RAFTK, a Novel Member of the Focal Adhesion Kinase Family, Is Phosphorylated and Associates with Signaling Molecules upon Activation of Mature T Lymphocytes

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Summary

The related adhesion focal tyrosine kinase (RAFTK), a recently discovered member of the focal adhesion kinase family, has previously been reported to participate in signal transduction in neuronal cells, megakaryocytes, and B lymphocytes. We have found that RAFTK is constitutively expressed in human T cells and is rapidly phosphorylated upon the activation of the T cell receptor (TCR). This activation also results in an increase in the autophosphorylation and kinase activity of RAFTK. After its stimulation, there was an increase in the association of the src cytoplasmic tyrosine kinase Fyn and the adapter protein Grb2. This association was mediated through the SH2 domains of Fyn and Grb2. RAFTK also co-immunoprecipitates with the SH2 domain of Lck and with the cytoskeletal protein paxillin through its COOH-terminal proline-rich domain. The tyrosine phosphorylation of RAFTK after T cell receptor-mediated stimulation was reduced by the pretreatment of cells with cytochalasin D, suggesting the role of the cytoskeleton in this process. These observations indicate that RAFTK participates in T cell receptor signaling and may act to link signals from the cell surface to the cytoskeleton and thereby affect the host immune response.

Transduction of signals from cell surface receptors involves the activation of numerous molecules having catalytic activity related to phosphorylation. In the case of signaling after the ligation of the TCR/CD3, there is the associated release of calcium, activation of intracytoplasmic protein tyrosine kinases including Fyn and Lck, modulation in phosphatidylinositol metabolism with phosphorylation of PLC-γ, and changes in the R as pathway (1–13). Associated with these activation events, there are changes within the cytoplasm that include alterations in the cytoskeleton (14–17).

Although considerable knowledge has been generated on such pathways in the T cell response to antigen, there is still much to be learned, particularly regarding the interactions of kinase signaling molecules with the cytoskeletal proteins. One recent study revealed that activation via TCR/CD3 led to important associations and changes in the actin cytoskeleton (17).

We and others have recently identified a novel signaling molecule known as related adhesion focal tyrosine kinase (RAFTK), Pyk2, or CAK-β (18–20). This molecule is most closely related to the focal adhesion kinase (FAK). FAK has been shown to associate with several signaling molecules and cytoskeletal structures that form in the so-called focal adhesions upon cell attachment to extracellular substrates (21–24). RAFTK has considerable deduced amino acid and structural similarity with FAK, including consensus motifs in the central catalytic domain, the absence of a transmembrane region, myristylation sites, and SH2 and SH3 domains. Also, similar to FAK, RAFTK has a proline-rich region in the COOH-terminal domain (18–20).

We have observed that RAFTK is phosphorylated in response to the activation of certain integrins in B lymphocytes (25) and megakaryocytes (26). The induced phosphorylation of RAFTK via calcium-mediated ion channel pathways and after stress activation has been shown in PC-12 pheochromocytoma cells (19, 27, 28).

We report the finding that RAFTK is expressed in human T lymphocytes and participates in signaling events triggered by the ligation of the TCR/CD3 complex. We have characterized several of the interacting molecules that associate with RAFTK in human T cells, including the cytoskeletal protein paxillin. These studies provide new information regarding the regulation of pathways of T cell

Abbreviations used in this paper: FAK, focal adhesion kinase; GST, Glutathione S transferase; RAFTK, related adhesion focal tyrosine kinase; RIPA, radioimmunoprecipitation assay; RT, room temperature.

1055 J. Exp. Med. © The Rockefeller University Press • 0022-1007/97/03/1055/09 $2.00
Volume 185, Number 6, March 17, 1997 1055–1063
receptor signaling and the associated cytoskeletal changes that may mediate the cellular immune response.

Materials and Methods

Cells and Cell Cultures. The permanent human T cell lines Jurkat and H9 were obtained from the American Type Culture Collection (Rockville, MD) and shown to be mycoplasma-free before their expansion in culture. The cells were carried in DMEM with 10% FCS, 2 mM glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Primary human PBLs were obtained from normal volunteers after obtaining their informed consent and then isolated by Ficoll Hypaque density centrifugation as previously described (29). The anti-CD3–producing hybridoma (OKT-3) was obtained from the American Type Culture Collection and grown in IMDM with 20% FCS. For antibody production, cells were grown in serum- and protein-free hybridoma medium (Sigma Chemical Co., St. Louis, MO) containing Nutridoma-HU 1% (Boehringer Mannheim, Indianapolis, IN).

Reagents and Materials. The lectin PHA was obtained from Pharmacia Biotech (Piscataway, NJ) and the nitrocellulose membrane from BioRad Laboratories (Hercules, CA). The anti-CD3 antibody X35 was obtained from Immunotech (Westbrook, ME), and OKT-3 was purified from OKT-3–producing hybridoma supernatants on protein A-Sepharose columns. Antibodies to Fyn were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Transduction Laboratories (Lexington, KY). Anti-paxillin antibody was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Specific polyclonal antibodies to RAFTK were generated by immunizing New Zealand white rabbits with a bacterially expressed fusion protein consisting of GST and the COOH terminus (amino acids 681–1,009) of the human RAFTK cDNA subcloned into the pGEX-2T expression vector as described (26). The sera were titrated against the GST-RAFTK COOH-terminal fusion protein by ELISA, and the serum (R-4250) that revealed the highest titer was used in the subsequent experiments. This antiserum was shown to be specific and not cross-reactive with FAK in earlier experiments (26). The anti-raftk antibody was obtained from BioRad Laboratories. The protease inhibitors leupeptin, apropin, and alpha-I antiprson and all other reagents were obtained from Sigma Chemical Co.

Stimulation of Cells. Cells were washed twice with HBSS (GIBCO BR L, Gaithersburg, MD) and resuspended at 5 × 10⁶ cells/ml in DMEM. Cells were stimulated with either PHA (10 μg/ml), αCD3 antibodies ×35 (10 μg/ml), or OKT-3 (10 μg/ml) at 37°C for various time periods. In some experiments, cells were pretreated with EGTA (5 mM) for 5 min at 4°C or cyclohexalin D (2 μM) for 60 min at 37°C before stimulation. After stimulation, 2 × 10⁸ cells were microfuged for 10 s and lysed in 1 ml of modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM PMF, 10 μg/ml of aprogin, leupeptin, and pepstatin, 10 mM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). Total cell lysates were centrifuged at 10,000 g for 10 min. Protein concentrations were determined by protein assay (BioRad Laboratories).

Immunoprecipitation and Western Blot Analysis. For immunoprecipitation studies, identical amounts of protein from each sample were clarified by incubation with protein A-Sepharose CL-4B (Pharmacia Biotech) for 1 h at 4°C. After the removal of protein A-Sepharose by brief centrifugation, the solution was incubated with different primary antibodies as detailed below for each experiment for 4 h or overnight at 4°C. Immunoprecipitations of the antibody–antigen complexes were performed by incubation for 2 h at 4°C with 75 μl of protein A-Sepharose (10% suspension). Nonspecific bound proteins were removed by washing the Sepharose beads three times with modified RIPA buffer and one time with PBS. Bound proteins were solubilized in 40 μl of 2× Laemmli buffer and further analyzed by immunoblotting. Samples were separated on 7.5% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk protein and probed with primary antibody for 3 h at room temperature (RT) or 4°C overnight. Immunoreactive bands were visualized using HRP-conjugated secondary antibody and the enhanced chemiluminescent system (Amerham Corp., Arlington Heights, IL). Monoclonal antibody (4G10, lgg2a) was used for Western blot analysis of phosphotyrosine protein.

Glutathione S Transferase Fusion Protein Binding Studies. The RAFTK COOH-terminal domain (amino acids 681 through 1,009) Glutathione S transferase (GST) fusion protein was amplified by the PCR technique and cloned into the pGEX-2T expression vector (Pharmacia Biotech) as previously described (26). The GST–fusion protein was produced by 1 mM isopropyl β-thiogalactopyranoside induction and purified on a Glutathione-Sepharose column by affinity chromatography according to manufacturer’s recommendations (Pharmacia Biotech). GST–fusion protein Grb2–SH3 3 NH2-terminal domain, Grb2–SH3 3 COOH-terminal domain, Grb2–SH2 domain, and Fyn–SH2 and -SH3 domains were purchased from Santa Cruz Biotechnology. For the binding experiments, 1 mg of cell lysate was mixed with 5 μg of GST–fusion protein and incubated for 1 h at 4°C on a rotatory shaker. GST protein (Santa Cruz Biotechnology) was used as control. 50 μl of Glutathione–Sepharose 4B beads (Pharmacia Biotech) were added to preabsorb the complex. After incubation for 3 h at 4°C on a rotatory shaker, the beads were centrifuged and washed three times with modified RIPA buffer. The bound proteins were eluted by boiling in Laemmli sample buffer and subjected to 7.5% SDS-PAGE and Western blot analysis.

mTOR Assays. The immunoprecipitated complexes obtained by immunoprecipitating cell lysates with RAFTK antiserum were washed twice with RIPA buffer and once in kinase buffer (20 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 100 mM Na2VO4, and 5 μM ATP). For in vitro kinase assays, the immune complex was incubated in kinase buffer containing 25 μg of poly (Glu/Tyr, 4:1) 20–50 KD; Sigma Chemical Co.) and 5 μCi [32P]ATP at RT for 30 min.

A utophosphorylation Assay. This assay was carried out by incubating the immune complex in a kinase buffer containing 5 μCi [32P]ATP at RT for 30 min. The reaction was stopped by adding 4× SDS sample buffer and by boiling the sample for 5 min. Proteins were then separated on SDS-PAGE and detected by autoradiography.

Results

RAFTK Is Expressed in Human T Lymphocytes and Is Phosphorylated upon T Cell Activation. To further characterize the signaling pathways in human T cells involved in the immune response, we used as models two permanent T cell lines, Jurkat and H9, as well as primary human PBLs. Analysis by immunoblotting (data not shown) or immunoprecipitation revealed abundant RAFTK protein in these T cells (Fig. 1).

The stimulation of human T cell lines with T cell receptor ligation induces the tyrosine phosphorylation of a phos-
phoprotein around 115 kD (5, 7). We therefore investigated whether various stimuli associated with such T cell activation modulated the phosphorylation of RAFTK, which has a deduced molecular weight of \( \sim 120 \) kD. As seen in Fig. 1, A and B, an increase in the tyrosine phosphorylation of RAFTK could be specifically observed in the T cell lines Jurkat or H9 after T cell receptor ligation or treatment with the lectin PHA. The membrane was then stripped and re-probed with anti–RAFTK antibody to confirm that equivalent amounts of RAFTK were loaded in each lane (Fig. 1, A and B, bottom). Stimulation of primary PBLs with anti–T cell receptor antibody also induced an increase in the tyrosine phosphorylation of RAFTK (Fig. 1 C).

To determine the time course of tyrosine phosphorylation of RAFTK, Jurkat cells were stimulated with anti–T cell receptor antibody \( \times 35, \) OKT-3, or with the lectin PHA. Ligation of the TCR/CD3 by monoclonal antibody \( \times 35 \) or OKT-3 reached a maximum by 2.5–5 min, and declined thereafter (Fig. 2, A and B). PHA stimulation resulted in an increased tyrosine phosphorylation by 5 min that declined slightly thereafter with substantial phosphorylation still detectable at 20 min (Fig. 2 C). Anti-RAFTK immunoblotting of the anti-RAFTK immunoprecipitates showed that the \( \sim 115 \)-kD phosphotyrosine polypeptide corresponds to the RAFTK protein (Fig. 2, A–C, bottom). Depending on the resolution of the gels, RAFTK was seen to migrate either as a single band or as a doublet (Figs. 1 and 2).

T Cell Receptor Stimulation Results in Increased RAFTK Autophosphorylation and Kinase Activity. The autophosphorylation and kinase activities of protein tyrosine kinases can be activated upon their tyrosine phosphorylation, which is essential for their role in signal transduction. We therefore performed an autophosphorylation assay and an in vitro kinase assay in which poly (Glu/Tyr, 4:1) was used as an exogenous substrate to determine the intrinsic tyrosine kinase activity of RAFTK. As shown, T cell receptor stimulation resulted in an increase in the autophosphorylating (Fig. 3 A) as well as the kinase activity of RAFTK (Fig. 3 B). In the autophosphorylation assays, maximal activity was observed at \( \sim 2.5–5 \) min. However, for the in vitro kinase assay, maximal activity was observed at \( \sim 5–10 \) min. The different kinetics observed in autophosphorylation versus in vitro kinase assay could be attributed to the differences in the assays used. The in vitro kinase activity observed in the RAFTK immune complexes using exogenous substrate

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**Figure 1.** Tyrosine phosphorylation of RAFTK by T cell receptor cross-linking in Jurkat and H9 human T cell lines or primary human PBLs. Cells were lysed in RIPA buffer. Lysates obtained from \( 2 \times 10^7 \) unstimulated (–), \( \alpha \)-CD3-stimulated or PHA-stimulated Jurkat cells (A), H9 cells (B), or primary human PBLs (C) were immunoprecipitated with RAFTK polyclonal antibody. Immunoprecipitates were size-fractionated on 7.5% SDS-PAGE gels, transferred to nitrocellulose membranes, and then subjected to serial immunoblotting with anti–phosphotyrosine antibody (4G10; top) and anti-RAFTK antibody (bottom). Normal rabbit serum (NRS) was used as a negative control. TCL, total cell lysates.

**Figure 2.** Time course of tyrosine phosphorylation of RAFTK upon T cell activation. Jurkat cells (\( 5 \times 10^6 \) ml) were incubated at 37°C for different time periods with either (A) anti-CD3 antibodies \( \times 35 (10 \mu g/ml) \), (B) OKT-3 \( (10 \mu g/ml) \), or (C) PHA \( (10 \mu g/ml) \). Stimulated or unstimulated cells were lysed in RIPA buffer, immunoprecipitated with anti-RAFTK antibody, resolved on SDS-PAGE gels, and subjected to serial immunoblotting with anti–phosphotyrosine antibody (top) and RAFTK antibody (bottom).
could be the result of RAFTK and other co-associated kinases such as Fyn.

RAFTK Associates with the Signaling Molecules Fyn and Grb2. To further characterize the role that RAFTK might play in T cell signaling after its activation via TCR/CD3 ligation, we performed immunoprecipitation studies followed by immunoblotting. We observed a specific association of RAFTK with Fyn, a Src family kinase that is known to be capable of associating with TCR (12, 30–32). A small fraction of Fyn was readily detected as associating with RAFTK before the TCR/CD3 activation of Jurkat cells, and this association increased after their stimulation (Fig. 4 A).

Using GST–fusion proteins corresponding to the SH3 and SH2 domains of Fyn, we observed an association only with the GST-SH2-Fyn protein by immunoprecipitation of the fusion protein followed by immunoblotting with anti-RAFTK antibody (Fig. 4 B). The SH2 domain of Lck, another Src family member, has recently been shown to play an important role in the initiation of signaling events after TCR stimulation (33). We also examined the association of the GST-SH2 domain of Lck with RAFTK. As shown (Fig. 4 B), RAFTK also co-immunoprecipitates with the GST-SH2 domain of Lck.

We then examined the ability of RAFTK to form in vitro complexes with another SH2 and SH3 domain-containing protein, Grb2, which has been shown to interact with RAFTK in other cells (19, 26). As shown in Fig. 5 A, RAFTK immunoprecipitates from activated T cell lysates associated with Grb2. To further characterize this interaction, GST–fusion proteins of the RAFTK-COOH-terminal domain, of Grb2-SH2, or of Grb2-SH3 domains were added to the lysates of the stimulated Jurkat cells. The complexes were immunoprecipitated with Glutathione-conjugated beads and the bound proteins were detected by anti-

**Figure 3.** RAFTK activation after T cell receptor stimulation. Unstimulated or stimulated Jurkat cell lysates were immunoprecipitated with RAFTK antibody. The immune complexes were either subjected to autophosphorylating activity (A) or in vitro kinase assays, using poly(γGlu/Tyr, 4:1) substrate (B). The 32P-incorporated proteins were resolved on 7.5% SDS-PAGE followed by autoradiography.

**Figure 4.** Association of RAFTK with Fyn and the SH2 domain of Lck. (A) Jurkat cells unstimulated or stimulated with anti-CD3 antibody OKT-3 (10 μg/ml) for different time periods were lysed in RIPA buffer and immunoprecipitated with RAFTK antibody. Immunoprecipitated complexes were resolved on 7.5% SDS-PAGE and immunoblotted with either anti-Fyn antibody (A, top) or RAFTK antibody (A, bottom). (B) Stimulated Jurkat cell lysates were incubated with GST-Fyn SH2 or SH3, or GST-Lck SH2 fusion proteins which were immunoprecipitated with Glutathione beads. The immunoprecipitates were run on 7.5% gels, transferred onto nitrocellulose membranes, and blotted with RAFTK antibody. GST protein only was used as a control.
tibodies to RAFTK or paxillin, we found a constitutive association of these two molecules (Fig. 6 A). Using the GST-COOH-terminal RAFTK protein, we observed a modestly increased association with paxillin after TCR/CD3 stimulation with anti-CD3 antibody (Fig. 6 B).

RAFTK Phosphorylation Is Inhibited by EGTA and Cytochalasin D. To examine the effect of calcium influx on TCR-induced activation of RAFTK, we treated Jurkat cells with anti-CD3 antibody in the presence or absence of the calcium chelator EGTA. The TCR-induced phosphorylation of RAFTK was partially reduced in the presence of EGTA (Fig. 7 A). This result suggests that the phosphorylation of RAFTK can be mediated in part by the increase in intracellular calcium concentration that is mediated by these stimuli.

To further investigate the role of the cytoskeleton in the tyrosine phosphorylation of RAFTK, Jurkat T cells were preincubated for 60 min at 37°C with media alone (−CD) or with media plus cytochalasin D (+CD) before TCR stimulation. Cytochalasin D is known to disrupt the cytoskeletal structure in T lymphocytes and other cells (14, 17, 34). The phosphorylation of RAFTK was reduced after the cytochalasin D treatment of cells (Fig. 7 B). No change in the levels of RAFTK proteins was observed under these conditions (Fig. 7 B). These results suggest that an intact cytoskeleton is important in RAFTK phosphorylation after TCR stimulation.

Discussion

Our studies indicate that RAFTK, a novel signaling molecule that appears to be a member of the FAK family, is present in human T lymphocytes and participates in signaling pathways after T cell activation. After the ligation of the TCR/CD3, there was a robust phosphorylation of RAFTK in both the model permanent T cell lines Jurkat and H9, as well as in primary human PBLs. Furthermore,
RAFTK was also activated, which was apparent both in the enhanced autophosphorylation of RAFTK as well as the increase in its in vitro kinase activity as determined using a poly (Glu/Tyr, 1:4) substrate. Parallel studies using other T cell activators, specifically the lectin PHA, revealed a similar phosphorylation of RAFTK in a time- and concentration-dependent manner. Furthermore, it appears that calcium influx may be required for the TCR-mediated tyrosine phosphorylation as well as the depletion of extracellular calcium by EGTA partially blocked the RAFTK phosphorylation induced by these stimuli. Pyk2/RAFTK phosphorylation has also been shown to be associated with changes in calcium in other cell types (19, 27, 28).

It is noteworthy that after phosphorylation, RAFTK was found to be capable of associating with the TCR/CD3 complex, and is also believed to play an important role in initiating the changes in phosphorylation that lead to further downstream signaling. This role has been most clearly demonstrated in studies showing the impaired development of CD4+ CD8+ thymocytes from double mutant mice rendered null for Fyn and FAK through homologous recombination (35). In addition, thymocytes from transgenic mice overexpressing Fyn were hyperstimulatable, and overexpression of a catalytically inactive form of Fyn substantially inhibited TCR-mediated activation in otherwise normal thymocytes (36). Grb2 is a well-characterized adapter molecule that is capable of associating with a number of kinases and substrates and may also act to facilitate signaling through the enhancement of the physical association of such partners in enzymatic reactions (37–39). Grb2 and Src have also been shown to play an important role in T cell signaling (5, 40–42). Recently, in PC12 pheochromocytoma cells, Pyk2/RAFTK has been shown to associate with activated src kinase. This interaction occurred through the SH2 domain of src, which bound to the COOH terminus of Pyk2/RAFTK (28). The SH2 domain of Fyn has also been shown to associate with RAFTK in other cell types (26). Also in PC12 cells, Grb2 has been reported to be associated with Pyk2/RAFTK (19). Thus, there appears to be similarities in the associations of activated RAFTK with certain signaling molecules in different cell types. RAFTK also associates with the SH2 domain of Lck, which has been shown to play an important role in TCR-induced signal transduction (33).

Our observations on RAFTK suggest that this novel signaling molecule could play a variety of roles in the transduction of T cell signals. Although further work is required to elucidate such functions of RAFTK, the association of RAFTK with the cytoskeletal protein paxillin provides a direction for these future efforts. The confluence of signaling molecules and cytoskeletal components may provide a platform for the regulated interactions of kinases and substrates and may lead to important changes in cell morphology that enable other aspects of the immune response such as adhesion or migration. Work in adherent mesenchymal cells indicates that the formation of the so-called focal adhesions may facilitate the creation of these platforms and mediate cell attachment and transduction of signals (23, 24, 43). Relatively little is known about similar mechanisms in hematopoietic cells like T lymphocytes. Recently, another member of the FAK family, termed FAK B, was identified. Initial studies indicated that FAK B may associate with ZAP-70, an intracytoplasmic protein tyrosine kinase also capable of associating with TCR (44). Amino acid sequence analysis demonstrates that RAFTK is not FAK B; however, future studies of the possible association of RAFTK with FAK B or with stimulatory molecules like ZAP-70 will be of value in elucidating these signaling mechanisms.

There is relatively limited information available on the convergence of protein tyrosine kinases and cytoskeletal elements in T lymphocytes. Several T cell surface structures, including CD11a/CD18 and CD44, associate with the cy-
toskeleton upon receptor cross-linking. Recently, the interaction of the \( \zeta \) chain of TCR with the actin cytoskeleton upon T cell activation was demonstrated (17). Our results revealed that RAFTK co-associates with paxillin, a major component of the cytoskeleton. Like FAK, the proline-rich COOH terminus of RAFTK binds to paxillin (24). Furthermore, the pretreatment of cells with cytochalasin D results in the reduced tyrosine phosphorylation of RAFTK upon T cell receptor activation. This result suggests that RAFTK phosphorylation may require the formation of a cytoskeletal complex, which provides a foundation for the interactions and compartmentalization of kinases and substrates. Future studies will further define whether other cytoskeletal components are associated with RAFTK upon T cell activation.

It has been suggested that in addition to FAK B, RAFTK may also participate in regulatory events after T lymphocyte activation (45). These earlier data, in conjunction with the results from our study, strongly suggest that molecules like FAK and RAFTK are important in coordinating a repertoire of kinases and adapter molecules in T cells, which then are capable of transmitting regulated signals downstream to the transcriptional activators that modulate gene expression. In this regard, in the PC12 neuronal cell model, Pyk2/RAFTK activation coupled stress signals to the c-Jun-NH\(_2\) terminal kinase pathway (27). This pathway is known to activate AP-1 and has been previously reported to participate in T lymphocyte signaling after costimulation (46, 47). Our future focus will be to assess whether inherited or acquired abnormalities in T cell function involve the activation and subsequent associative functions of RAFTK as it colocalizes signaling molecules with the cytoskeleton, and how this may be linked to transcriptional activation.

We thank our colleagues Zhong-Ying Liu and Jian-Feng Wang for their technical assistance. We would also like to thank Dr. David J. McConkey and Dr. Dananagoud Hiregowdara for their helpful discussions. We are grateful to Janet Delahanty for her editing and preparation of the figures and to Jennifer McGrath and Evelyn Gould for their assistance with the figures for this manuscript. Finally, we appreciate Tee Trac for her typing assistance and Delroy Heath for facilitating our receipt of the needed reagents for the experiments. This paper is dedicated to the memory of our colleague Dr. Dananagoud Hiregowdara.

This work was supported in part by National Institutes of Health grants HL 53745-02, HL 43510-07, HL 55187-01, HL 51456-02, and HL 55445-01, and the David Geffen Foundation.

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Received for publication 4 November 1996 and in revised form 15 January 1997.

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