RANTES Induces Tyrosine Kinase Activity of Stably Complexed p125^{FAK} and ZAP-70 in Human T Cells

By Kevin B. Bacon,* Margit C. Szabo,‡ Hans Yssel,* Joseph B. Bolen,* and Thomas J. Schall*

*From the *DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California 94304; and the ‡Department of Pathology, Stanford University School of Medicine, Stanford, California 94305*

Summary

The chemokine RANTES is a chemoattractant and activating factor for T lymphocytes. Investigation of the signal transduction mechanisms induced by RANTES in T cells revealed tyrosine phosphorylation of multiple protein species with prominent bands at 70–85 and 120–130 kD. Immunoprecipitation and Western analyses revealed that a protein of 125 kD was identical to the focal adhesion kinase (FAK) pp125^{FAK}. RANTES stimulated phosphorylation of FAK as early as 30 seconds, and immunoblots using antiphosphotyrosine monoclonal antibodies revealed that there was consistent phosphorylation of a 68–70 kD species in the pp125^{FAK} immunoprecipitates. Immunoblotting and kinase assays showed this to be two separate proteins, the tyrosine kinase zeta-associated protein (ZAP) 70, and the focal adhesion protein paxillin. These results indicate a potentially important role for RANTES in the generation of T cell focal adhesions and subsequent cell activation via a molecular complex containing FAK, ZAP-70, and paxillin.

Chemokines are a growing family of small molecular mass proteins (8–16 kD) that were originally classified based on the conservation of a four-cysteine motif and on their ability to cause the directed migration of leukocytes in vitro (1–3). RANTES, originally characterized as a potent chemoattractant of memory T lymphocytes and monocytes (4) has also been shown to have a role in the biology of eosinophils and basophils (1–3). In addition to its T cell migratory activity, some reports detail RANTES-stimulated adhesion of T lymphocytes to endothelial monolayers and purified adhesion molecules (5–7). Most recently, RANTES has been demonstrated to activate T lymphocytes in an antigen-independent fashion (8).

The activation signals for T lymphocytes after stimulation by chemokines have not been well defined. The nature of the RANTES receptors on T cells and signal tranduction machinery engaged by this chemokine have not been extensively detailed. Recent evidence has demonstrated a role for G protein–dependent calcium influx and protein tyrosine kinase (PTK)–induced signal transduction pathways (8), as well as potent stimulation of phospholipase D activity (Bacon, K.B., and T.J. Schall, manuscript in preparation). The activation of adhesion in leukocytes has been shown to involve members of the family of both β1 and β2 integrins (9, 10). In fibroblasts and smooth muscle cells, the regulation of intracellular focal adhesion complexes and their interaction with α-actinin, talin, and vinculin of the cytoskeleton has also been given much attention with respect to adhesion, cell migration, and cell structure control (11–15). Recent studies have centered on the focal adhesion kinase (FAK)[1] protein pp125^{FAK} since this protein has been localized in focal adhesions with actin bundles after receptor-mediated activation of cell shape change or reorientation (12–14). FAK is also autophosphorylated and capable of phosphorylating SH2 domain-containing proteins which it may bind (16–20), potentially localizing numerous signal transduction components to a specific cytosolic compartment. This phosphorylation of associated proteins serves to increase their kinase potential, and in some cases, to bring other proteins into the macromolecular complex.

One report has demonstrated a functional coassociation of the newly identified FAK B with the T cell tyrosine kinase zeta-associated protein (ZAP) 70 (21). ZAP-70 tyrosine kinase has been shown to be a fundamental component of the signal transduction apparatus involved in the activation of T cells after antigen receptor ligation. ZAP-70 binds via its SH2 domains, to the phosphotyrosine in the immunoreceptor tyrosine-based activation motif (ITAM) domains of TCR γ, δ, ε, and ζ in a process catalyzed by p56^{lck} or p59^{fyn} (22–26). This molecular association results

[1] *Abbreviations used in this paper:* ECL, enhanced chemiluminescence; FAK, focal adhesion kinase; HA, herbimycin A; ITAM, immunoreceptor tyrosine-based activation motif; ZAP-70, zeta-associated protein 70.

in phosphorylation of tyrosine in the kinase domain of ZAP-70 by the *lck* or *fyn* kinases, and ultimately results in activation of, among other substrates, phospholipase C γ and *vav*.

To investigate RANTES-induced T cell activation at the molecular level, we have attempted to characterize signal transduction events that may subserve the migratory and adhesive functions. In this report, we demonstrate that RANTES induces homotypic adhesion of an antigen-specific human $CD4^+$ T cell clone. This adhesion response is measurable as early as 10 min and maximal after overnight stimulation. Moreover, the response is associated with tyrosine phosphorylation of multiple protein species but can be abrogated by the tyrosine kinase inhibitor herbimycin A (HA). The phosphorylation of $pp125^{FAK}$ in vitro, as well as stimulation of its kinase potential, is one of the earliest events occurring after RANTES stimulation. Interestingly, immunoprecipitates from cells stimulated with RANTES reveal that the chemokine induces a functional molecular complex consisting of FAK, paxillin, and ZAP-70.

Materials and Methods

Cells. Studies were performed with a $CD4^+$ (Th0) tetanus toxin–specific T cell clone (27). Cells were grown in culture as described, however, before assay for phosphorylation, they were starved of IL-2 and serum (0.5% serum used) for at least 48 h. Cells were stimulated with increasing concentrations of human recombinant RANTES or PBS alone, (R&D Systems, Inc., Minneapolis, MN) for up to 24 h after starvation before lysis for biochemical analyses.

Antibodies. TCR-mediated stimulation of cells was performed using anti-CD3 antibody (UCHT-1, IgG1; Sigma Chemical Co., St. Louis, MO). Murine isotype-matched control (IgG1) was also obtained from Sigma Chemical Co. mAbs to $pp125^{FAK}$ (mAb 2A7, IgG1), ZAP-70 mAb (2F3.2, IgG2a), and antiphosphotyrosine (4G10), were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit polyclonal antiserum BC3 (anti-$pp125^{FAK}$) was kindly provided by Dr. J.T. Parsons (University of Virginia, Charlottesville, VA). Rabbit polyclonal antiserum STL91 (anti-TCR-ζ) has been previously described (28) and mAb to paxillin (Z035, IgG1) was obtained from Zymed Laboratories Inc. (South San Francisco, CA).

Immunoprecipitation and Immunoblotting. After stimulation (human rRANTES, or cross-linked mAb to CD3), starved cells were rapidly centrifuged to remove excess agonist. The pellets were lysed in lysis buffer (1% NP40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.25% deoxycholate, and 5 mM EDTA) containing protease and phosphatase inhibitors (1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 1 mM EGTA, 100 μM β-glycerophosphate, 10 mM sodium fluoride, and 1 mM tetrasodium pyrophosphate) for 15 min on ice with periodic vortexing. After lysis, the samples were centrifuged and the supernatants were either used immediately or frozen at −70°C before assay. Protein concentration of the lysates was assessed using the BCA reagent kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Whole phosphotyrosine analysis was performed with each sample by SDS-PAGE on 10% gels (NOVEX, San Diego, CA). Protein was immunoprecipitated from the lysate (100 μg total protein) and immune complexes (2 h, 4°C with constant rotation) were captured with species-specific, agarose-coupled anti-IgG (10 μl; Sigma Chemical Co.) for 2–18 h at 4°C with constant rotation. The immunoprecipitates were washed three times with excess lysis buffer, then resuspended in Laemmli sample buffer before electrophoresis.

Samples from all gels were transferred to activated Immobilon P^{TM} membranes (Millipore Corp., Bedford, MA) and nonspecific sites were blocked with block buffer (5% BSA in Tris-buffered saline [TBS] containing 0.1% [vol/vol] Tween 20 and 0.05% [vol/vol] thimerosol). The blots were then stained with primary antibody (1 μg/ml mAb or 1/500–1/1,000 polyclonal antisera) overnight at 4°C, washed extensively with wash buffer (TBS containing 0.5% NP-40), stained for 1 h with 2° antibody coupled to horseradish peroxidase (Amersham Corp., Arlington Heights, IL; 1:10,000 in block buffer), washed extensively, and revealed with enhanced chemiluminescence (ECL) reagent (Amersham Corp.) and Biomax™ MR film (Eastman Kodak Co., Rochester, NY).

Immune Complex Kinase Assays. Immune complex kinase assays were performed according to published methods analyzing the intrinsic kinase activity of $pp125^{FAK}$ or the ability of immunoprecipitated $pp125^{FAK}$ or ZAP-70 to phosphorylate the substrate (poly:Glu,Tyr [1:4]; Sigma Chemical Co.). In all instances, protein was immunoprecipitated from lysates as outlined above, washed twice in lysis buffer, and once in kinase assay buffer (20 mM Pipes, pH 7.2, containing 5 mM each of $MgCl_2$ and $MnCl_2$).

Kinase assays to assess phosphorylation were performed by addition of 10 μCi γ-[^{32}P]ATP (6,000 Ci/mmol specific activity; Amersham Corp.) for 20 min at 30°C. After assay, the immune complexes were washed to remove excess radioactivity and analyzed on 8% SDS gels (NOVEX). After electrophoresis, the bands corresponding to $pp125^{FAK}$ and ZAP-70 were localized, excised from the gel, and the radioactivity quantitated by liquid scintillation counting.

Immune complex kinase assays were also performed to assess the ability of immunoprecipitated $pp125^{FAK}$ or ZAP-70 to phosphorylate an exogenous substrate, poly:Glu:Tyr. Immunoprecipitations were performed as outlined above. Washed immune complexes were resuspended in kinase buffer containing 10 μCi γ-[^{32}P]ATP, 5 mM ATP, and 0.4 mg poly(Glu:Tyr) according to published methods (17). After a 20-min incubation at 30°C, the supernatant was removed from the immune complex and the poly(Glu:Tyr) precipitated with 10% cold TCA. The supernatants were removed and the incorporation of radioactivity in the precipitates was assessed by liquid scintillation counting.

Results

RANTES Stimulation of Cell Adhesion. Cells were starved of IL-2 and serum to render them quiescent. After 48-h starvation, levels of IL-2R were reduced and cytokine production (IL-2, IL-3, IL-4, IL-5, IFN-γ, and GM-CSF) was below detection limits of ELISA assay (Bacon, K.B., unpublished data). Fig. 1 *A* shows the morphology of cells in the quiescent state. RANTES (0.1–100 nM), when added to the cells for up to 24 h, had no effect on the survival or activation of the T cells ($n = 3$, data not shown; 8). However, at a concentration of 1 μM, RANTES induced profound changes in the activation of the T cells. Fig. 1, *B–E* shows that RANTES not only maintained the survival of the cells in serum- and IL-2–free culture, but stimulated progressive homotypic adhesion of the cells. This phenom-

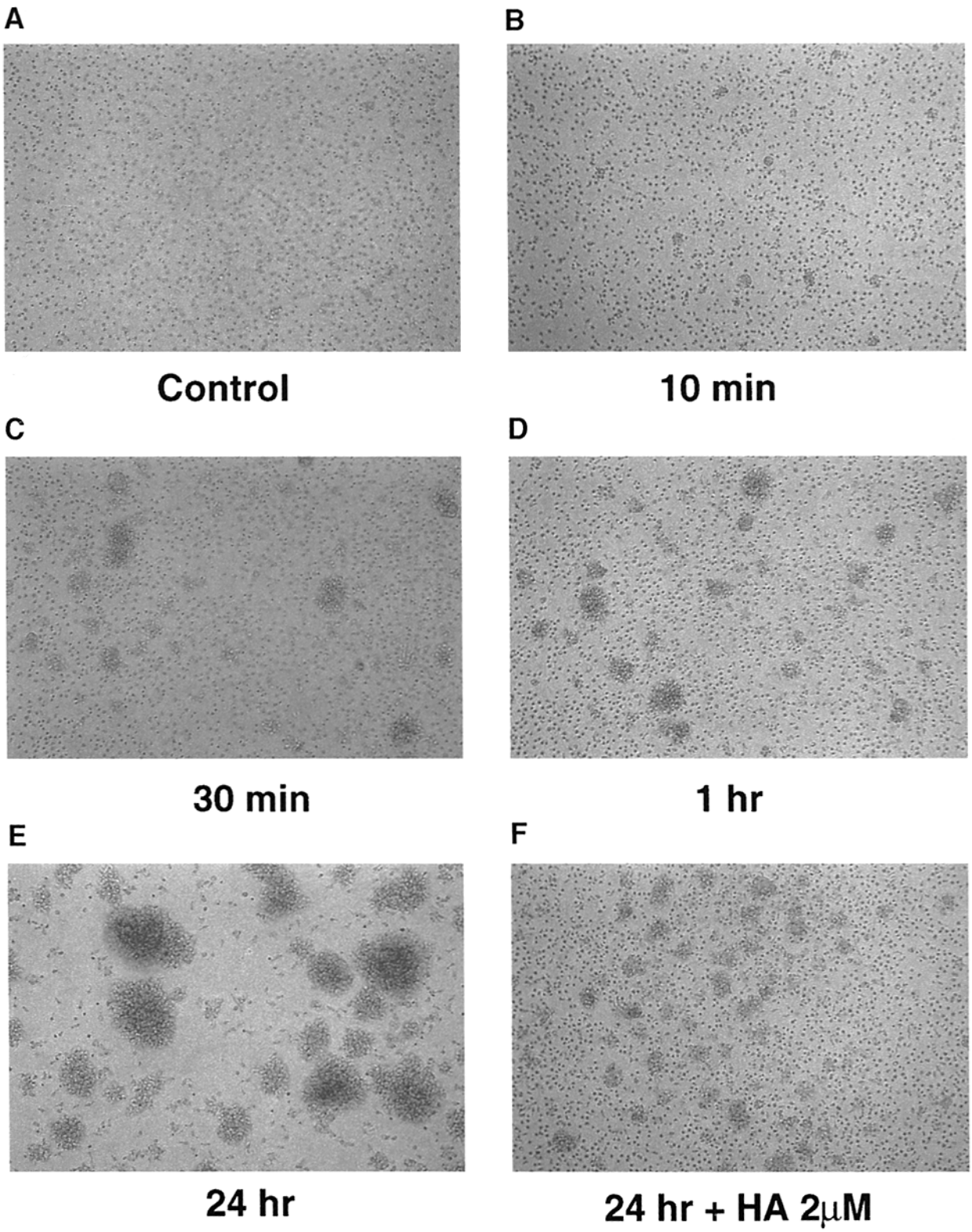

Figure 1. Time course of RANTES-induced homotypic aggregation in the serum- and IL-2–starved T cell clone. Cells were seeded under sterile conditions in 6-well culture plates (Costar Corp., Cambridge, MA) in serum- and IL-2–free IMDM for 48 h before addition of RANTES at 10^{-6} M. Cells were subsequently photographed after the times indicated. HA treatment was for 18 h before RANTES addition.

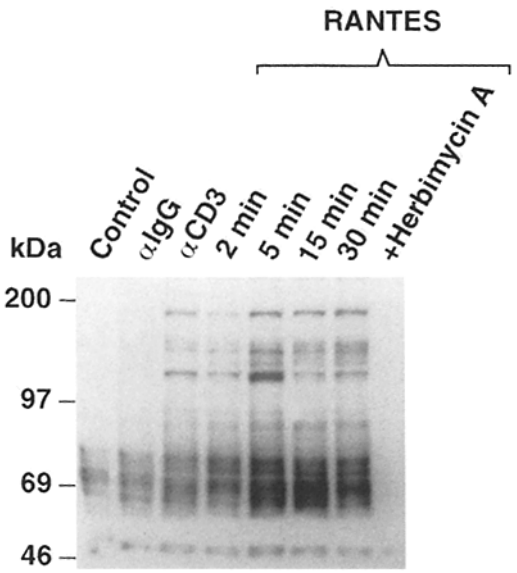

Figure 2. Time course stimulation of tyrosine phosphorylation in starved T cell clones using 10^{-6} M RANTES. Cross-linked antibody to CD3 and an isotype match were used as positive controls. (*Control*) Cells incubated in the presence of PBS alone. Cells were stimulated for the indicated times, then lysed and soluble protein isolated as described in the Materials and Methods section. After SDS-PAGE on 10% Tris-glycine SDS gels and transfer to Immobilon membranes, Western blots were stained with antiphosphotyrosine antibody 4G10 and visualized with ECL reagent.

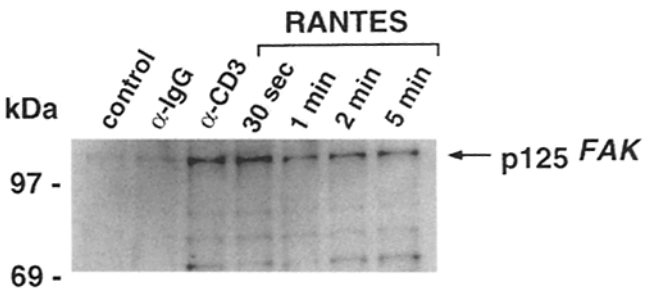

Figure 3. Whole cell lysates from cells stimulated with anti-CD3 (3 min) or RANTES for the time course indicated, were incubated overnight with monoclonal anti-FAK (2A7; see Materials and Methods), and immunecomplexes were precipitated with anti–mouse IgG-agarose for 2 h at 4°C. Western blots of electrophoresed immunecomplexes (8% Tris-glycine SDS gels) were stained with antiphosphotyrosine antibody 4G10 and revealed with ECL reagent.

enon was visible when using T cell clones or normal peripheral blood T cells (not shown) as early as 10 min, becoming maximal at 24 h, but was inhibitable if cells were pretreated with 2 μM HA for 18 h. Other chemokines (IL-8, MCP-1 [monocyte chemoattractant protein-1], MIP-1α [macrophage inflammatory protein-1α], MIP-1β, and lymphotactin) did not induce the adhesive effect (data not shown). No heterotypic adhesion of T cells to cytokine-activated endothelial cells was observed (Szabo, M.C., E. Butcher, B. McIntyre, T.J. Schall, and K.B. Bacon, manuscript submitted for publication).

RANTES Stimulates Phosphorylation of Multiple Intracellular Proteins in the Antigen-specific T Cell Clone. Initial experiments were performed to investigate signal transduction occurring after RANTES-induced homotypic adhesion in the T cell clone. In numerous experiments, using the concentration of RANTES that induced homotypic adhesion, Western blots of whole cell lysates stained with antiphosphotyrosine antibodies revealed intensly stained bands in two main areas of the gel, 50–75 and 120–130 kD, as early as 30 s and up to 5 min. Fig. 2 shows a representative blot where maximal phosphorylation was observed at 5 min. Interestingly, the pattern of phosphorylation observed closely parallelled that induced by stimulation of the clone with antibody to CD3 (Fig. 2, lane *3*). There was no stimulation of the cells with PBS alone or isotype-matched IgG as a control for the α-CD3 (Fig. 2, lanes *1* and *2*) and pretreatment of the T cell clone with HA (2 μM) completely inhibited the phosphorylation induced by RANTES (lane *8*).

RANTES Induces Phosphorylation of T Cell pp125^{FAK}. To test whether the stimulation of adhesion receptors or focal adhesion complexes accompanied the increase in homotypic adhesion observed in response to RANTES, studies were performed to define the phosphorylated proteins at 120–130 kD. Fig. 3 shows that mAb to pp125^{FAK} immunoprecpitated a phosphorylated species that migrated on gel in a similar fashion to that observed with the phosphotyrosine antibody blot of whole cell lysates. Surprisingly, phosphotyrosine blots of the immunoprecipitated pp125^{FAK} consistently revealed additional bands including species of 70 kD. In all cases, there was no immuoprecipitation by nonspecific control antibodies (mouse anti–human IgG; data not shown) and levels of pp125^{FAK} in each lane were equivalent as assessed by parallel immunoblots (not shown).

pp125^{FAK} Antibody Coimmunoprecipitates Paxillin and the T Cell–specific Kinase ZAP-70. Phosphoprotein pp125^{FAK} participates in the formation of focal adhesions during cytoskeletal reorganization in cell motility and adhesion. In addition to pp125^{FAK}, focal adhesions are characterized by the presence of α-actinin, vinculin, talin, and paxillin (14, 15, 29–31). We therefore sought to define if any of these proteins coimmunoprecipitated from the lysates using anti-pp125^{FAK} antibody. Fig. 4 *A* shows results from a representative experiment using anti-FAK antisera. After electrophoresis of immunoprecipitated proteins, Western blots were stained with antipaxillin antibody, and as controls for 68–70 kD proteins, anti-p72^{syk}, anti-ZAP-70, and anti-p70^{S6k}. In RANTES-stimulated cells, paxillin coimmunoprecipitated with the FAK complex (Fig. 4 *A*). Levels of paxillin were slightly increased between 1 and 5 min RANTES stimulation. Surprisingly, the kinase ZAP-70 was also found in the FAK immunoprecipitates (Fig. 4 *A*) but not p72^{syk} or p70^{S6k} (not shown). Levels of ZAP-70 appeared to remain constant, independent of the nature or length of time of the stimulus.

To confirm these results, reciprocal immunoprecipitations were performed using specific paxillin and ZAP-70 mAbs. Fig. 4 *B* demonstrates that FAK and ZAP-70 were present in these complexes. Reciprocal immunoprecipita-

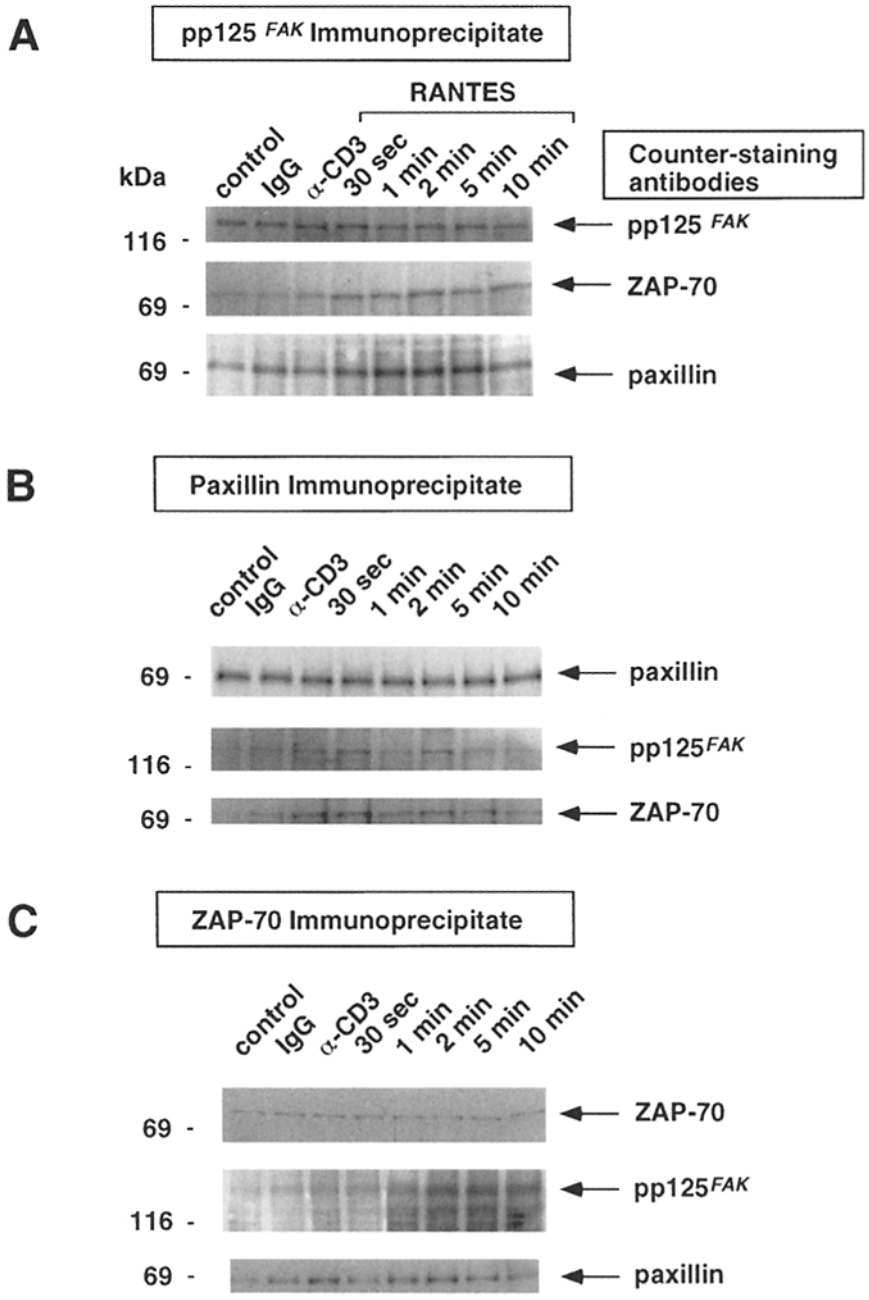

Figure 4. (*A*) Composite representation of FAK immunoprecipitations and coimmunoprecipitated paxillin and ZAP-70. FAK was immunoprecipitated from whole cell lysates as described using mAb 2A7. After SDS-PAGE using 8% gels and 100 μg protein per lane, Western blots were stained with 2A7 (anti-$pp125^{FAK}$), antipaxillin, and anti ZAP-70 mAbs as indicated. Stained species were revealed using ECL reagent as described. (*B*) Reciprocal immunoprecipitations using mAb to paxillin. Experiments were performed as described in *A* except that mAb to paxillin was the immunoprecipitating antibody. (*C*) Reciprocal immunoprecipitations using mAb anti-ZAP-70.

tions using anti-ZAP-70 mAb (Fig. 4 *C*) show that paxillin, ZAP-70, and $pp125^{FAK}$ may preexist in a complex even in the absence of RANTES stimulation, but that the levels of $pp125^{FAK}$ phosphorylation within this complex change markedly after stimulation with the chemokine.

RANTES Stimulates Kinase Activity of $pp125^{FAK}$. Kinase assays were employed to characterize the molecules in the macromolecular complex. Complexes were immunoprecipitated using either mAb to $pp125^{FAK}$ or ZAP-70; the immune complex was then incubated with γ-[^{32}P]ATP alone or in conjunction with the exogenous substrate poly(Glu:Tyr).

Phosphorylation of $pp125^{FAK}$ immunoprecipitated by FAK mAb was measurable as early as 30 s, becoming maximal at 5 min (Fig. 5 *A*). Little if any incorporation of labeled phosphate into FAK was observed after 5 min. Anti-CD3 stimulation also appeared to be suboptimal for FAK phosphorylation, mirroring results obtained in Western analyses. Similarly, in FAK immune complexes, there was incorporation of ^{32}P into ZAP-70, being measurable at 30 s and maximal after 2–5 min (Fig. 5 *B*). Reciprocal immunoprecipitates showed increased phosphorylation in ZAP-70 and FAK after 30 s and maximal by 2 min (Fig. 5, *C* and *D*, respectively).

As both $pp125^{FAK}$ and ZAP-70 function as kinase enzymes, experiments were performed to confirm this potential and the specificity of the immunoprecipitations. Immune complexes obtained using anti-$pp125^{FAK}$ mAb were

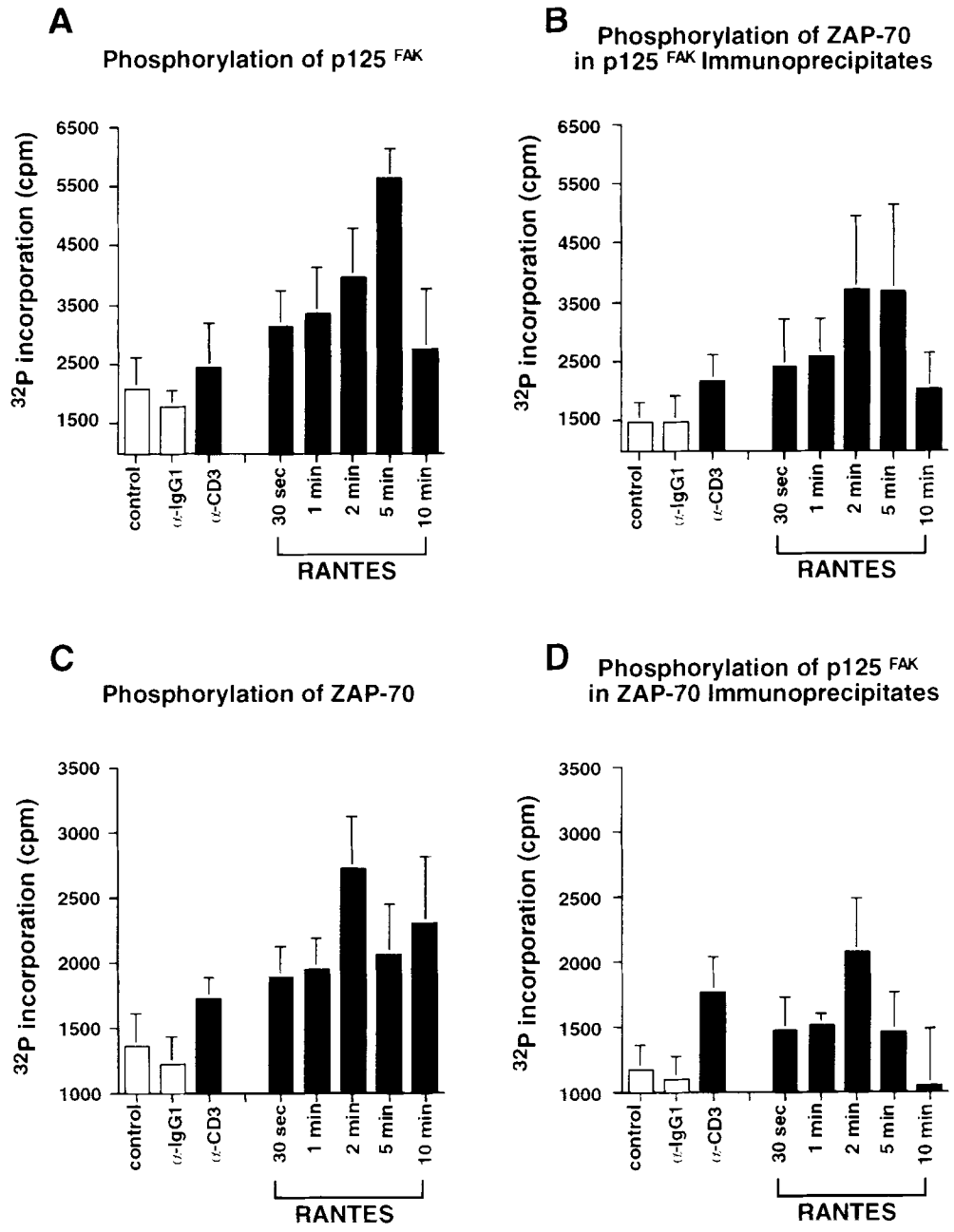

Figure 5. Phosphorylation of pp125^{FAK} and ZAP-70. Immunoprecipitations were carried out as described in Materials and Methods from cells stimulated with RANTES or anti-CD3. Immune complexes were then used for kinase assay as described and after electrophoresis the bands corresponding to pp 125^{FAK} or ZAP-70 were excised from the gel. Incorporation of radioactive phosphate was assesed by liquid scintillation counting.

capable of phosphorylating the exogenous substrate with rapid kinetics (maximal at 1-min RANTES stimulation). The level of phosphorylation was greater than threefold background (Fig. 6 *A*). In contrast, there was little if any significant phosphorylation by the anti-ZAP-70–derived immune complexes during the first 2 min of RANTES stimulation. Maximal phosphorylation occurred at 10 min after RANTES stimulation (Fig. 6 *B*).

RANTES-induced Partial Phosphorylation of TCR-ζ. The activation of the T cell–specific kinase ZAP-70 by RANTES implied a mechanism that employs components of the TCR. Since ZAP-70 is known to preferentially associate with the phospho-ITAMs of TCR-ζ, we analyzed the phosphorylation of this polypeptide after stimulation with RANTES. Immunoprecipitates were performed using mAb 4G10 (antiphosphotyrosine) followed by staining Western blots with the ζ chain polyclonal antisera. Fig. 7 demonstrates the levels of phosphorylation of TCR-ζ by cross-linked anti-CD3 or RANTES. The concentrations of agonist used were equivalent to that inducing homotypic adhesion and the proliferative activity observed in earlier studies (8). Anti-CD3 caused phosphorylation to higher molecular weight species of TCR-ζ, represented by the band shift (Fig. 7, lane *3*). In contrast, whereas RANTES

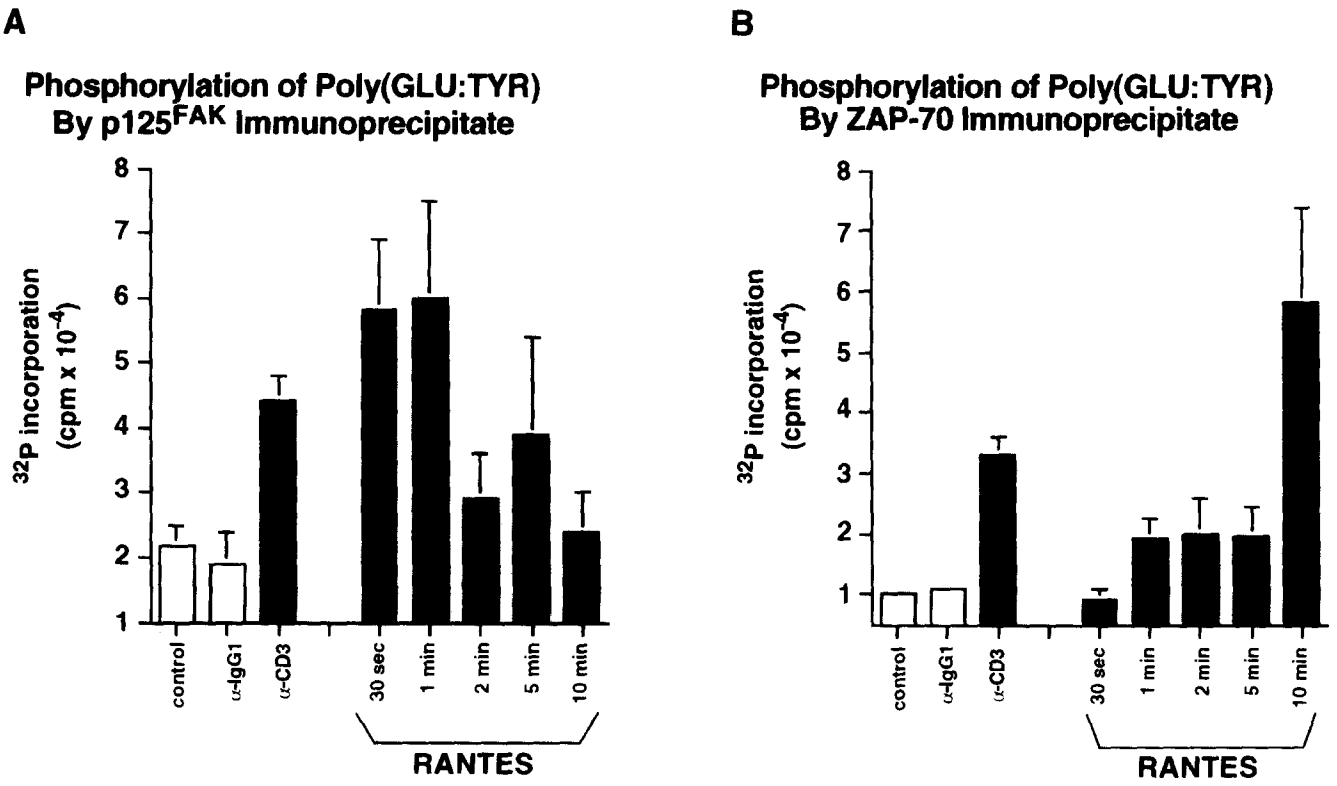

Figure 6. Immune complexes immunoprecipitated with the same antibodies were also used in kinase assays to phosphorylate exogenous enzyme. The immune complexes were added to the reaction buffer containing radiolabeled phosphate and exogenous substrate poly(Glu:Tyr). Optimal conditions dictated 20-min incubation with exogenous substrate. Supernatants were then removed to separate substrate from immune complex and the poly(Glu:Tyr) was precipitated with cold TCA. Phosphorylated substrate was then assessed by liquid scintillation counting.

phosphorylated TCR-ζ to levels slightly above those from unstimulated cells, this chemokine failed to hyperphosphorylate TCR-ζ to a level similar to anti-CD3 (Fig. 7, lanes *4–8*).

Discussion

We have attempted to define, at least in part, the signal transduction mechanisms induced by RANTES in the stimulation of T lymphocyte homotypic adhesion. We have demonstrated that an immune modulator distinct from antigen is capable of promoting activation of the machinery to assemble focal adhesion macromolecular complexes and induce homotypic adhesion. In dissecting this phenomenon, we have shown that FAK-containing complexes immunoprecipitated from T cells stimulated by RANTES demonstrate a rapid upregulation of pp125^{FAK} activity, typified by its phosphorylation and its capacity to phosphorylate the exogenous substrate poly(Glu:Tyr). The kinetics of phosphorylation measured in these experiments suggest an important role for RANTES in the activation of kinase enzymes responsible for both T cell activation and cytoskeletal control. Western blot analyses revealed early phosphorylation patterns on FAK and ZAP-70, with slightly slower kinetics for paxillin. This pattern was mir-

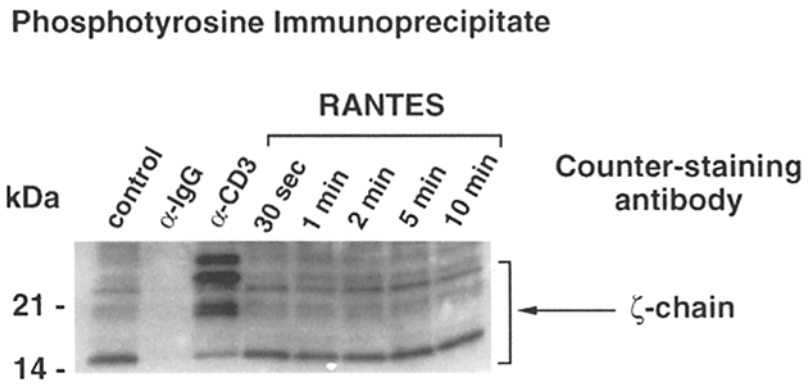

Figure 7. Phosphotyrosine analysis of TCR-ζ. Tyrosine phosphorylated proteins were immunoprecipitated from whole cell lysates stimulated with either anti-CD3 (3 min) or RANTES for the indicated amount of time. Western blots were stained with anti-ζ chain polyclonal antibody (STL91) and visualized using ECL reagent.

rored by the phosphorylation kinetics measured by ^{32}P incorporation. Whereas we postulate that autophosphorylation of FAK is occurring, it is possible that the additional, as yet uncharacterized, bands in the FAK immunoprecipitates (50–70 kD) may have contributed to the phosphorylation of pp125^{FAK}. Analyses of the phosphorylation of the exogenous substrate poly(Glu:Tyr) however, demonstrated rapid ^{32}P incorporation induced by the FAK immunoprecipitates but slower incorporation induced by ZAP-70 immunoprecipitates. This slower kinetic may be indicative of the need for ZAP-70 to be optimally phosphorylated before exhibiting maximal kinase activity. Alternatively, there may be less FAK in the ZAP-70 immunoprecipitates resulting in a slower rate in achieving maximal kinase activity. One hypothesis suggests that the level of phosphotyrosine indicated by Western analysis may simply not reflect the efficiency of kinase activity.

It has been well characterized that paxillin can bind to or associate with pp125^{FAK} either directly or indirectly (14, 15, 32, 33). Direct binding occurs at a COOH-terminal site proximal to the focal adhesion targeting (FAT) site, whereas indirect binding occurs by paxillin binding through SH3 domains to a SH2-containing kinase such as *c-src*. (20). In a similar manner to Hildebrand et al. (32), we demonstrate that FAK and paxillin exist as a preformed complex. In our hands there was slight upregulation of phosphotyrosine content of paxillin in the T cell clone when stimulated with RANTES. This protein binds vinculin and metavinculin (molecular mass of 150 kD) and most likely functions in cell structural control. Recent data have emerged to demonstrate that paxillin, which itself has no known enzymatic activity, binds to a class of proteins known as the adaptor proteins. This group of proteins includes members such as Crk. Crk contains one SH2 and two SH3 domains and is known to bind the guanine nucleotide exchange factors C3G and SOS, through the SH3 domains (34). Crk is also known to participate in the phosphorylation of a p130 (p130^{cas}) substrate in oncogenically transformed cells through its association with the *cAbl* protooncogene (35). Tyrosine phosphorylation of p130^{cas} is a consistent finding after integrin-mediated cell adhesion to extracellular matrix (36). Cas contains 15 possible SH2 binding sites, an SH3 domain, and regions of proline-rich domains likely to function as SH3 binding sites (37). Interestingly, we observed the appearance of a tyrosine phosphorylated protein of ~130 kD (Fig. 2) in RANTES-treated cells. If this molecule represents p130^{cas}, it may function as an adaptor protein to bring various signal transduction components together at the focal adhesion. Therefore it is conceivable that paxillin functions as a potential kingpin in the assembly of multiple signal transduction components within a focal adhesion site. In support of the idea for focal assembly of signal transduction components, it has also been shown that FAK binds the adaptor protein Grb2 (38), and we have found that FAK immunoprecipitates coimmunoprecipitate the small GTP-binding proteins p21rho and rac1 (Bacon, K.B., G. Aversa, J. Carballido, J. de Vries, and T.J. Schall, manuscript in preparation) in both normal peripheral blood T cells and another antigen-specific T cell clone. That this macromolecular complex mediates a single function is unlikely. The assembly of signaling molecules involved in the potential activation of the tyrosine kinases, the Ras/mitogen-activated protein kinase (MAPK) and calcium homeostasis pathways, indicates that the stimulation of T lymphocytes by RANTES induces a full program of growth and differentiation, and not simply the regulation of adhesion mechanisms.

It is intriguing that the FAK complex also contained the T cell–specific kinase ZAP-70. Whereas the association of FAK and ZAP-70 appeared to be preformed in a similar fashion to paxillin, there was an increase in the phosphotyrosine content of the ZAP-70. The association of FAK, as opposed to FAK B, and ZAP-70 is in contrast to the results of Kanner et al. (21). The differences obtained in these two studies may represent important features of the cells used. The phosphorylation of ZAP-70 is critical for the activation of the kinase potential of this molecule. Our data demonstrate fairly rapid phosphorylation kinetics of ZAP-70, but much slower phosphorylation of exogenous substrate poly(Glu:Tyr) (Fig. 7). Fig. 4 shows that ZAP-70 immunoprecipitates FAK. However, the maximal kinase activity is less than that observed from the FAK immunoprecipitates, thus the low concentration of FAK in the ZAP-70 immunoprecipitates may account for the slower kinetics observed in phosphorylation of exogenous substrate.

The association of ZAP-70 with the macromolecular complex and its activation implies an important role for RANTES in the antigen-independent activation of the T lymphocyte. These findings also beg the question of whether ZAP-70 becomes activated according to standard mechanisms or by components within the FAK macromolecular complex. Phosphotyrosine immunoblots of TCR-ζ in this T cell clone indicate that concentrations of anti-CD3 antibody that induce proliferation and homotypic aggregation can augment the amount of phosphorylated ζ chain, whereas concentrations of RANTES that have similar effects, fail to increase phosphorylated TCR-ζ to similar levels (Fig. 7), nor is there any hyperphosphorylation at lower concentrations (10^{-9}–10^{-7} M) of RANTES (Bacon, K.B., unpublished data). These results infer the possibility that RANTES is capable of inducing the activation of ZAP-70 at high concentration by making use of the basal level of phosphorylated ζ and are supported by the presence of coimmunoprecipitated ZAP-70 from RANTES-stimulated clones in experiments using anti-ζ antisera (Bacon, K.B., unpublished data). This activation phenomenon may be sustained and/or augmented by assembling numerous signal transduction molecules in, for example, a focal adhesion complex formed by adhesion receptors cocapped with RANTES receptors or other surface moieties.

It is interesting to note that there was a consistant dephosphorylation of pp125^{FAK} and ZAP-70 after 5-min stimulation with RANTES (Figs. 4–6). This raises important questions concerning the biology of RANTES as well

as the potential action of protein tyrosine phosphatases in these cells. It has been suggested that there are low numbers of RANTES receptor(s) on the surface of T cells. Indeed the mRNA for CC-CKR1 (39) is present in T lymphocytes, and there is surface expression of this receptor on ~30% of the cells of the T cell clone used in these studies, as measured using a specific mAb (Bacon, K.B., Szabo, M.C., K. Soo, and T.J. Schall, unpublished data). It is therefore surprising that no further phosphorylation occured even in the presence of high concentrations of RANTES over prolonged periods of time. It is unlikely that all of the RANTES was bound but it is reasonable to assume that all of the available receptors were complexed with RANTES. Thus, knowledge of the fate of receptor–ligand interactions will help us understand why no further phosphorylation was possible. This fate may be related to the trafficking and recycling of receptor–ligand complexes or the action of specific phosphatases that may be activated downstream of the kinase signals.

We previously demonstrated the proliferation-inducing capacity of RANTES which was associated with calcium flux and tyrosine kinase signal transduction qualitatively similar to that induced by anti-CD3. We have now shown a potential mechanism for this T cell activation, after homotypic aggregation, in the complexing and activation of ZAP-70 tyrosine kinase activity. It has been suggested that the partial phosphorylation of TCR-ζ chain underlies the anergic state in T cells by preventing adequate recruitment of ZAP-70, as well as the inability to stimulate full kinase activity in ZAP whose SH2 domains were not structurally in an optimal configuration after ITAM binding (40–42). The ability of RANTES to provide the framework for T cell proliferation in the absence of hyperphosphorylated TCR-ζ chain therefore implies an important role for RANTES in generalized T cell activation, bypassing the requirement for specific antigen-mediated activation. This then represents an important route of activation for lymphocytes in pathologies characterized by high concentrations of RANTES but lacking obvious α/β or γ/δ TCR restriction.

We acknowledge the gift of anti-pp125^{FAK} from Dr. J. Thomas Parsons. We thank Drs. M. Howard and J. Chiller for critical review of the manuscript.

DNAX is supported by Schering-Plough Corporation.

Address correspondence to Dr. Kevin B. Bacon, Department of Immunology, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304.

Received for publication 2 April 1996 and in revised form 22 May 1996.

References

1. Schall, T.J., and K.B. Bacon. 1994. Chemokines, leukocyte trafficking and inflammation. *Curr. Opin. Immunol.* 6:865–873.
2. Bacon, K.B., and T. J. Schall. 1996. Chemokines as mediators of allergic inflammation. *Int. Arch. Allergy Immunol.* 109: 97–109.
3. Baggiolini, M., B. Dewald, and B. Moser. 1994. Interleukin 8 and related chemotactic cytokines—CXC and CC chemokines. *Adv. Immunol.* 55:97–179.
4. Bacon, K.B., B.A. Premack, P. Gardner, and T.J. Schall. 1995. Dual T cell signal transduction pathways activated by RANTES. *Science (Wash. DC).* 269:1727–1730.
5. Schall, T.J., K. Bacon, K.J. Toy, and D.V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature (Lond.).* 347:669–671.
6. Lloyd, A.R., K. Conlon, J.M. Wang, J.R. Ortaldo, A. Harada, K. Matsushima, D.J. Kelvin, and J.J. Oppenheim. 1993. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J. Exp. Med.* 177:1809–1814.
7. Brezinschek, R.L., P.E. Lipsky, P. Galea, R. Vita, and N. Oppenheimer-Marks. 1995. Phenotypic characterization of CD4$^+$ T cells that exhibit a transendothelial migratory capacity. *J. Immunol.* 154:3062–3077.
8. Gilat, D., R. Hershkoviz, Y.A. Mekori, I. Vlodavsky, and O. Lider. 1994. Regulation of adhesion of CD4$^+$ T lymphocytes to intact or heparinase-treated subendothelial extracellular matrix by diffusable of anchored RANTES and MIP-1β. *J. Immunol.* 153:4899–4906.
9. Butcher, E.C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell.* 67: 1033–1036.
10. Springer, T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell.* 76:301–314.
11. Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–525.
12. Hildebrand, J.D., M.D. Schaller, and J.T. Parsons. 1993. Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125^{FAK}, to cellular focal adhesions. *J. Cell Biol.* 123:993–1005.
13. Schaller, M.D., C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds, and J.T. Parsons. 1992. pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* 89:5192–5196.

14. Clark, E.A., and J.S. Brugge. 1995. Integrins and signal transduction pathways: The road taken. *Science (Wash. DC).* 268: 233–239.
15. Miyamoto, S., H. Teramoto, O.A. Coso, J.S. Gutkind, P.D. Durbelo, S.K. Akiyama, and K.M. Yamada. 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol.* 131:791–805.
16. Gutkind, J.S., and K.C. Robbins. 1992. Activation of transforming G protein–coupled receptors induces rapid tyrosine phosphorylation of cellular proteins, including $p125^{FAK}$ and the p130 v-src substrate. *Biochem. Biophys. Res. Commun.* 188:155–161.
17. Schaller, M.D., J.D. Hildebrand, J.D. Shannon, J.W. Fox, R.R. Vines, and J.T. Parsons. 1994. Autophosphorylation of the focal adhesion kinase, $pp125^{FAK}$ directs SH2-dependent binding of $pp60^{src}$. Mol. Cell. Biol. 14:1680–1688.
18. Cobb, B.S., M.D. Schaller, T.-H. Leu, and J.T. Parsons. 1994. Stable association of $pp60^{src}$ and $pp59^{fyn}$ with the focal adhesion-associated protein tyrosine kinase, $pp125^{FAK}$. *Mol. Cell. Biol.* 14:147–155.
19. Calalb, M.B., T.R. Polte, and S.K. Hanks. 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for src family kinases. *Mol. Cell. Biol.* 15:954–963.
20. Zachary, I., J. Sinnet-Smith, C.E. Turner, and E. Rozengurt. 1993. Bombesin, vasopressin and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. *J. Biol. Chem.* 268:22060–22065.
21. Kanner, S.B., A. Aruffo, and P.-Y. Chan. 1994. Lymphocyte antigen receptor activation of a focal adhesion kinase-related tyrosine kinase substrate. *Proc. Natl. Acad. Sci. USA.* 91: 10484–10487.
22. Chan, A.C., B.A. Irving, J.D. Fraser, and A. Weiss. 1991. The ζ chain is associated with a tyrosine kinase and upon T cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. *Proc. Natl. Acad. Sci. USA.* 88:9166–9170.
23. Strauss, D.B., and A. Weiss. 1993. The CD3 chains of the T cell antigen receptor associate with the ZAP-70 tyrosine kinase and are tyrosine phosphorylated after receptor stimulation. *J. Exp. Med.* 178:1523–1530.
24. Chan, A.C., D.M. Desai, and A. Weiss. 1994. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu. Rev. Immunol.* 12:555–592.
25. Wange, R.L., S.N. Malek, S. Desiderio, and L.E. Samelson. 1993. Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor ζ and CD3ε from activated Jurkat T cells. *J. Biol. Chem.* 268:19797–19801.
26. Irving, B.A., A.C. Chan, and A. Weiss. 1993. Functional characterization of a signal transducing motif present in the T cell antigen receptor ζ chain. *J. Exp. Med.* 177:1093–1103.
27. Roncarolo, M.-G., H. Yssel, J.-L. Touraine, R. Bacchetta, L. Gebuhrer, J.E. de Vries, and H. Spits. 1988. Antigen recognition by MHC-incompatible cells of a human mismatched chimera. *J. Exp. Med.* 168:2139–2148.
28. Burkhardt, A.L., B. Stealey, R.B. Rowley, M. Prendergast, J. Fargnoli, and J.B. Bolen. 1994. Temporal regulation of non-transmembrane protein tyrosine kinase enzyme activity following T cell antigen receptor engagement. *J. Biol. Chem.* 269:23642–23647.
29. Turner, C.E., J.R. Glenney, and K. Burridge. 1990. Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol.* 111:1059–1068.
30. Burridge, K., C.E. Turner, and L.H. Romer. 1992. Tyrosine phosphorylation of paxillin and $pp125^{FAK}$ accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell. Biol.* 119:893–903.
31. Chen, H.-C., P.A. Appeddu, J.T. Parsons, M.D. Schaller, and J.L. Guan. 1995. Interaction of focal adhesion kinase with cytoskeletal protein talin. *J. Biol. Chem.* 270:16995–16999.
32. Hildebrand, J.D., M.D. Schaller, and J.T. Parsons. 1995. Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxy terminal domain of focal adhesion kinase. *Mol. Biol. Cell.* 6:637–647.
33. Turner, C.E., and J.T. Miller. 1994. Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and $pp125^{FAK}$ binding region. *J. Cell Sci.* 107:1583–1591.
34. Schaller, M.D., and J.T. Parsons. 1995. $pp125^{FAK}$-dependent tyrosine phosphorylation of paxillin creates a high affinity binding site for crk. *Mol. Cell. Biol.* 15:2635–2645.
35. Mayer, B.J., H. Hirai, and R. Sakai. 1995. Evidence that SH2 domains promote processive phosphorylation by protein tyrosine kinases. *Curr. Biol.* 5:296–305.
36. Vuori, K., and E. Ruoslahti. 1995. Tyrosine phosphorylation of $p130^{cas}$ and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix. *J. Biol. Chem.* 270:22259–22262.
37. Sakai, R., A. Iwamatsu, N. Hirano, S. Ogawa, T. Tanaka, H. Mano, Y. Yazaki, and H. Hirai. 1994. A novel signaling molecule, p130, forms stable complexes in vivo with v-crk and v-src in a tyrosine phosphorylation-dependent manner. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3748–3756.
38. Schlaepfer, D.D., S.K. Hanks, T. Hunter, and P. Van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. *Nature (Lond.).* 372:786–790.
39. Neote, K., D. DiGregorio, J.Y. Mak, R. Horuk, and T.J. Schall. 1993. Molecular cloning, functional expression and signaling characteristics of a C-C chemokine receptor. *Cell.* 72:415–425.
40. Sloan-Lancaster, J., A.S. Shaw, J.B. Rothbard, and P.M. Allen. 1994. Partial T cell signalling: altered phospho-ζ and lack of ZAP-70 recruitment in APL-induced T cell anergy. *Cell.* 79:913–922.
41. Van Oers, N.S.C., N. Killeen, and A. Weiss. 1994. ZAP-70 is constitutively associated with tyrosine-phosphorylated TCR ζ in murine thymocyte and lymph node T cells. *Immunity.* 1:675–685.
42. Hatada, M.H., X. Lu, E.R. Laird, J. Green, J.P. Morgenstern, M. Lou, G.S. Marr, T.B. Phillips, M.K. Ram, K. Theriault et al. 1995. Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T cell receptor. *Nature (Lond.).* 377:32–38.