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Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 16, Issue of April 17, pp. 9361–9364, 1998
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Printed in U.S.A.

A Calcium-dependent Tyrosine Kinase Splice Variant in Human Monocytes

ACTIVATION BY A TWO-STAGE PROCESS INVOLVING ADHERENCE AND A SUBSEQUENT INTRACELLULAR SIGNAL*

(Received for publication, February 5, 1998)

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Freshly isolated human monocytes do not express p125^{FAK} but upon adherence to substrata activate the highly related calcium-dependent tyrosine kinase (CADTK), also known as Pyk2, CAKβ, RAFTK, and FAK2. The monocyte CADTK was 5 kDa smaller than protein from epithelial cells; isolation and sequencing of the monocyte CADTK cDNA revealed a predicted 42-amino acid deletion between the two proline-rich domains of the enzyme. The nucleic acid sequence suggests that the deletion is caused by alternative RNA splicing. This species was also found in T and B lymphocytes and appears to be the predominant form of cytoskeletal associated tyrosine kinase in non-neoplastic, circulating, hematopoietic cells. CADTK was not activated when monocytes maintained in suspension were treated with agents that produce an intracellular calcium (thapsigargin) or protein kinase C (phorbol 12-myristate 13-acetate) signal including a chemokine, RANTES, that binds to the HIV co-receptor, CCK5. In contrast, monocyte adherence to tissue culture plastic-stimulated CADTK tyrosine phosphorylation, a process that was enhanced by thapsigargin, phorbol 12-myristate 13-acetate, and RANTES but that was completely blocked by preincubation with cytochalasin D. When compared with plastic, adherence to fibronectin- or collagen-coated surfaces produced only minimal CADTK activation but permitted significant stimulation by added thapsigargin. These data suggest that in a cell type that lacks p125^{FAK}, CADTK plays an early role in post-adherence signaling. Its activation involves two stages, cytoskeletal engagement, which is permissive, and co-stimulatory signals (calcium or protein kinase C) generated by extensive cell surface engagement, agonists, or inflammatory chemokines.

* This work was supported in part by grants from the American Cancer Society (to H. S. E.) and the National Institutes of Health (to J. S. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Peripheral monocytes circulate until they encounter an injured or activated endothelial surface to which the receptors of the monocyte adhere (1, 2). This results in shape changes initiating migration as well as altered transcription and mRNA stability, which in turn change gene expression and produce a more differentiated phenotype (3–5). Adherence to extracellular matrix or engagement of fibroblast or epithelial cell surface integrins activates the focal adhesion kinase, p125^{FAK} (6–8). We have purified (9) and then sequenced (10) another member of the p125^{FAK} family whose regulation by calcium led us to call it the calcium-dependent tyrosine kinase (CADTK).[1] Four other groups isolated this kinase by molecular techniques, naming it Pyk2 (11), CAKβ (12), RAFTK (13), and FAK2 (14). CADTK is 45% identical and 66% similar to p125^{FAK}, but unlike p125^{FAK}, CADTK is not tyrosine phosphorylated in adherent, epithelial (10), neural (11), and smooth muscle cells (15). Rather CADTK is rapidly activated and tyrosine phosphorylated when an intracellular calcium or protein kinase C signal is generated (10, 11, 15). p125^{FAK}, but not CADTK, is detected in well studied fibroblast cell lines (*e.g.* NIH 3T3), whereas both enzymes are expressed in many neural and epithelial cells (10, 11, 16). In this report we demonstrate a third type of cell exemplified by freshly isolated monocytes, which express CADTK but not p125^{FAK}. In addition, CADTK activation in monocytes and epithelial cells is apparently a two-stage process involving a permissive cytoskeletal engagement step and an additional intracellular calcium or PKC signal. The concept of a hierarchy in adherence-dependent signaling in monocyte endothelial interactions is well established and may be reflected in the two-stage activation of CADTK.

EXPERIMENTAL PROCEDURES

Isolation and Adherence of Monocytes—Human monocytes were isolated from randomly selected, healthy donors as described previously (17). Purified monocytes were cultured in RPMI 1640 supplemented with 5% autologous serum at 37 °C under 5% CO_2. When cultured adherently, 5×10^6 to 5×10^7 monocytes were plated on polystyrene tissue culture dishes (Corning) or fibronectin (Becton Dickenson) or collagen Type IV (Sigma) coated culture dishes. Nonadherent monocytes were incubated in polystyrene tubes (Falcon) at cell concentration 10^6 cells/ml. Rat liver epithelial cells (GN4) were cultured as described (9).

Immunoprecipitation and Immunoblotting—Lysates were immunoprecipitated with CADTK (21) or p125^{FAK} antibody (A-17 or C-20, Santa Cruz Biotechnology) and analyzed as described (10).

PCR Analysis—Total cellular RNA from purified monocytes was isolated by the guanidimium isothiocyanate-CsCl method, and subsequently reverse transcribed with random hexamers as primers (17). Three sets of PCR primers, which cover N-terminal (5′-CTTAGCTGCTGCCTGAGAGG-3′, 5′-CAGCTGAAGTACTGCCTGGC-3′), catalytic (5′-GCCAGGCAGTACTTCAGCTG-3′, 5′-CCAGCAGCGGGTCATGAGGG-3′), and C-terminal domains (5′-CCCTCATGACCCGCTGCTGG-3′, 5′-GGTGGCCCCCACCCTCCGTC-3′) of human CADTK (Pyk2), were used to amplify the entire first strand cDNA from monocytes. PCR-amplified products were then cloned and sequenced. Cellular RNA of T and B cell lines were kindly provided by Drs. Beverly Mitchell and Nancy Raab-Traub, respectively. First strand cDNAs were made by a Superscript™ preamplification system according to the manufacturer's (Life Technologies, Inc.) protocol. The C-terminal PCR primers were

[1] The abbreviations used are: CADTK, calcium-dependent tyrosine kinase; Ang II, angiotensin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

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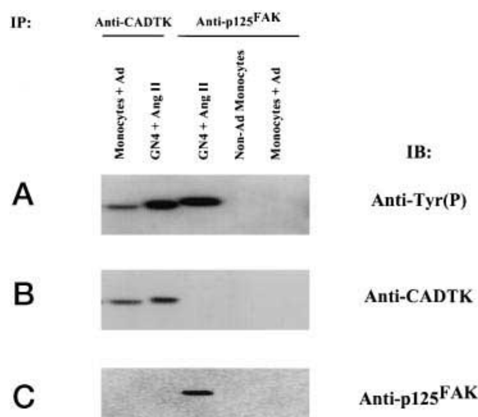

FIG. 1. **CADTK is tyrosine phosphorylated in freshly isolated adhered monocytes but p125^{FAK} is absent.** CADTK or p125^{FAK} was immunoprecipitated (*IP*) from adhered (30 min) human monocytes or Ang II-treated rat liver epithelial cells (GN4). *A*, anti-Tyr(P) immunoblot (*IB*) showed tyrosine phosphorylated CADTK in adhered monocytes and Ang II-treated GN4 cells, but Tyr(P) p125^{FAK} was only seen in GN4 cells. *B*, reprobing with anti-CADTK antibody demonstrated a slight difference in the electrophoretic mobility of CADTK protein from human monocytes and GN4 cells. *C*, p125^{FAK} immunoblotting demonstrated p125^{FAK} in GN4 cells but not in human monocytes.

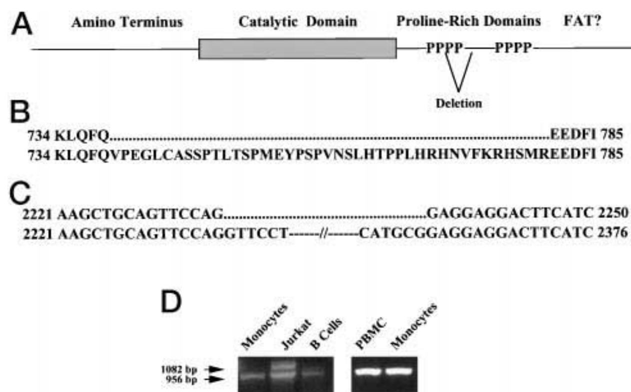

FIG. 2. **Structure and expression of a putative monocyte CADTK splice variant.** *A*, schematic of CADTK showing the N-terminal domain of unknown function, the tyrosine kinase domain, the two proline-rich motifs separated by 63 amino acids, and a potential focal adhesion targeting domain that is 70% similar by amino acid sequence to p125^{FAK}. The area of deletion in the proline-rich domain is shown. *B*, the predicted monocyte amino acid sequence is identical to the published human Pyk2 sequence with the exception of the deletion shown aligned with the full-length sequence. *C*, the nucleic acid sequence surrounding the splice variant reveals potential RNA splice sites for acceptance and donation. *D*, PCR primers designed to distinguish the full-length and deleted isoforms were synthesized (see "Experimental Procedures") and were predicted to yield PCR products of 1082 or 956 base pairs from the full-length and deleted isoform, respectively. Monocytes and a B cell line express the deleted isoform; Jurkat cells expressed both forms in roughly similar amounts. Subcloning and sequencing confirmed the identity of both PCR products. First strand cDNA from human peripheral blood mononuclear cells (*PBMC*), a mixture of monocytes, and B and T cells demonstrated only the 956-base pair fragment.

used to distinguish between the full-length and 126-base pair-deleted isoforms.

RESULTS AND DISCUSSION

Monocytes Do Not Express p125^{FAK} but Activate CADTK on Adherence—We adhered freshly isolated, lymphocyte-depleted monocytes (an approximately 95% pure population) to plastic tissue culture dishes for 30 min and immunoprecipitated CADTK and p125^{FAK}. Comparison with parallel immunoprecipitates from angiotensin II (Ang II)-treated GN4 rat liver epithelial cells revealed tyrosine phosphorylated CADTK in both cell types; however, the monocyte CADTK immunoreactive species had a faster electrophoretic mobility (Fig. 1, *A* and *B*). In contrast to GN4 cells, monocytes lacked p125^{FAK} (Fig. 1*C*). We have noted, under less stringent conditions, *i.e.* those not involving preplating with autologous serum, that p125^{FAK}-containing, large, platelet-like entities were found in the monocyte preparations. With our technique, these were eliminated and p125^{FAK} was absent.

Monocytes as Well as T and B Lymphocytes Express a Putative CADTK Splice Variant—Monocyte CADTK had an estimated molecular mass of 110 kDa *versus* 115 kDa in GN4 cells. This could result from cell type-specific phosphorylation, antibody cross-reaction with yet another member of the p125^{FAK} family, proteolysis, or an encoded isoform. We used RNA from freshly prepared human peripheral monocytes to isolate and sequence the entire CADTK coding region. The monocyte CADTK cDNA sequence was almost identical to human Pyk2 with an exception, a 126-base pair deletion resulting in a molecule missing 42 amino acids between the two CADTK SH3 binding proline-rich domains (Fig. 2, *A* and *B*). Examination of the nucleic acid sequence reveals potential splice donor sites at the junctions of the deletion, suggesting that this isoform may be generated by alternative RNA splicing (Fig. 2*C*). A similar set of splice donor acceptor sites is found in the rat CADTK sequence, suggesting the capability of forming this splice variant in other species. CADTK (and p125^{FAK}) has two proline-rich domains; this deletion could influence the local tertiary structure, thereby changing individual CADTK-SH3 interactions (*e.g.* with p130^{Cas}). Alternatively, the simultaneous interaction of SH3 domains from different molecules with the two proline-rich regions of CADTK would be influenced by the different spacing between the two regions due to the 42-amino acid deletion. In addition, the deleted sequence is rich in prolines and serines characteristic of PEST-like sequences whose deletion might change the susceptibility to proteolysis (18), *i.e.* foreshortened CADTK could be more long-lived.

Lymphocyte CADTK (Pyk2/RAFTK) is activated by T cell receptor engagement (19, 20), a finding that we have replicated in mouse T lymphocytes activated with specific antigens.[2] Therefore, we investigated CADTK isoforms using first strand cDNA from human T, B, and epithelial cell lines. A PCR strategy designed to detect full-length and spliced CADTK revealed the short (splice variant) form in monocytes as well as T and B cell lines (Fig. 2*D*). Jurkat T cells also expressed a normal sized CADTK species (Fig. 2*D*). The identify of the PCR products from Jurkat and the B cell line were confirmed by subcloning and sequencing. The short form was identical to that of monocytes; the sequence of the larger Jurkat product was identical to full-length human CADTK/Pyk2. Analysis of multiple neoplastic T, B, and myelomonocytic leukemia cell lines reveals the presence of both the deleted and full-length isoforms. The epithelial lines (C33–1B cervical) and MCF10 (breast) do not exhibit the deleted isoform (Fig. 3*A*). Normal, monocyte-depleted, peripheral, blood, mononuclear cells exhibit either the deleted isoform alone (Fig. 2*D*) or a preponderence of the deleted isoform with a smaller proportion of the full-length isoform. The proportion of the full-length isoforms appears to be donor-specific. In summary, the deleted isoform is found in all hematopoietic cells tested with the amount of full-length isoform being variable but appearing to be increased with neoplastic transformation. Close examination of Figs. 2*D* and 3*A* reveals one other band in some T, B, and mononuclear cell line samples (*e.g.* CEM-LD, H-9, BL41, RAJ1, and U937). Currently, we have not identified a third isoform, but this possibility exists. Furthermore, PCR analysis of adherent monocyte

[2] A. Villette and H. S. Earp, unpublished results.

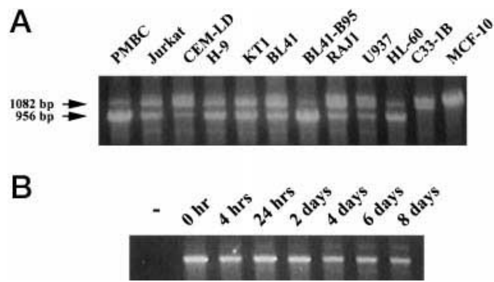

FIG. 3. **PCR analysis of CADTK isoform expression in mononuclear cell lines and adherent monocytes.** First strand cDNA from monocyte-depleted, peripheral blood mononuclear cells (*PBMC*, >70% T cells), human T cell lines (Jurkat, CEM-LD, H-9, and KT-1), B cell lines (BL41, BL41–95, and RAJ1), myelomonocytic leukemia lines (U937 and HL-60), and epithelial cell lines (C33–1B and MCF-10) were amplified with C-terminal CADTK primers. As shown in *A*, the deleted isoform of CADTK was expressed in all tested cell lines of hematopoietic origin, but not in human epithelial cell lines. Although the deleted isoform of CADTK predominates in monocytes, a small amount of the full-length isoform is seen after one day of monocyte adhered to plastic (*B*).

CADTK expression showed that expression of the longer isoform increased somewhat with long term monocyte adhesion (Fig. 3*B*).

Characterization of CADTK Tyrosine Phosphorylation Following Monocyte Adherence—CADTK tyrosine phosphorylation occurs within 5 min of adherence to tissue culture plastic (data not shown) and appeared maximal at 30 min. In adherent epithelial (10), neural (11, 16), and smooth muscle cells (15), CADTK is tyrosine phosphorylated upon addition of agonists. Thus, it was surprising that we failed to activate CADTK in freshly isolated monocytes in suspension with PMA treatment (Fig. 4, *A* and *C*). However, addition of PMA to adherent monocytes produced an additional increment in CADTK tyrosine phosphorylation above that of adherence alone (Fig. 4*A*). Continued adherence for 20 h or 4 days resulted in persistent CADTK tyrosine phosphorylation (Fig. 4*A*). We repeated the adherent/nonadherent experiment using PMA, the chemokine RANTES (which produces a distinct calcium signal) (21, 22), and the tumor promoter thapsigargin, which results in an intracellular calcium signal by blocking the intracellular calcium reuptake mechanism. Again, in suspended monocytes, agonists failed to stimulate significant tyrosine phosphorylation, although, in an occasional experiment, low but detectable levels of CADTK tyrosine phosphorylation were seen with thapsigargin (*e.g.* Fig. 4*C*). In adherent cells, the addition of PMA or, particularly, thapsigargin resulted in increased CADTK tyrosine phosphorylation (usually from 50% to 2–3-fold above that seen with adherence alone). The increase caused by thapsigargin was present whether thapsigargin was present during the entire 30 min adherence or during the last 5 min of the 30-min adherence protocol (data not shown). These results suggest two phases of activation, adherence followed by additional amplifying signals. Each experiment was performed with individual donors, and there were some donors in which adherence to tissue culture plastic produced near maximal CADTK tyrosine phosphorylation without additional agonists (data not shown).

The importance of cytoskeletal engagement was emphasized by a complete absence of CADTK tyrosine phosphorylation in cells adhered to plastic in the presence of cytochalasin D (Fig. 4*E*), even though similar amounts of CADTK were immunoprecipitated from both samples (Fig. 4*E*). In contrast, adherence in the presence of the microtubular inhibitor, colchicine (10 μM, 30 min), did not inhibit CADTK tyrosine phosphorylation (data not shown). Thus, it appears that engagement or involvement of the actin cytoskeleton but not the microtubules is necessary

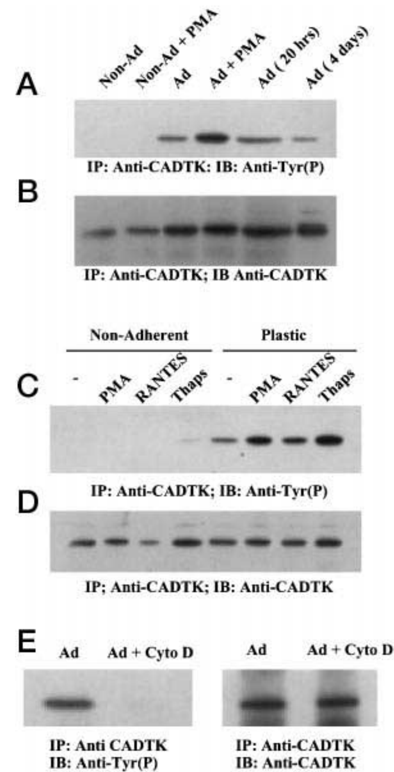

FIG. 4. **CADTK is tyrosine phosphorylated in adherent but not nonadherent monocytes.** Freshly isolated monocytes were prepared and either kept suspended or adhered to tissue culture dishes for 30 min, 20 h, or 4 days. Some samples were treated with PMA (100 nM), thapsigargin (*Thaps*, 2 μM), or RANTES (1 μM). Cells were lysed, immunoprecipitated (*IP*) with anti-CADTK antibody, and subjected to SDS-PAGE and immunoblotting. In one experiment, the anti-Tyr(P) immunoblot (*IB*, *A*) was reprobed with anti-CADTK (*B*), which revealed that PMA slightly increased CADTK tyrosine phosphorylation in adherent (*Ad*) but not in nonadherent (*Non-Ad*) monocytes. In a second experiment, the Anti-Tyr(P) (*C*) and reprobed anti-CADTK immunoblots (*D*) revealed that PMA, RANTES, and thapsigargin increased tyrosine phosphorylation of CADTK in adherent cells to a much greater extent than in nonadherent cells. In a third experiment, monocytes were adhered in the presence or absence of cytochalasin D (*Cyto D*, 2 μM, 30 min). Immunoblotting (*E*) revealed that cytochalasin D blocked adherence-dependent CADTK tyrosine phosphorylation.

for the CADTK activation.

Adherence to activated or injured endothelium is a multistage process that appears to involve several sets of monocyte-endothelial surface protein interactions. Experimentally, monocyte activation and subsequent gene expression differ depending on the substrata to which cells adhere (3–5, 17). To investigate any hierarchy in adherence signaling, we compared adherence to tissue culture plastic, a strong stimulus, with a more physiologic substratum, culture dishes coated with fibronectin or collagen. Adherence to the latter substrate produced low level CADTK tyrosine phosphorylation, but addition of thapsigargin to monocytes on fibronectin and collagen stimulated CADTK tyrosine phosphorylation to the level near that seen with adherence to plastic (Fig. 5*A*). Adherence to fibronectin and collagen appears to be a weak stimulus per se but is permissive, allowing thapsigargin, which has little or no effect in nonadhered monocytes, to fully promote CADTK tyrosine phosphorylation (Fig. 5*A*, *Exp. 1*). Similarly, adherence to fibronectin followed by RANTES resulted in enhanced CADTK tyrosine phosphorylation (Fig. 5*A*, *Exp. 2*). To further test the idea that cytoskeletal engagement is necessary for CADTK activation, we examined the effect of agonists on rat liver epithelial cells adhered to culture dishes and then suspended in same tissue culture medium for 15 min. Ang II, thapsigargin,

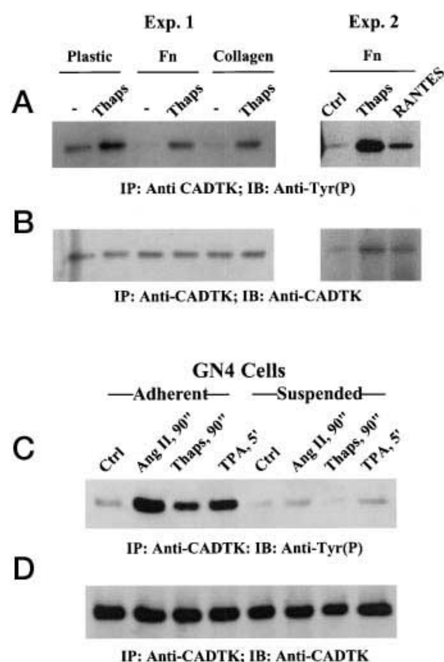

FIG. 5. **Adherence on fibronectin and collagen is less efficacious in activating CADTK tyrosine phosphorylation.** Freshly isolated monocytes were adhered on tissue culture plastic or dishes coated with fibronectin (*Fn*) or collagen. Adherent cells, treated with or without 2 μM thapsigargin (*Exp. 1* and *Exp. 2*) or RANTES (*Exp. 2*) were lysed, and CADTK immunoprecipitates (*IP*) were analyzed. The anti-Tyr(P) (*A*) and reprobed anti-CADTK (*B*) immunoblots (*IB*) revealed that adherence on plastic induced CADTK tyrosine autophosphorylation to a greater extent than adherence to fibronectin or collagen. Thapsigargin-stimulated CADTK tyrosine phosphorylation in monocytes adhered on all three substratum, but the effect was greater on fibronectin and collagen because basal tyrosine phosphorylation was lower. On fibronectin, RANTES also resulted in CADTK tyrosine phosphorylation. Adherent GN4 cells were suspended after a short trypsin treatment and washing three times. With culture medium, they were kept in suspension for 15 min. Both suspended and adherent GN4 cells treated with Ang II (1 μM), thapsigargin (*Thaps*, 2 μM), and TPA (100 nM) for the indicated time were lysed and CADTK immunoprecipitates were analyzed. The anti-Tyr(P) (*C*) and reprobed anti-CADTK (*D*) immunoblots showed that calcium and/or PKC signal significantly activate CADTK only in adherent but not in suspended cultured GN4 cells. *Ctrl*, control.

and TPA stimulate CADTK in adherent GN4 cells but fail to do so when added to GN4 cells in suspension, even though CADTK expression is very similar in these two conditions (Fig. 5, *C* and *D*). These data further support the hypothesis that cytoskeleton engagement is required for CADTK activation.

The discovery of a second member of the $p125^{FAK}$ tyrosine kinase family independently by five groups (10–14) has raised questions as to their similarities and differences between these proteins. In epithelial and smooth muscle cells, adherence results in sustained $p125^{FAK}$ tyrosine phosphorylation, whereas CADTK is dephosphorylated. Agonists stimulate CADTK, and additional paxillin and $p130^{Cas}$ tyrosine phosphorylation follows (23–25). The monocyte provides a slightly different model. CADTK is expressed but is not activated by thapsigargin, PMA, or RANTES in nonadhered peripheral monocytes. Adherence by itself produces a range in monocyte CADTK tyrosine phosphorylation depending on the substratum and, to some extent, the donor, which was enhanced by acute treatment with thapsigargin, PMA, or RANTES. The effect of cytochalasin D and the minimal tyrosine phosphorylation when monocytes were adhered to fibronectin and collagen support a potential two-stage process for CADTK tyrosine phosphorylation (Figs. 4 and 5). A similar deficit in thapsigargin or TPA-dependent activation is observed in GN4 epithelial cells (Fig. 5). This indicates that in adherent cells the first stage, engagement of the cytoskeleton, has already occurred and CADTK activation simply awaits a second cue, calcium or PKC activation. Whether this two-stage hypothesis implies distinct mechanisms, alignment to a cellular locale or structure followed by a calcium or PKC-dependent activating phosphorylation, or a continuum in which cytoskeletal engagement is followed by another cytoskeletal step that is indirectly influenced by calcium or PKC remains to be determined.

Acknowledgments—We thank Ruth Dy and Tim Harding for excellent technical assistance and Darla Nichols for manuscript preparation.

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