. We next asked whether the selective SDF-1α costimulation of AP-1 regulates downstream gene expression. PBMC T cells markedly elevated their per cell cell-surface expression of CD69 when costimulated by SDF-1α (Figures 6D and 6E). In contrast to its effects on CD69, SDF-1α costimulation had no effect on the cell-surface expression of CD25 (Figure 6F). SDF-1α costimulation increased the proportion of TCR-stimulated T cells expressing IL-10 (Figure 6G), as well as the amount of IL-10 secreted into the supernatants (Figure 6H). SDF-1α costimulation also significantly increased the number of TCR-stimulated T cells expressing IL-2 (Figures 6I and 6J) ($p < 0.05$). In contrast to its effects on IL-10 and IL-2 expression, SDF-1α costimulation failed to consistently increase the expression of IFN-γ by TCR- or TCR+CD28-stimulated PBMC T cells (data not shown). Thus, SDF-1α costimulation selectively enhances TCR-mediated activation of the AP-1 transcription factor and the expression of CD69, IL-2, and IL-10.

Model of SDF-1α Signaling via CXCR4 and the TCR

Based on our results, we propose the model shown in Figure 7. SDF-1α binding to CXCR4 induces its association with the TCR, via a mechanism involving reorganization of the actin cytoskeleton and PI3 kinase activity. Colocalization of the receptors permits CXCR4 to signal via pre-existing, constitutively phosphorylated TCR-ZAP-70 complexes, leading to more robust Ras activation in response to SDF-1α. Both TCR and ZAP-70 are required for SDF-1α to stimulate prolonged ERK activation. Prolonged ERK activation leads to AP-1 transcriptional activation, which mediates gene expression in response to SDF-1α alone and which also mediates SDF-1α's ability to selectively costimulate cytokine gene expression by TCR-stimulated T cells.

Discussion

We describe here close, physical interactions between CXCR4 and the TCR that are formed in response to stimulating CXCR4 with SDF-1α. Two complementary

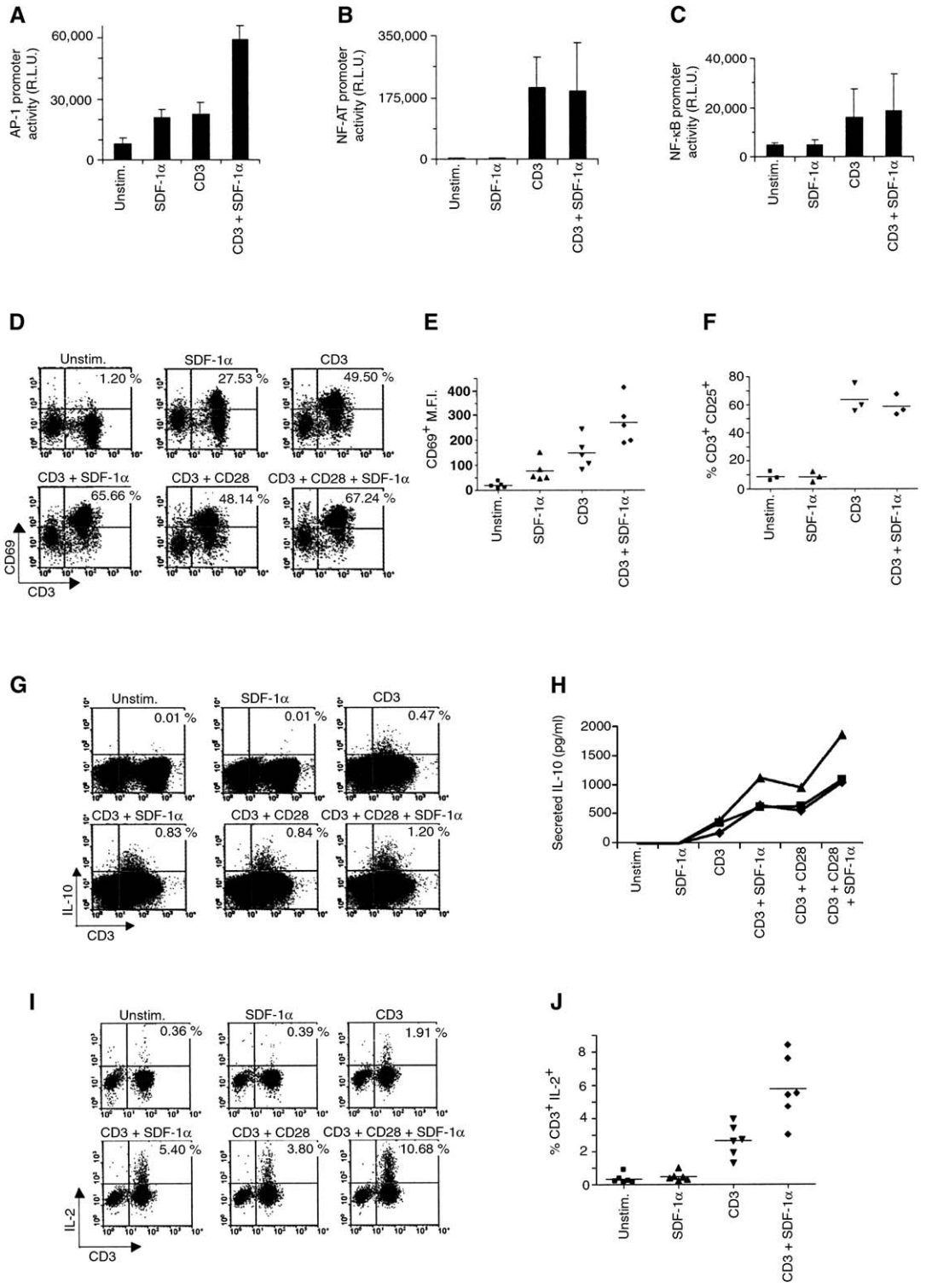

Figure 6. SDF-1α Costimulation of TCR-Stimulated T Cells Selectively Increases AP-1-Dependent Transcriptional Activity and Enhances Expression of CD69, IL-10, and IL-2

(A–C) Jurkat T cells were transiently transfected with the indicated reporter constructs and assayed for luciferase activity as in Figure 5A (n = 3).

(D) PBMC T cells were stimulated in vitro and assayed for CD69 by flow cytometry.

(E) Summary of results as in (D) for five different PBMC donors; M.F.I., mean fluorescent index.

(F) Summary of CD25 expression on human T cells for three different PBMC donors.

(G) PBMC T cells were stimulated in vitro and assayed for IL-10 by intracellular cytokine staining.

(H) Summary of IL-10 secreted by three different PBMC donors.

(I) PBMC T cells were stimulated in vitro and assayed for IL-2 by intracellular cytokine staining.

(J) Summary of IL-2 production for six different PBMC donors.

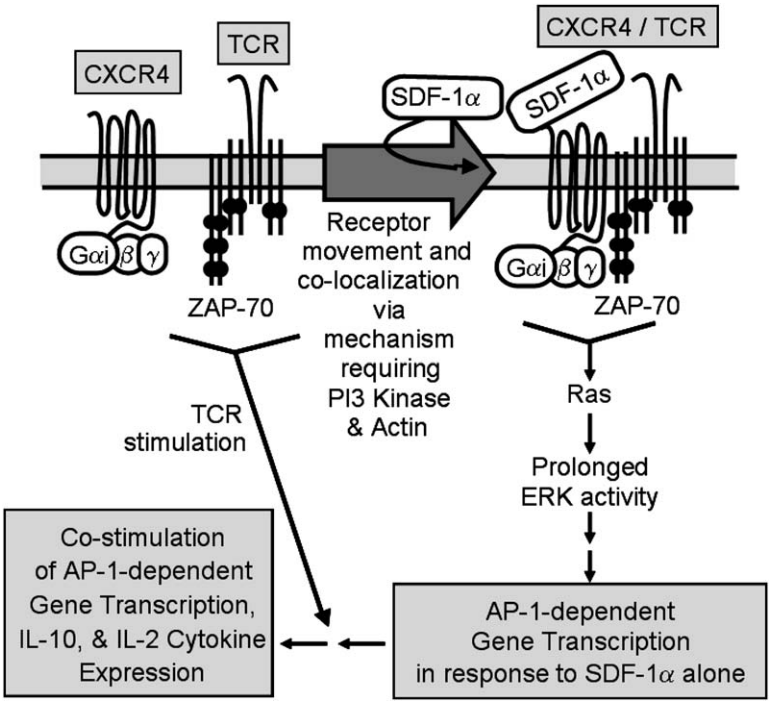

Figure 7. Model of SDF-1α Signaling via CXCR4 and the TCR

SDF-1α binding to CXCR4 induces its association with the TCR via a mechanism involving reorganization of the actin cytoskeleton and PI3 kinase activity. The association of CXCR4 with the TCR permits CXCR4 to signal via pre-existing, constitutively phosphorylated TCR-ZAP-70 complexes, leading to more robust Ras and prolonged ERK activation in response to SDF-1α. Prolonged ERK activation enhances AP-1 transcriptional activity, which mediates gene expression in response to SDF-1α alone and which also mediates SDF-1α's ability to selectively costimulate cytokine expression by TCR-stimulated T cells.

fluorescence resonance energy transfer (FRET) approaches and coimmunoprecipitation were used to show that SDF-1α treatment induces the close association of CXCR4 with both TCR-CD3-ζ and TCR-CD3-ε subunits. The FRET method used to measure CXCR4-TCR-CD3-ε interactions additionally allows us to conclude that the interacting receptor molecules were originally located on the cell surface. FRET occurs when specific pairs of chromophores come within approximately 10 nm of each other (Stryer, 1978). Because the vast majority of cell-surface TCR-CD3-ε and TCR-CD3-ζ subunits reside within the TCR holoreceptor (Alarcon et al., 1988), our results indicate that SDF-1α treatment induces the close and perhaps direct association of CXCR4 and the TCR. We further show here that Ras activation, $[Ca^{2+}]_i$ mobilization, and the prolonged ERK activation previously noted to be typical of SDF-1α signaling via CXCR4 in T cells (Kremer et al., 2003; Tilton et al., 2000) depend on expression of the TCR β-subunit and correlate with cell-surface expression levels of the TCR. Together, our results indicate that the physical interaction between CXCR4 and the TCR functionally modulates SDF-1α signaling via CXCR4 in T cells.

The TCR has not previously been shown to heterodimerize with CXCR4 or any other GPCR; therefore, we analyzed the molecular mechanisms responsible for this signal transduction pathway. The TCR signals via its cytosolic ITAM domains binding the tyrosine kinase, ZAP-70 (Hatada et al., 1995; Iwashima et al., 1994; Cambier, 1995). We have shown that ZAP-70 expression is required for SDF-1α to stimulate prolonged ERK activation (Kremer et al., 2003), and we show here that ZAP-70 is also required for SDF-1α to stimulate robust Ras activation. We also show here that plasma membrane localization of the intracellular region of the TCR-CD3-ζ subunit, which contains three ITAM domains, is sufficient to restore SDF-1α-dependent prolonged ERK activation in cells deficient in TCR expression. In addition, we show that the mutation of one or both ITAM tyrosines abrogates the ability of the ITAM domain to participate in SDF-1α signaling. To bind ZAP-70, an ITAM domain must be phosphorylated on both of its canonical tyrosine residues (Iwashima et al., 1994). Thus, our results indicate that SDF-1α signals via the TCR ITAM domains, most likely because these domains bind ZAP-70. These results support previous reports that tyrosine kinases critically participate in CXCR4 signal transduction in T cells and suggest that, in addition to ZAP-70, the Src family tyrosine kinase Lck participates in CXCR4 signaling. Indeed, CXCR4 has previously been shown to activate Lck (Ganju et al., 1998), and Lck can phosphorylate TCR ITAM domains (Iwashima et al., 1994).

Our results as well as previous studies (Nanki and Lipsky, 2000; Molon et al., 2005) make clear that CXCR4 signaling does not simply recapitulate TCR signaling. For example, although CXCR4 stimulates the ERK pathway to a similar extent as does the TCR, the TCR but not CXCR4 also activates NF-κB and NF-AT luciferase reporter constructs. It is therefore perhaps not surprising that in contrast to direct stimulation of the TCR, our results indicate that CXCR4 signaling via the TCR does not detectably increase the phosphorylation of either the TCR or ZAP-70. Because we were unable to detect inducible tyrosine phosphorylation of either the TCR or ZAP-70 in response to CXCR4, and because we detected constitutively phosphorylated and associated TCR and ZAP-70 within unstimulated cells, we suggest that CXCR4 signaling, in contrast to TCR signaling, utilizes pre-existing constitutively tyrosine phosphorylated TCR-ZAP-70 complexes. Jurkat T cells as well as normal resting T cells and murine thymocytes have previously been noted to contain low but detectable amounts of constitutively associated TCR and ZAP-70 (Chan et al., 1991; van Oers et al., 1994; Kersh et al., 1998).

Alternatively, it is possible that CXCR4 signaling increases the tyrosine phosphorylation of a subset of TCR and ZAP-70 molecules that we were unable to detect. Our results further suggest that SDF-1α treatment induces the association of CXCR4 and TCR molecules detectable at the cell surface and within intracellular compartments via a mechanism partly sensitive to inhibitors of cellular polarization. GPCRs heteromultimerized with receptor tyrosine kinases have been shown to promote activation of endosomal Ras-ERK pathways in other cell types (Mor and Philips, 2006). It is therefore possible that the subcellular localization of CXCR4-TCR-ZAP-70 complexes mediates more robust and prolonged activation of Ras and ERK.

CXCR4 signaling via this TCR- and ZAP-70-dependent pathway contributes to several physiological effects of SDF-1α on T cells. First, expression of both the TCR and ZAP-70 is required for robust AP-1-dependent transcriptional activity in response to SDF-1α. AP-1 is most likely activated in response to prolonged ERK activity, because AP-1 activation was abrogated by a MEK-1 inhibitor and because prolonged ERK activity prolongs the half-life of AP-1 subunits (Murphy et al., 2002). SDF-1α signaling via the TCR would therefore be expected to mediate the transcription of AP-1-dependent gene products during the migration of resting T cells through the lymph nodes, bone marrow, and other anatomical locations that constitutively express SDF-1α (Muller et al., 2001). This SDF-1α signaling pathway may be important for promoting the survival of resting T cells, because SDF-1α treatment of a T cell line stimulates the expression of several antiapoptotic gene products via the MEK-1-ERK pathway (Suzuki et al., 2001). SDF-1α signaling via the TCR may also modulate thymocyte development, which critically depends on the Ras-ERK pathway (Hernandez-Lopez et al., 2002). Second, our results indicate that the AP-1 activity induced via this novel CXCR4-TCR signaling pathway mediates SDF-1α costimulation of cytokine secretion by activated T cells. SDF-1α selectively costimulates the TCR-stimulated expression of CD69, IL-10, and IL-2, which are AP-1 dependent (Jain et al., 1995; Wang et al., 2005; D'Ambrosio et al., 1994). IFN-γ costimulation may critically depend on the relative timing of the SDF-1α and TCR signals, since while SDF-1α costimulatory effects on IFN-γ production failed to reach significance in our experiments, SDF-1α was previously reported to costimulate IFN-γ production by $CD4^+$ T cells when added 2 hr prior to TCR ligation (Nanki and Lipsky, 2000) and after APC-mediated T cell stimulation (Molon et al., 2005). A remarkable feature of SDF-1α costimulation is its selectivity, which corresponds with SDF-1α selectively enhancing the AP-1, but not NF-κB or NF-AT, -dependent transcription of either TCR- or TCR+CD28-stimulated T cells. Selective costimulation of cytokine secretion may allow SDF-1α to promote T cell tolerance in two ways: by enhancing T cell activation within SDF-1α-rich lymph nodes as opposed to SDF-1α-poor tissues and by biasing against Th1 responses by enhancing the production of IL-10.

Our results broaden paradigms for understanding CXCR4 and GPCR signal transduction in immune cells. CXCR4 signal transduction in other cell types is likely to be modulated and regulated by other cell-surface receptors that contain ITAM domains, including the B cell receptor, the Fc-ε receptor I, and NK activating receptors that signal via DAP-12 (Cambier, 1995). Signaling by CXCR4 that depends on coexpression of an ITAM-containing protein may help explain, for example, the different functions of CXCR4 at different stages of B cell development (D'Apuzzo et al., 1997). In addition, our results suggest that other GPCR may, like CXCR4, be capable of utilizing ITAM-containing receptors for signal transduction. Thus, among immune cells that express ITAM-containing receptors and either ZAP-70 or its homolog, SYK, the roles and functions of CXCR4 and other chemokine receptors and GPCRs may be distinct as compared to other cell types.

Experimental Procedures

Cells

PBMC were isolated from healthy volunteers with approval of the Mayo IRB. T cells were purified with the RosetteSep T cell enrichment cocktail (Stem Cell Technologies, Canada). Jurkat T cell lines were maintained as described (Kremer et al., 2003). R. Abraham (Burnham Institute, La Jolla, CA) provided ZAP-70-deficient Jurkat cells (P116) and P116 cells stably reconstituted with ZAP-70 (P116-C40) (Williams et al., 1998). Jurkat cells deficient in TCR-β (JRT3) were obtained from the American Type Culture Collection (Rockville, MD). JRT3 stably reconstituted with TCR-β (PF-2.4) was a gift of A. Weiss (University of California, San Francisco, CA) (Ohashi et al., 1985; Weiss and Stobo, 1984).

Reagents and Stimulation Conditions

Unless otherwise indicated, stimulations were at 37°C with 5×10^{-8} M SDF-1α (R&D Systems, Minneapolis, MN). TCR ligation was achieved by crosslinking TCR mAbs: for reporter assays and cytokine production assays, the TCR was ligated via plate bound 1 μg/ml CD3 mAb (OKT3, Ortho Biotech, Bridewater, NJ); for biochemical assays, OKT3 was crosslinked as described (Kremer et al., 2003). Where indicated, cells were pretreated for 5 min with the CXCR4 antagonist, CXCR4-4-2 (Tarasova et al., 1999), 16 hr with 100 ng/ml PTX or PTX-B (RBI/Sigma, Natick, MA), 90 min with 50 μM PD098059 (Sigma, St. Louis, MO), 60 min with 10 μM cytochalasin D, or 16 hr with 50 μM LY294002 (Calbiochem).

Confocal FRET Imaging and Fluorescence Spectroscopic Analysis of CXCR4 and TCR Fluorescent Fusion Proteins Transiently Expressed in Live Cells

cDNA encoding human CXCR4, TCR-CD3-ζ, and TCR-CD3-ε was amplified by PCR (primers available upon request) and subcloned into pEYFP-N1, pDsRed2-N1, or pECFP-N1 (Clontech, Mountain View, CA). Plasmids were transiently transfected into Jurkat cells (Kremer et al., 2003) or purified human T cells (Bell et al., 2001), as described. The same, live cells were imaged with a Model 510 inverted laser scanning confocal microscope with a 100× objective and a 37°C heated stage (Carl Zeiss, Germany) ± SDF-1α for 30 min. Confocal FRET assays employed respective excitation lasers and emission filters of 440 nm and 455–525 nm (for CFP), 532 nm and 560–675 nm (for YFP), and 440 nm and LP550 nm (for FRET). Jurkat T cells expressing fluorescent fusion proteins individually were employed as controls; no FRET signals were detected in these control samples (Figure S3). Spectroscopic analysis of steady-state fluorescence of cells in a quartz cuvette in response to 433 nm excitation before and after 30 min of SDF-1α stimulation (SPEX Fluorolog Spectrofluorophotometer, Model 1680-0.22, SPEX Industries, Edison, NJ) confirmed that SDF-1α-induced increases in FRET fluorescence were accompanied by decreased CFP fluorescence (Figure 1D). Cells expressing either one or the other of the fluorescent fusion proteins were employed as controls.

FRET of Fluorescent mAbs Bound to Endogenous, Cell-Surface CXCR4 and TCR-CD3-ε

Cells were incubated with excess CXCR4 mAb conjugated to PE (Clone #44717; R&D Systems) and excess CD3-ε mAb conjugated

to APC (Clone #UCHT1; Pharmingen, San Diego, CA) for 20 min at 4°C, then washed in cold FACS buffer without azide (HBSS with 10 mg/ml BSA and 10 mM HEPES [pH 7.5]). Where indicated, CD45 mAb conjugated to APC (Pharmingen, San Diego, CA) was used instead of CD3-ε-APC mAb. Stained cells were either kept on ice or incubated at 37°C ± SDF-1α. FRET was assayed by flow cytometry either immediately or after fixation in 2% paraformaldehyde/PBS. Flow cytometry utilized a dual-laser (488 nm Ar and 635 nm He-Ne) FACS Caliber (Becton Dickinson, Franklin Lakes, NJ), as described (Batard et al., 2002). Excitation/emission windows were 488 nm/530 ± 15 nm (FL1), 488 nm/585 ± 21 nm (FL2), 488 nm/>670 nm (FL3), and 635 nm/661 ± 8 nm (FL4). Where indicated, an acid wash was employed as follows. Cells were prepared as above for FRET analysis, stimulated with SDF-1α for 30 min to induce FRET signals, then incubated at 4°C for 5–10 min either in cold FACS buffer or in 20 mM HCl/HBSS (pH 2.0). After washing, the cells were resuspended in cold FACS buffer, fixed, and analyzed for FRET signals.

Coimmunoprecipitation of CXCR4/TCR Complexes

Jurkat T cells were transiently transfected with a plasmid encoding the CXCR4-YFP fusion protein. Cells were stimulated at 37°C with SDF-1α for 20 min, then lysed in Buffer A (150 mM NaCl, 20 mM TRIS-Cl [pH 7.4], 5 mM EDTA, 1 mM $CaCl_2$, 1% Triton X-100, 5% glycerol, 10 mM NaF, and 1 mM sodium orthovanadate). TCR-CD3-ζ was immunoprecipitated with anti-CD3-ζ-agarose or control IgG agarose (Santa Cruz Biotechnology, Santa Cruz, CA), washed with Buffer A, and analyzed by immunoblotting with anti-YFP (Clontech, Mountain View, CA) or anti-TCR-CD3-ζ.

Assays of Active Ras and Active, Phosphorylated ERK1 and ERK2

Cells were stimulated as indicated, and levels of active, GTP bound Ras were assayed with an affinity purification kit (Upstate; Waltham, MA). Active ERK was assayed by immunoblotting as described (Kremer et al., 2003). Where indicated, JRT3 cells were transiently transfected with plasmids encoding chimeric CD8-CD3-ζ proteins (gifts of P. Allen, Washington University, St. Louis, MO) and stimulated with SDF-1α, and ERK was assayed by flow cytometry of fixed, permeabilized cells stained with anti-CD8 and phospho-specific p44/p42 ERK1 and ERK2 (Thr-202/Tyr-204) mAb conjugated to either Alexa Fluor-488 or Alexa Fluor-647 (BD Pharmingen). Gating was used to compare ERK activities of cells expressing similar levels of CD8.

Immunoprecipitation and Immunoblot Analyses of Tyrosine Phosphorylated Proteins

Cells were stimulated as indicated and lysed as described (Kremer et al., 2003), and endogenous signaling proteins were immunoprecipitated with P-Tyr mAb 4G10 (Upstate; Waltham, MA) or anti-TCR-ζ (Santa Cruz Biotechnology). Proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-P-Tyr mAb (4G10), anti-ZAP-70-pTyr-493 (Cell Signaling Technology, Danvers, MA), or anti-total ZAP-70 (Cell Signaling Technology). Quantification was via autoradiography and densitometry (AMBIS; Molecular Dynamics, Sunnydale, CA).

Reporter Assays, $[Ca^{2+}]_i$ Analysis, and Assays of Cytokine and Cell-Surface Marker Expression

Assays of NF-AT- or NF-κB-dependent transcriptional activity in transiently transfected cells were performed as described (Hedin et al., 1997). The AP-1 reporter plasmid (gift of D. Billadeau, Mayo Clinic, Rochester, MN; Cao et al., 2002) was used in a similar manner. $[Ca^{2+}]_i$ assays were performed as described (Kremer et al., 2003). The Cytoperm/Cytofix intracellular cytokine staining kit (BD Pharmingen, San Diego, CA) was used after cells were stimulated for 24 hr (for CD69 and IL-10 detection) or 6 hr (for CD25 and IL-2). Secreted IL-10 was assayed after 24 hr by ELISA (BD Pharmingen).

Supplemental Data

Three Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/2/213/DC1/.

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