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, p125FAK (6–8). We have purified (9) and then sequenced (10) another member of the p125FAK 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), CAKb (12), RAFTK (13), and FAK2 (14). CADTK is 45% identical and 66% similar to p125FAK but unlike p125FAK, 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). p125FAK, 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 p125FAK. 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% CO2. When cultured adherently, 5 × 105 to 5 × 106 monocytes were plated on polylysine tissue culture dishes (Corning) or fibronectin (Becton Dickinson) or collagen Type IV (Sigma) coated culture dishes. Nonadherent monocytes were incubated in polylysine tubes (Falcon) at cell concentration 106 cells/ml. Rat liver epithelial cells (GN4) were cultured as described (9).

**Immunoprecipitation and Immunoblotting—**Lysates were immunoprecipitated with CADTK (21) or p125FAK 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 guanidium isothiocyanate-CsCl method, and subsequently reverse transcribed with random hexamers as primers (17). Three sets of PCR primers, which cover N-terminal (5′-CTTACGTGCTGCCGTGAGG-3′, 5′-CAAGCTGAAGTACTGCGTGCC-3′), catalytic (5′-GCAGCGGCAATCTTCAGCTG-3′, 5′-CAACGCGGCTTCATGAGGG-3′), and C-terminal domains (5′-GCTTCGACCGCCGCTGCG-3′, 5′-GCTCCCTCCACCCCACCTGCCG-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 SuperscriptII preamplification system according to the manufacturer’s (Life Technologies, Inc.) protocol. The C-terminal PCR primers were

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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-tetradecanoylphorbol-13-acetate.
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Fig. 1. CADTK is tyrosine phosphorylated in freshly isolated adhered monocytes but p125FAK is absent. CADTK or p125FAK 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) p125FAK 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, p125FAK immunoblotting demonstrated p125FAK in GN4 cells but not in human monocytes.

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

RESULTS AND DISCUSSION

Monocytes Do Not Express p125FAK 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 p125FAK. 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 p125FAK (Fig. 1C). We have noted, under less stringent conditions, i.e., those not involving preincubating with autologous serum, that p125FAK-containing, large, platelet-like entities were found in the monocyte preparations. With our technique, these were eliminated and p125FAK 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 p125FAK 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 (Ang II) sequence the entire CADTK coding region. The monocyte amino acid sequence is identical to the published human Pyk2 sequence. CADTK (and p125FAK) has 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. 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. 2D). Jurkat T cells also expressed a normal sized CADTK species (Fig. 2D). The identity 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. 3A). Normal, monocyte-depleted, peripheral, blood, mononuclear cells exhibit either the deleted isoform alone (Fig. 2D) 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. 2D and 3A reveals one other band in some T, B, and mononuclear cell lines (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...
CADTK expression showed that expression of the longer isoform increased somewhat with long term monocyte adhesion (Fig. 3B).

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. 4A). Continued adherence for 20 h or 4 days resulted in persistent CADTK tyrosine phosphorylation (Fig. 4A). We repeated the adherence/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. 4C). 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 above that seen with adherence alone (Fig. 4A).

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 RAJI), 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).

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 probed 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 multi-stage 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. 5A).

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. 4E), even though similar amounts of CADTK were immunoprecipitated from both samples (Fig. 4E). 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...
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 p125FAK 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 p125FAK tyrosine phosphorylation, whereas CADTK is dephosphorylated. Agonists stimulate CADTK, and additional paxillin and p130Cas 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.

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