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Membrane cofactor protein (MCP; CD46): Isoform-Specific Tyrosine Phosphorylation

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Membrane cofactor protein (MCP; CD46) is a widely expressed type 1 transmembrane glycoprotein that inhibits complement activation on host cells. It also is a receptor for several pathogens including measles virus, Streptococcus pyogenes, Neisseria gonorrhoea, and Neisseria meningitidis. That MCP may have signaling capability was suggested by its microbial interactions. That is, binding of MCP on human monocytes by measles virus hemagglutinin or cross-linking by an anti-MCP Ab resulted in IL-12 down-regulation, while binding to MCP by Neisseria on epithelial cells produced a calcium flux. Through alternative splicing, MCP is expressed on most cells with two distinct cytoplasmic tails of 16 (CYT-1) or 23 (CYT-2) amino acids. These play pivotal roles in intracellular precursor processing and basolateral localization. We investigated the putative signal transduction pathway mediated by MCP and demonstrate that CYT-2, but not CYT-1, is phosphorylated on tyrosine. We examined MCP tail peptides and performed Ab cross-linking experiments on several human cell lines and MCP isoform transfectants. We found an MCP peptide of CYT-2 was phosphorylated by a src kinase system. Western blots of the cells lines demonstrated that cells bearing CYT-2 were also phosphorylated on tyrosine. Additionally, we provide genetic and biochemical evidence that the src family of kinases is responsible for the latter phosphorylation events. In particular, the src kinase, Lck, is required for phosphorylation of MCP in the Jurkat T cell line. Taken together, these studies suggest a src family-dependent pathway for signaling through MCP. The Journal of Immunology, 2000, 164: 1839–1846.

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1 Abbreviations used in this paper: MCP, membrane cofactor protein; MCP CYT-1, MCP bearing cytoplasmic tail one; MCP CYT-2, MCP bearing cytoplasmic tail two; MV, measles virus; CHO, Chinese hamster ovary cell line; GB24 and TRA-2–10, anti-MCP mAbs; anti-pTyr, anti-phosphotyrosine; PY20, anti-pTyr with or without HRP mAb; MOPC-21, IgG1 mAb of unknown antigenic specificity; CCP, complement control protein.

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pathways is likely to determine the nature of the cellular response to its natural ligands and to the three pathogens that bind MCP. In this study, tyrosine phosphorylation of MCP bearing CYT-2 but not CYT-1 was induced in several human cell lines by cross-linking with anti-MCP mAbs. In contrast with the tyrosine phosphorylation of MCP observed in the Jurkat T cell line, tyrosine phosphorylation of MCP was markedly reduced in a Lck-deficient T cell line (JCaM1.6). The latter taken together with data that an MCP peptide of CYT-2 could be in vitro phosphorylated by p60src implicates this family of kinases in the signaling pathway of MCP.

Materials and Methods

Cell lines

Cloned Chinese hamster ovary (CHO) cell lines expressing each of the four isoforms of MCP have been previously described (25). These cells were grown in Ham’s F12 medium supplemented with 10% FBS, 0.5 mg/ml geneticin, and 1 mM L-glutamine. We also used a T cell leukemia cell line, Jurkat (American Type Culture Collection, Manassas, VA), and three mutant Jurkat cell lines. The latter are P116 (deficient in ZAP-70 kinase; Ref. 38), JCaM1.6 (deficient in Lck kinase; Ref. 39), and J45.01 (deficient in CD45 phosphatase; Ref. 40). Both parental and mutant cell lines were grown in RPMI 1640 supplemented with 10% FBS, L-glutamine, and antibiotics. The human cervical epithelial cell line ME-180 (American Type Culture Collection) was maintained in McCoy’s 5A medium supplemented with 10% FBS, L-glutamine, and antibiotics. Tissue culture reagents were obtained from the Tissue Culture Support Center at Washington University School of Medicine.

Abs

Two mAbs against MCP were employed. TRA-2–10, a gift of P. W. Andrews, binds to an epitope in CCP 1 and does not block complement regulatory function (29, 41). GB24, a gift from B. L. Hsi, binds to an epitope in CCP 3/4 and does block function (27, 42). The anti-MCP rabbit polyclonal antiserum was produced by CytoMed (Cambridge, MA). Two anti-phosphotyrosine (anti-pTyr) mAbs were employed (both from Transduction Laboratories, Lexington, KY): PY20 with and without a HRP label. The control mAb was MOPC-21 (Sigma, St. Louis, MO).

MCP peptide-binding ELISA

Four peptides, MCP CYT-1 (TYYLTDTHREVKFTSL), CYT-2 (KADG GAEYATQKTSSITPA), CYT-3 (KIKKNGKHTFSEVE) were used for the cell-free phosphorylation experiments. The peptides were coated overnight at 4°C on microtiter wells (Nunc modules, Fisher Scientific, St. Louis, MO) at 80 μg/ml in PBS. Wells were blocked with 1% BSA in 0.01% Tween 20 in PBS for 1 h at 37°C. A polyclonal rabbit anti-MCP Ab was used to establish that the peptides had been adsorbed onto the solid phase in equivalent amounts. Wells were rinsed with kinase reaction buffer (50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.015% Brij-35, 50 μg/ml BSA, 0.075% 2-ME, 0.05 mM ATP, 10 mM MgCl2) followed by incubation with the kinase p60src. The ELISA results indicated that CYT-2 but not CYT-1 was phosphorylated in a cell-free system for tyrosine phosphorylation by src kinase, protein kinase C, and casein kinase 2 are shown in Fig. 1, A and B. CYT-1 has one tyrosine residue, and CYT-2 possesses two. To examine if the cytoplasmic tails of MCP are phosphorylated, CYT-1 and CYT-2 peptides containing these tyrosines were assayed in a cell-free system for tyrosine phosphorylation by p60src. The ELISA results indicated that CYT-2 but not CYT-1 can serve as a substrate for p60src kinase (Fig. 2). CYT-2 peptide was phosphorylated by p60src in a dose-dependent fashion (Fig. 2A). Two other MCP-derived peptides, one possessing two tyrosine residues (C-UN) and one without (CCP 3), were not phosphorylated (Fig. 2B).

RT-PCR

Total RNA was isolated using the RNeasy mini protocol (Qiagen, Valencia, CA). Isolated RNA was quantitated by OD and was checked with a 1% agarose gel. Both cDNA synthesis and PCR were performed in a single test tube from RNA using gene-specific primers and Superscript One-Step RT-PCR system (Applied Biosystems with Perkin-Elmer GeneAmp PCR System 9600, Foster City, CA). The sequence of the 5′ MCP-CCP 4 primer was GGTGTCAAATGTCGATTTCCAGTAGTCG and the 3′ untranslated primer was CAAGGCCCCATTGGAATATAGCTAAGGCCCACA. These primers allow MCP isoforms, which arise through alternative splicing, to be distinguished (23). DNA in a total volume of 50 μl (1 μl of 1 μg/ml RNA; 25 μl of 2X reaction mix; 1 μl of 1 μg/ml sense and anti-sense primers, respectively; 1 μl of reverse transcription/heat mix; 21 μl of distilled water) was incubated at 50°C for 30 min to prepare cDNA. The PCR amplification was initiated by denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. At the completion of the cycling reactions, a final extension was performed at 72°C for 10 min. The PCR products were analyzed on a 3% agarose gel. This semiquantitative PCR is linear at least within 25–45 cycles (Ref. 23 and our unpublished observations).

Results

CYT-2 peptide serves as a substrate for p60src

The structure of MCP isoforms, the amino acid sequence of the two tails, and putative signals for phosphorylation by src kinase, protein kinase C, and casein kinase 2 are shown in Fig. 1. A and B. CYT-1 has one tyrosine residue, and CYT-2 possesses two. To examine if the cytoplasmic tails of MCP are phosphorylated, CYT-1 and CYT-2 peptides containing these tyrosines were assayed in a cell-free system for tyrosine phosphorylation by p60src. The ELISA results indicated that CYT-2 but not CYT-1 can serve as a substrate for p60src kinase (Fig. 2). CYT-2 peptide was phosphorylated by p60src in a dose-dependent fashion (Fig. 2A). Two other MCP-derived peptides, one possessing two tyrosine residues (C-UN) and one without (CCP 3), were not phosphorylated (Fig. 2B).
Tyrosine phosphorylation of MCP is induced in three cell lines by pervanadate

Treatment of cells with pervanadate inhibits tyrosine phosphatases and mimics receptor engagement in T, B, and NK cells. Therefore, we asked if pervanadate treatment promoted phosphorylation of MCP on Jurkat, ME-180, and CHO cells (Figs. 3 and 4). Cell lysates derived from treated cells were immunoprecipitated with GB24 (anti-MCP), PY20 (anti-pTyr), or MOPC-21. The precipitates derived from treated cells were immunoprecipitated with MCP on Jurkat, ME-180, and CHO cells (Figs. 3 and 4). Cell lysates were solubilized and electrophoresed on SDS-polyacrylamide gels and then transferred and blotted with a rabbit polyclonal anti-MCP antibody (Figs. 3 and 4). As shown in Fig. 3, the CHO cell lines expressing BC2 or C2 MCP isoforms (lanes 8 and 14) were tyrosine phosphorylated. These phosphorylated bands aligned with MCP in lanes 7 and 13. Based on scanning of the gels, 5–30% of the MCP of BC2 and C2 isoforms was phosphorylated. There was no detectable tyrosine phosphorylation of BC1 or C1 (lanes 1 and 5). Under the same experimental conditions, Fig. 4A demonstrates that Jurkat and ME-180 cells are also tyrosine phosphorylated (lanes 2 and 5) and that these phosphorylated proteins align with MCP as expressed by these two cell lines. In these two cell lines, 50–80% of the MCP was phosphorylated. In addition, blotting with anti-pTyr on Jurkat and ME-180 cells (Fig. 4B, lanes 1 and 5) indicated that MCP was phosphorylated, consistent with the results shown in Fig. 4A. Protein tyrosine phosphorylation was not detected in unstimulated cells (Fig. 4B, lanes 3 and 7) or in cells immunoprecipitated with a control mAb (lanes 2 and 6).

Three additional points facilitate the identification of the protein bands in Fig. 4. First, for both cell lines MCP migrated identically following precipitation by either anti-MCP or anti-pTyr mAbs (compare lanes 1 and 2 and lanes 4 and 5). Second, upon reduction and separation by SDS-PAGE (compare Fig. 4, A and B), protein bands characterized more narrowly and migrated more slowly (~5 kDa greater in molecular mass). The latter is due to the disruption of the two disulfide bonds in each of four CCP modules. Third, the difference in M, between the upper bands in Jurkat and ME-180 is secondary to variations in glycosylation (see next section).

MCP isoforms expressed by Jurkat and ME-180 cells

The previous data (Fig. 4) establishes that MCP was tyrosine phosphorylated on Jurkat and ME-180 cell lines. Because most human cells and cell lines express four isoforms of MCP, we next undertook the identification of the specific isoforms in these cell lines that were phosphorylated. To do this, we used a semiquantitative RT-PCR, previously developed in our laboratory (23), which allows for easy separation of the product coding for each isoform. The RT-PCR was performed in parallel with the two cell lines and with MCP-transfected CHO cells expressing a single isoform (Fig. 5). The PCR bands derived from Jurkat and ME-180 mRNA were identical in mobility with those obtained from the four CHO...
cells lines. For Jurkat cells, BC2 was predominant, representing 50%, while BC1 represented 15%, C2 20%, and C1 5%. For ME-180, the BC2 isoform also was predominant, representing 50%, while C2 represented 30%, BC1 15%, and C1 5%. Thus, for both Jurkat and ME-180, CYT-2-containing isoforms were the major species (70 and 80%, respectively), with the majority of the protein being BC2. Taken together with data presented in Figs. 2 and 3 and the fact that 50% or greater of MCP was phosphorylated in these two cell lines, we conclude that isoforms bearing CYT-2, but not CYT-1, likely undergo tyrosine phosphorylation.

We next compared these data to that of the Western blots in Fig. 4. Both Jurkat and ME-180 express a predominant upper band phenotypic MCP pattern. In a survey of normal individuals, the quantity of upper bands (BC1 and BC2) expressed versus that of the lower band forms (C1 and C2) is an inherited trait, with 65% of the population expressing predominantly the upper band forms, 29% equal quantities, and 6% predominantly the lower band (23, 43). Thus, the Jurkat cell line is derived from an individual with the most common upper band phenotype (the lower molecular mass is visible upon longer exposures). This upper band of Jurkat migrates faster than that of ME-180. MCP displays a characteristic microheterogeneity of $M_r$ due to variations in glycosylation among cell types (34, 44), and most epithelial-derived cells are a few kDa larger than peripheral blood cells or cell lines. Thus, the RT-PCR results in Fig. 5 and the protein data of Fig. 4 strongly suggest that the BC2 isoform is the dominant isoform tyrosine phosphorylated in Jurkat. Similarly for ME-180, we conclude that both BC2 and C2 are phosphorylated.

Cross-linking of cell-surface MCP induces rapid tyrosine phosphorylation

MCP on Jurkat cells was cross-linked with GB24 and evidence for tyrosine phosphorylation was sought. Jurkat cells were incubated with GB24 at 37°C for 1–30 min. The anti-MCP immunocomplexes were electrophoresed on SDS-polyacrylamide gels, transferred, and then immunoblotted with anti-pTyr mAb (Fig. 6). MCP was rapidly tyrosine phosphorylated, with the peak occurring between 1 and 2 min, which was followed by a steady decrease for the next 10 min. The Jurkat cells were also incubated over the same time periods with GB24, but this time at 4°C. Similar results were observed, although the staining was less intense (not shown). In addition, the Jurkat cells were stimulated with pervanadate for

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Western blot analysis of MCP isoforms treated with pervanadate. CHO cells were incubated with pervanadate for 10 min at room temperature. The cells ($4 \times 10^7$) were then lysed and immunoprecipitated with GB24 (mAb to MCP), PY20 (mAb to pTyr), or MOPC-21 (control mAb). The solubilized precipitates were electrophoresed under nonreducing conditions. Following transfer, immunoblotting was performed with a polyclonal rabbit anti-MCP antiserum. The heavy bands in each lane with an $M_r$ of $\sim 160$ kDa represent a cross-reaction between the Ab used for immunoprecipitation and the Ab used for detection.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Tyrosine phosphorylation of MCP expressed by Jurkat and ME-180 cells. General design of the experiment was as described in Fig. 3. Cells ($1 \times 10^6$) were lysed, and the lysates were immunoprecipitated employing the Ab noted. A. Electrophoresis was performed under nonreducing conditions, and immunoblotting was done with a polyclonal rabbit anti-MCP antiserum. GB24, mAb to MCP; PY20, mAb to pTyr; MOPC-21, mAb of unknown specificity. B. Electrophoresis was performed under reducing conditions, and immunoblotting was performed with HRP-PY20 (anti-pTyr) mAb. The band at 50 kDa in lane 5 is unidentified. “Stim” refers to conditions where the cells were or were not incubated with pervanadate. Under reducing conditions, MCP migrates slower in gels and the major bands focus less broadly (3).
10 min after incubation with GB24. In this case, there was also rapid tyrosine phosphorylation (not shown) in the presence of a phosphatase inhibitor, although as expected it persisted for up to at least 30 min.

**Tyrosine phosphorylation of MCP is dependent upon Lck**

To further characterize the tyrosine phosphorylation of MCP, mutant Jurkat cell lines deficient in a kinase or phosphatase were employed. The Jurkat (parental), P116 (deficient in ZAP-70 kinase), JCaM1.6 (deficient in Lck kinase), and J45.01 (deficient in CD45 phosphatase) expressed similar quantities of MCP (Fig. 7). MCP on these cells was cross-linked with GB24 (Fig. 8A) or TRA-2–10 (Fig. 8B) at 37°C for 2 or 20 min. The lysates were immunoprecipitated with these same mAbs. Following electrophoresis and transfer, the gels were immunoblotted with anti-pTyr mAb (top panels) or anti-MCP polyclonal Ab (bottom panels). The parental Jurkat, P116, and J45.01 cell lines demonstrated tyrosine phosphorylation of MCP at 2 min, which was abrogated by 20 min. However, this phosphorylation at 2 min was absent or markedly reduced (on longer exposures a faint band could be seen) in JCaM1.6 cells. Similar results were obtained with both mAbs.

**Discussion**

MCP regulates complement activation by serving as a membrane-bound cofactor for the plasma serine protease factor I to cleave C3b and C4b (reviewed in Refs. 2, 3, and 7). MCP binds these two ligands efficiently if they are attached to the same cell on which MCP itself is expressed (45). But MCP lacks receptor function. It inefficiently binds C3b- or C4b-bearing fluid-phase or cell-bound immune complexes (1, 45). Thus, MCP’s role is to transiently interact with either of these ligands bound to its “home” cell. This cofactor activity is an absolute requirement for cleavage by factor I of C3b to C3bi and C4b to C4c and C4d. The resulting C3bi or C4d cleavage fragments cannot form C3 or C5 convertases. This effectively halts further complement activation. In this scenario, there is no a priori reason why MCP is required to send a signal to the cell interior. On the other hand, C3b on a host cell is abnormal and potentially injurious. This does occur, for example, at a site of inflammation featuring complement activation. In this view, one function of MCP would be to provide a signal to the cell within this localized inflammatory process. The epithelial, endothelial, or blood cell in such a microenvironment would then respond by synthesizing proteins to protect its integrity and facilitate an inflammatory response. This line of reasoning provides a physiologic, sentry-like purpose for signaling through MCP.

A second reason for suspecting an important role for MCP in cell signaling relates to its cytoplasmic structure. A surprise in the molecular analysis of MCP was the identification of two cytoplasmic tails that arise by alternative splicing (23, 24). Analysis by RT-PCR indicated that both tails were expressed by most cells and that both carried motifs for interactions with kinases (23, 35). A third reason relates to tissue-specific isoform expression, most noteworthy in the brain, where cytoplasmic tail 2 is expressed predominantly, if not exclusively, on neural tissue (32, 33). Also, some cell lines, such as the ME-180 cells used in this study, express predominantly one tail or the other.
The fourth reason for pursuing signaling activity mediated by MCP is the recently published experiments on biological effects of microbes that interact with MCP. Karp et al. demonstrated that IL-12 production was down-regulated in primary human monocyte cultures following exposure to MV (20). Because MCP is a receptor for MV hemagglutinin (4–6), the likely interpretation of these results is that hemagglutinin on the viral envelope cross-links MCP to initiate a signaling event causing reduced synthesis of IL-12. Moreover, in the same experimental system, dimeric C3b and a mAb to MCP mimicked the effects of viral infection on IL-12 production, providing more evidence for MCP being critical to this response. Neisseria gonorrhea and Neisseria meningitidis have also been shown to use MCP as a receptor (9). Kallstrom and colleagues demonstrated that an MCP-dependent cell signaling event (induction of Ca²⁺ flux) occurred upon exposure of human epithelial cells to the purified pili of these pathogenic Neisseria (22). Additionally, employing a mouse macrophage cell line transfected with human MCP, Hirano et al. observed changes in NO production following stimulation with IFN-γ that were related to which tail of MCP was expressed (46). Finally, intracellular domains were a critical factor for effective virus-cell fusion and subsequent MV replication (47, 48), again suggesting the possibility of a signal transducing event mediated by MCP.

CYT-1 possesses potential phosphorylation sites for protein kinases C and casein kinase 2, while CYT-2 possesses potential phosphorylation sites for src kinases and casein kinase 2 (35). To define a mechanism by which MCP could mediate signal transduction events, we analyzed if CYT-1 or CYT-2 was phosphorylated on tyrosine. After our initial studies indicated that a peptide derived from CYT-2 of MCP could be tyrosine phosphorylated by a src kinase, we sought to extend these results by asking if MCP demonstrated differential tyrosine phosphorylation of its tails in Jurkat T cell lines, Lck, but not ZAP-70 or CD45, was required for the downstream mediators of this phosphorylation. Lck is found predominantly in T cells (51–55), where it has been shown to play a critical role during T cell activation (51, 56, 57). Lck is a 56-kDa src-related protein tyrosine kinase that is attached to the inner face of the plasma membrane via amino-terminal myristylation and palmitoylation (58). Lck has a dicysteine motif at this N terminus that mediates its association with CD4 and CD8 T cell-surface Ags. As with other related src kinases, the remaining amino-half consists of Src homology domains 3 and 2 while the carboxyl end consists primarily of the kinase domain. Cross-linking of CD4 or stimulation of the TCR by Ag leads to a rapid increase of intracellular tyrosine phosphorylation (59, 60). Lck is felt, in turn, to be responsible for the phosphorylation of several proteins engaged in TCR activation. More recently, a role for Lck in TCR function in downstream events also has been proposed (57).

While there are no published studies on tyrosine phosphorylation of the cytoplasmic tails of MCP, Wong et al. showed that the intracellular domains of human MCP were associated with kinases in a transfected mouse macrophage cell line stimulated with IFN-γ (49). In the case of CYT-1, the association was dependent on a sequence motif proximal to the tail. However, kinase activity remained upon deletion of this same region when CYT-2 was present. It may be that this “residual kinase” activity associated with CYT-2 reflects what we have observed in this study.

Many src family protein tyrosine kinases have been associated with membrane proteins involved in cell activation including Lck, Fyn, ZAP-70, syk, and others (50). Lck is found predominantly in T cells (51–55), where it has been shown to play a critical role during T cell activation (51, 56, 57). Lck is a 56-kDa src-related protein tyrosine kinase that is attached to the inner face of the plasma membrane via amino-terminal myristylation and palmitoylation (58). Lck has a dicysteine motif at this N terminus that mediates its association with CD4 and CD8 T cell-surface Ags. As with other related src kinases, the remaining amino-half consists of Src homology domains 3 and 2 while the carboxyl end consists primarily of the kinase domain. Cross-linking of CD4 or stimulation of the TCR by Ag leads to a rapid increase of intracellular tyrosine phosphorylation (59, 60). Lck is felt, in turn, to be responsible for the phosphorylation of several proteins engaged in TCR activation. More recently, a role for Lck in TCR function in downstream events also has been proposed (57).

The extensive data relating to a role for src kinases in general and Lck in particular in TCR activation can be used to facilitate interpretation of our studies of MCP. First, as shown in this report, MCP demonstrated differential tyrosine phosphorylation of its tails following cross-linking by Ab in the Jurkat T cell line. As noted, others have recently reported IL-12 down-regulation (20), Ca²⁺ fluxes (22), and NO (46) changes in association with experimental procedures that cross-link MCP. Src kinases are known to play a role in these types of signaling events as they do in T cell activation, which is accompanied by a calcium flux (50, 52, 61). Second, in Jurkat T cell lines, Lck, but not ZAP-70 or CD45, was required for tyrosine phosphorylation of MCP. The straightforward explanation is that Lck directly phosphorylates CYT-2 or that it is required for the downstream mediators of this phosphorylation. Lck is unlikely to be the only src kinase involved because it is not expressed in ME-180 cells or CHO cells (51, 60). Because T cell activation requires Lck, ZAP-70, and CD45, the signaling by MCP likely involves a distinct, albeit partially shared, signal transducing pathway. Thus, a comparison of these two pathways in a T cell line...
such as Jurkat could be informative relative to how this cell mediates signal transducing events from different effectors. Two widely expressed complement regulatory proteins, CD55 and CD59, are GPI linked and upon cell activation can associate with src family tyrosine kinases in specialized coated pits in the membrane (58, 62, 63). Signal transducing events leading to cell activation or, more commonly, the enhancement of other activating agents has been demonstrated for T lymphocytes, PBMC, granulocytes, and other cell types. Of interest, then, is our observation of a signaling event that also uses src kinase(s) and involves a transmembrane complement regulatory protein. These studies raise many intriguing questions relative to the potential role of physiologic and pathologic ligands in mediating MCP signaling and the identification of the intracellular pathways involved in these responses. For example, the penchant for two of three MCP-reacting pathogens (MV and Neisseria) to induce a cellular signal may parallel native ligands such as C4b and C3b. The finding that neuronal tissue primarily expresses CYT-2 suggested a physiologic relevance. Finally, other possible signaling events mediated by CYT-1 and CYT-2 remain to be discerned. In the future, we plan to determine the kinase(s) involved in other cell lines, to define the site of phosphorylation, and to connect the phosphorylation event(s) to a biologic response such as a calcium flux. We plan to focus initially on T cells and T cell lines where much information and potentially informative reagents are available to dissect a tyrosine phosphorylation event mediated by src kinases.

References


