Activation of Mitogen-Activated Protein Kinase Kinase by v-Raf in NIH 3T3 Cells and in Vitro

Paul Dent, Wayne Haser, Timothy A. J. Haystead, Leigh Ann Vincent, Thomas M. Roberts, Thomas W. Sturgill*

Mitogen-activated protein (MAP) kinases are 42- and 44-kD serine-threonine protein kinases that are activated by tyrosine and threonine phosphorylation in cells stimulated with mitogens and growth factors. MAP kinase and the protein kinase that activates it (MAP kinase kinase) were constitutively activated in NIH 3T3 cells infected with viruses containing either of two oncogenic forms ($p35^{EC12}$, $p37^{22W}$) of the c-Raf-1 protein kinase. The v-Raf proteins purified from cells infected with EC12 or 22W viruses activated MAP kinase kinase from skeletal muscle in vitro. Furthermore, a bacterially expressed v-Raf fusion protein (glutathione S-transferase–$p37^{22W}$) also activated MAP kinase kinase in vitro. These findings suggest that one function of c-Raf-1 in mitogenic signaling is to phosphorylate and activate MAP kinase kinase.

Many serine-threonine protein kinases are activated upon stimulation of cells with insulin and other peptide growth factors. These enzymes include the Raf protein kinases, MAP kinase kinase, p42 and p44 MAP kinases, and the 90-kD family of ribosomal protein S6 kinases (termed RSKs) (*1*, *2*). MAP kinase kinase, MAP kinase, and RSK appear to function in vivo in a cascade, with MAP kinase kinase phosphorylating and activating MAP kinase and MAP kinase phosphorylating and activating RSK (*1*). MAP kinase kinase is a 45- to 46-kD dual specificity protein tyrosine and threonine kinase (*3*–*5*), and is itself activated by serine-threonine phosphorylation (*6*), but the responsible enzymes have not been identified.

The c-Raf-1 protein kinase belongs to a family of serine-threonine protein kinases encoded by three known genes in mammals termed A-raf-1, B-raf, and c-raf-1 (*2*). The Raf protein kinases show similarity to members of the protein kinase C family both in the conserved catalytic domain and in the regulatory NH_2-terminal domain. The regulatory domain is truncated in v-Raf, the transforming gene product of the mouse sarcoma virus 3611. Truncation of the regulatory domain causes constitutive activation of Raf enzymatic activity. The c-Raf-1 protein kinase, a 74-kD protein, has been found in all mammalian tissues tested (*2*).

MAP kinase will phosphorylate c-Raf-1 in vitro, generating a phosphopeptide that is also present in c-Raf-1 recovered from insulin-stimulated cells (*7*, *8*). However, phosphorylation by MAP kinase does not activate c-Raf-1 enzymatic activity assessed either by autophosphorylation or mobility shift on SDS–polyacrylamide gel electrophoresis (PAGE) (*7*, *8*). Consequently, we examined the possibility that c-Raf-1 acts upstream in the cascade of kinases.

If c-Raf-1 acts upstream of MAP kinase, then expression of active v-Raf might result in the activation of MAP kinase. We measured the activities of MAP kinase kinase and MAP kinase in NIH 3T3 cells that had been infected with the recombinant viruses EC12 and 22W (*9*), which encode constitutively active v-Raf proteins, termed $p35^{EC12}$ and $p37^{22W}$, respectively. The $p35^{EC12}$ protein is truncated from c-Raf-1 by deletion of 334 NH_2-terminal amino acids (*9*). Cells infected with recombinant virus EC13 express an inactive 33-kD v-Raf protein ($p33^{EC13}$), formed by truncation of ten more amino acids than those deleted to form $p35^{EC12}$ (*9*). The $p37^{22W}$ protein (which lacks the 306 NH_2-terminal amino acids of c-Raf-1) has the most transforming activity of a series of c-Raf-1 truncation mutants (*9*).

In unstimulated NIH 3T3 cells or NIH 3T3 cells infected with EC13 virus, only basal activities of MAP kinase kinase and MAP kinase were detected (Fig. 1, A and B). However, when those cells were stimulated with serum, both MAP kinase kinase and MAP kinase became activated (Fig. 1A) (*10*). In contrast, in unstimulated cells infected with EC12 or 22W viruses, both MAP kinase kinase and MAP kinase were activated constitutively (Fig. 1, B and C). The extent of activation of MAP kinase in cells infected with the EC12 virus was comparable to that achieved by serum-stimulation of NIH 3T3 cells infected with EC13 virus (Fig. 1, A and B) or uninfected NIH 3T3 cells (*10*). The activity of MAP kinase

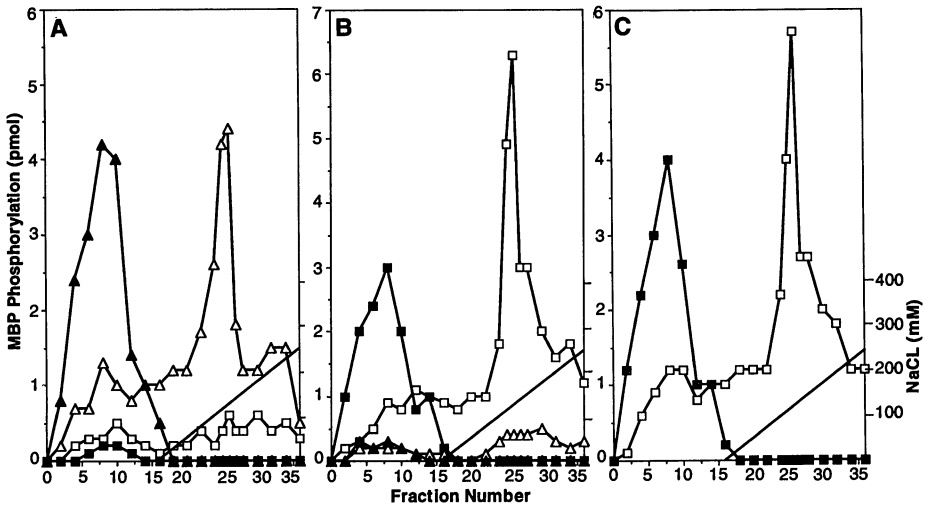

Fig. 1. MAP kinase kinase and MAP kinase activities in NIH 3T3 cells transformed by v-Raf. Cells were grown to confluence in Dulbecco's modified Eagle's medium containing fetal calf serum (10%). Cells were starved of serum for 2 hours in Kreb's Ringer bicarbonate–Hepes buffer (*20*). Cells from five 100-mm dishes were stimulated with fetal calf serum (10%, final) for 10 min or left unstimulated (control), washed with ice-cold 150 mM NaCl, and scraped into 0.5 ml of buffer A (*21*). The cells were homogenized by 20 strokes of a tight-fitting Dounce homogenizer, and centrifuged at 10,000*g* for 10 min. The resulting supernatant was made to 10 ml with buffer B (*21*) and applied to a HR5/5 Mono Q Fast Protein Liquid Chromatography (FPLC) column. Equal amounts of cell lysate protein (0.5 mg) were applied to each column. Chromatography was performed at a flow rate of 0.25 ml/min with a 20-ml linear gradient of 0 to 250 mM NaCl (fractions 16 through 36) in buffer B (*21*). Fractions (1 ml) were assayed for MAP kinase kinase activity (closed symbols), which stimulated incorporation of ^{32}P into MBP by activating recombinant MAP kinase that was present in the reaction mixture, or for MBP phosphotransferase activity (open symbols) as described (*4*). Shown is incorporation of ^{32}P into MBP catalyzed by fractions from Mono Q chromatography of extracts from: (**A**) cells infected with EC13 virus either serum-stimulated (triangular symbols) or unstimulated (square symbols); (**B**) unstimulated NIH 3T3 cells (triangular symbols) and unstimulated cells infected with EC12 virus (square symbols); or (**C**) unstimulated cells infected with 22W virus.

P. Dent, T. A. J. Haystead, L. A. Vincent, T. W. Sturgill, Departments of Internal Medicine and Pharmacology, University of Virginia, Charlottesville, VA 22908.
W. Haser and T. M. Roberts, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115.
*To whom correspondence should be addressed.

in cells infected with EC12 virus was not augmented by stimulation with serum (*10*). These differences in activity were not due to differences in expression of MAP kinase because similar amounts of MAP kinase immunoreactivity were detected in aliquots of Mono Q fractions taken across the peak of MAP kinase activity from cells infected with EC12, EC13, or 22W viruses (*11*, *12*).

Because these results implied that v-Raf was upstream of MAP kinase kinase in these cells and MAP kinase kinase activity is regulated by serine-threonine phosphorylation (6), we examined the possibility that v-Raf directly activates MAP kinase kinase. The v-Raf protein from cells infected with either EC12 or 22W viruses was partially purified by gel filtration chromatography on Superose-12 and detected by immunoblotting (Fig. 2, A and B). The v-Raf proteins usually eluted from gel filtration as a broad peak with an apparent molecular size of ~160 kD (Fig. 2, A and B), implying that they exist as a multimer or are complexed to other proteins. Infrequently, v-Raf eluted from gel filtration with an apparent molecular size of a monomer (*10*). Immunoprecipitates with antiserum to c-Raf-1 of Superose-12 fractions from cells infected with EC12 or 22W viruses (Fig. 2, A and B) contained $p35^{EC12}$ or $p37^{22W}$, respectively, as detected by protein immunoblotting with antiserum to c-Raf-1 (Fig. 2C). Control antiserum did not immunoprecipitate either protein (*10*).

Partially purified $p35^{EC12}$ and $p37^{22W}$ (Fig. 2, A and B) were concentrated and portions were used in reactivation experiments (*13*) either directly or after immunoprecipitation (Fig. 2C). MAP kinase kinase, partially purified from rabbit skeletal muscle and free of MAP kinase activity, was deactivated by treatment with the serine-threonine protein phosphatase 2A (*13*). Deactivated MAP kinase kinase was incubated with a portion of the concentrated peak fractions of $p35^{EC12}$ from Superose-12 chromatography (Fig. 2A), together with homogeneous recombinant $p42^{mapk}$, myelin basic protein (MBP), [γ-^{32}P]adenosine triphosphate (ATP), and Mg^{2+} (*13*). Incorporation of ^{32}P into MBP is catalyzed by $p42^{mapk}$, which becomes activated as a result of phosphorylation on Thr^{183} and Tyr^{185} (*14*). Reactivation of MAP kinase kinase was observed (Fig. 3) as measured by incorporation of ^{32}P into MAP kinase or by activation of MAP kinase enzymatic activity toward MBP. Because the specific MBP kinase activity of MAP kinase is proportional to the extent of MAP kinase phosphorylation, the curves are expected to be approximately equivalent, as observed. MAP kinase kinase activity was stimulated more than threefold by $p35^{EC12}$ after 40 min; the amount of ^{32}P incorporated into MBP increased from 14,000 to 51,000 cpm.

In separate experiments, partially purified $p35^{EC12}$ from cells infected with the EC12 virus (Fig. 2A) reactivated MAP kinase kinase, as measured by stimulation of MBP phosphotransferase activity of MAP kinase (*13*). Treatment of MAP kinase kinase with phosphatase 2A reduced its activity to 17 ± 9% (SD, n = 9) of its initial activity. The $p35^{EC12}$ protein restored MAP kinase kinase activity to 64 ± 13% of its initial activity. Similarly, partially purified $p37^{22W}$ from cells infected with the 22W virus (Fig. 2B) and $p35^{EC12}$ that eluted at a monomeric molecular size reactivated MAP kinase kinase to 74 ± 9% and 78 ± 10% of its initial activity, respectively. Fractions from cells infected with EC12 or 22W viruses that did not contain v-Raf detectable by immunoblotting did not reactivate MAP kinase kinase. Omission of v-Raf, MAP kinase kinase, or MAP kinase prevented the time-dependent increase in MBP phosphotransferase activity (*10*). Incubation of fractions containing v-Raf with protein phosphatase 2A did not affect the reactivation of MAP kinase kinase (*10*).

The v-Raf protein was immunoprecipitated from fractions after Superose-12 chromatography of extracts from cells infected with EC12 or 22W virus (pool b from Fig. 2, A and B, respectively). The immunoprecipitates were stringently washed with 0.5 M LiCl (*8*). These immunoprecipitates also reactivated MAP kinase kinase to 53 ± 7% (SD, n = 6) and 49 ± 6% of its initial

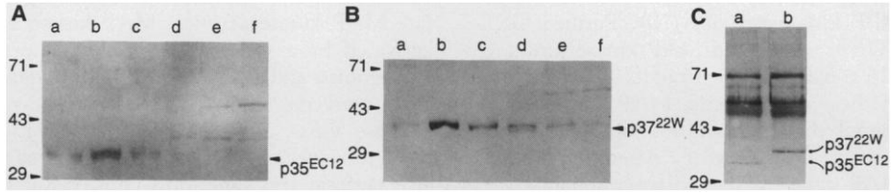

Fig. 2. Immunoblots of v-Raf. The v-Raf protein was detected by immunoblotting of protein from cells infected with EC12 (**A** and **C**) and 22W (**B** and **C**) viruses. Cell lysates (1 mg of protein in 0.5 ml) were subjected to Superose-12 (HR 10/16)-chromatography in buffer C (*21*). The flow rate was 0.25 ml/min, and 0.25-ml fractions were collected. The following fractions were pooled: 35–39, 40–44, 45–49, 50–54, 55–59, 60–64 (lanes a to f, respectively). Proteins were precipitated from portions (50 μl) of these fractions by adding TCA (10%, w/v) to each sample. The solution was left on ice for 10 min and centrifuged at 10,000*g* for 10 min. The sedimented proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (on 11% gels) and detected by immunoblotting with a Vectastain ABC kit according to the manufacturers instructions. Blots were developed with diaminobenzidine-cobalt chloride. The v-Raf proteins were detected with antiserum to a COOH-terminal peptide of c-Raf-1 (*8*). The other stained bands are nonspecific (*10*). Fractions containing v-Raf (pools b) from cells infected with EC12 and 22W viruses (A) and (B) were separately concentrated (Centricon C10) to ~0.1 ml (1.5 mg/ml final). (C) Proteins from portions (12 μg) of concentrated v-Raf (pools b) were immunoprecipitated with the same antiserum to Raf, washed as described (*8*), and subjected to SDS-PAGE (11% gel) and immunoblotting. Lane a, pool b from cells infected with EC12 virus. Lane b, pool b from cells infected with the 22W virus. The molecular size markers aldolase (160 kD), bovine serum albumin (68 kD), ovalbumin (43 kD), and carbonic anhydrase (29 kD) eluted primarily in fractions 46, 51, 56, and 60, respectively. Positions of molecular size markers (in kilodaltons) are shown to the left of the gels.

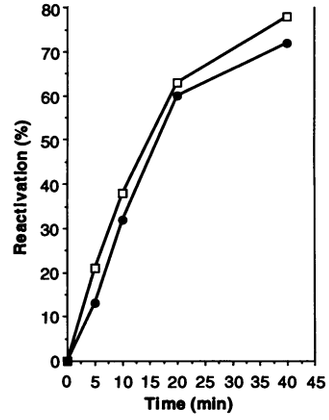

Fig. 3. Reactivation of MAP kinase kinase by $p35^{EC12}$. The ability of $p35^{EC12}$ to reactivate MAP kinase kinase was measured as described (*13*) except that the concentration of MAP kinase was increased to 50 μg/ml to permit measurement of the amount of ^{32}P incorporated into both MAP kinase (open squares) and MBP (closed circles). At the times indicated, a portion (10 μl) was removed and spotted onto P81 filter paper to determine the incorporation of ^{32}P into MBP that resulted from reactivation of MAP kinase kinase. Proteins were precipitated from the remaining material (30 μl) by addition of TCA (10%, w/v) and prepared for SDS-PAGE as described (Fig. 2). The gel was stained, the $p42^{mapk}$ protein bands were excised, and the amount of incorporation of ^{32}P into $p42^{mapk}$ was determined by liquid scintillation spectroscopy. The data were corrected for the amount of phosphorylation in control samples (*13*), which was <20% of the total at each time point. The percentage of reactivation in this figure only was calculated as the percent of the MAP kinase kinase activity that was lost during incubation with phosphatase 2A that was restored by incubation with $p35^{EC12}$. The MAP kinase kinase used in this experiment lost 85% of its activity as a result of treatment with protein phosphatase 2A (*13*). For untreated MAP kinase kinase, the total ^{32}P incorporation was ~10,000 cpm into MAP kinase and ~70,000 cpm into MBP.

activity, respectively. Nonimmune rabbit serum did not immunoprecipitate any activity that caused reactivation of MAP kinase kinase activity. Immunoprecipitates of $p33^{EC13}$ from unstimulated NIH 3T3 cells infected with EC13 virus did not reactivate MAP kinase kinase (*10*). These results imply that either v-Raf or, less likely, a protein that co-immunoprecipitates with v-Raf, can reactivate of MAP kinase kinase. The fact that v-Raf that eluted either as a complex or as a monomer from the gel filtration column reactivated MAP kinase kinase strengthens this supposition (*10*).

To confirm that v-Raf can activate MAP kinase kinase, we expressed a glutathione S-transferase fusion protein containing $p37^{22W}$ (GST-$p37^{22W}$) in bacteria and purified it by affinity chromatography (*15*). The purified protein had no detectable MBP kinase activity (*10*). Purified GST-$p37^{22W}$ reactivated MAP kinase kinase to 116 ± 8% (SD, $n = 6$) of its initial activity. In these experiments, MAP kinase kinase caused the incorporation of 7929 ± 153 cpm of ^{32}P into MBP. After treatment of MAP kinase kinase with phosphatase 2A, incorporation was reduced to 1100 ± 140 cpm. Addition of GST-$p37^{22W}$ resulted in incorporation of 9191 ± 680 cpm. Control preparations of GST alone were completely inactive. Reactivation of MAP kinase kinase by GST-$p37^{22W}$ was confirmed with other preparations (*10*). Preservation of GST-$p37^{22W}$ activity was variable. Some GST-$p37^{22W}$ preparations had reduced activity or were inactive, because of protein denaturation, errors in folding, or other factors not yet identified (*10*). Active GST-$p37^{22W}$ caused phosphorylation of a 68-kD band, corresponding to GST-$p37^{22W}$, and a 46-kD protein in the deactivated MAP kinase kinase preparation that may correspond to the MAP kinase kinase. Thus, v-Raf may directly phosphorylate MAP kinase kinase (*10*).

The c-Raf-1 protein is an essential transducer of mitogenic signals in NIH 3T3 cells downstream of serum growth factor receptors and Ras (*16*). Several studies indicate that MAP kinase is downstream of p21Ras (*17, 18*). For example, a dominant inhibitory mutant of Ras, c-Ha-Ras (Asn^{17}), inhibits activation of c-Raf, MAP kinase, and RSK in PC12 cells stimulated with nerve growth factor. Furthermore, expression of v-Ras activates MAP kinase (*18*). These findings are consistent with a common pathway or parallel pathways for activation of these three serine-threonine kinases by one or more signals generated by activation of Ras.

Constitutively active forms of c-Raf-1 serine-threonine protein kinase (such as v-Raf) cause constitutive activation of the MAP kinase pathway in NIH 3T3 cells. However, expression of v-Raf and other oncogenes has different effects on MAP kinase activity in other established cell lines. For example, in Rat 1a cells, expression of v-Raf or v-Ras does not cause constitutive activation of MAP kinase, whereas transfection of either oncogene into NIH 3T3 cells activates MAP kinase (*11, 19*). In contrast, expression of either the Gip2 or v-Src proteins constitutively activates MAP kinase in Rat 1a cells but fails to do so in NIH 3T3 cells (*19*). Cell type is expected to be an important variable because establishment as a continuous cell line requires loss of growth control at one or more steps, and these steps are unlikely to be equivalent in different cell lines. These apparently conflicting sets of data may be indicative of the complexity of the network of factors and feedback controls that regulate MAP kinase activity. MAP kinase is activated by a wide range growth factors and agonists and at some point these signals must converge to activate the pathway. Because v-Raf activates MAP kinase kinase, we expect that activated c-Raf-1 may also perform this function. Thus, c-Raf-1 appears to be one transducer that integrates mitogenic signals that activate the MAP kinase cascade in vivo.

Note added in proof: Kyriakis *et al.* (*23*) reported that MAP kinase kinase is constitutively active in v-Raf–transformed NIH 3T3 cells, and that c-Raf-1 purified from mitogen-stimulated NIH BXB cells reactivates MAP kinase kinase purified from H4 hepatoma cells. The authors reached conclusions similar to those discussed in this report.

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12. The p42 MAP kinase predominates in comparison to p44 MAP kinase in NIH 3T3 cells by immunoreactivity, or by activity after chromatographic separation of p42 and p44 MAP kinases (*10, 11*). The p42 and p44 proteins coelute from the MonoQ column under the conditions described in Fig. 1 (*10*).
13. MAP kinase kinase was purified to near homogeneity as described (*5*). Portions were dialyzed in a solution of 50 mM tris-Cl (pH 7.5 at 4°C), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM Benzamidine, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 50% (w/v) glycerol to remove protein phosphatase inhibitors. Portions were inactivated by incubation for 2 hours at 30°C with protein phosphatase 2A (200 mU/ml), which was subsequently irreversibly inactivated by addition of microcystin-LR (5 μM) (*22*). Effects of v-Raf on MAP kinase kinase activity were assessed in assays modified from those described (*4, 5*). Reactivation assays contained the v-Raf sample and recombinant, inactive MAP kinase (2.5 μg/ml), with or without dephosphorylated MAP kinase kinase (3 μg) in a total volume of 12 μl. Partially-purified v-Raf (12 μg, pools b, Fig. 2, A and B), washed immunoprecipitates (Fig. 2C), and GST-$p37^{22W}$ (~0.2 μg) were assayed (see text). The reaction was initiated by the addition of 28 μl of 25 mM Hepes (pH 7.5 at 30°C), 1 mM DTT, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (5000 cpm/pmol) containing MBP (0.5 mg/ml) and 1.5 μM okadaic acid, and incubated at 30°C. After 50 min (5 min with GST-$p37^{22W}$), a portion (38 μl) was removed and spotted onto P81 filter paper and immersed in 180 mM phosphoric acid to terminate the reaction. The amount ^{32}P incorporated was determined as described. Assays of MAP kinase kinase (not treated with phosphatase) alone usually yielded 1.5×10^5 to 3×10^5 cpm of ^{32}P incorporated into MBP, which was taken as 100% activity. Control assays were performed to measure MBP phosphotransferase activity of all individual components. MAP kinase kinase, inactive MAP kinase, and MBP did not stimulate incorporation of ^{32}P into MBP. In the presence of [δ-^{32}P]ATP alone, 0.05% of total ^{32}P remained associated with MBP. The MBP phosphotransferase activity of v-Raf samples was always <10 to15% of that of fully active MAP kinase kinase. Results were normalized to permit comparison of results obtained with different preparations of MAP kinase kinase and v-Raf. Percentage of activity for reactivation was calculated from the ratio of the difference between incorporation of ^{32}P measured in assays done with and without phosphatase-treated MAP kinase kinase to the amount of ^{32}P incorporated in the presence of fully active MAP kinase kinase. Any contribution from contaminating MBP kinases in the v-Raf preparations is thus canceled.
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21. Buffer A: 25 mM tris-Cl (pH 7.5 at 4°C), 1 mM DTT, 2 mM EDTA, 2 mM EGTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 40 mM sodium paranitrophenylphosphate, 1 mM PMSF, 1 mM benzamidine, leupeptin (0.04 mg/ml), pepstatin A (0.01 mg/ml), *N*-tosyl-L-phenylalaninechloromethyl ketone (0.1 mg/ml), Triton X-100, 0.5% (v/v), NP-40, 0.5% (v/v), and glycerol 10% (v/v). Buffer B: buffer A without leupeptin, pepstatin A, Triton X-100, and NP-40, containing 0.2 mM EDTA and 1.5 mM EGTA. Buffer C: buffer B without protein phospha-

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A 90-Kilodalton Endothelial Cell Molecule Mediating Lymphocyte Binding in Humans

Marko Salmi* and Sirpa Jalkanen

Interactions between leukocyte surface receptors and their ligands on vascular endothelial cells control lymphocyte traffic between the blood and various lymphoid organs, as well as extravasation of leukocytes into sites of inflammation. A heretofore undescribed 90-kilodalton human endothelial cell adhesion molecule (VAP-1) defined by a monoclonal antibody 1B2 is described. The expression pattern, molecular mass, functional properties, and an amino-terminal amino acid sequence define VAP-1 as an endothelial ligand for lymphocytes. VAP-1 helps to elucidate the complex heterotypic cell interactions that direct tissue-selective lymphocyte migration in man.

Most mature lymphocytes continuously recirculate between the blood and lymphatic organs (*1*). Lymphocyte trafficking allows the full repertoire of lymphocyte specificities to be available for immune reactions throughout the body, and it also facilitates the cell-cell interactions required for the generation and control of immune responses. Lymphocytes leave the blood by recognizing and binding to the vascular endothelial cells. Thereafter, they migrate between the endothelial cells into the surrounding tissues. Lymphocyte adherence to endothelial cells is dependent on interactions between complementary adhesion molecules expressed on both cell types (*2–7*). Under normal conditions, lymphocytes mainly bind to specialized postcapillary venules called high endothelial venules (HEV). Functionally separate lymphocyte-HEV recognition systems mediate lymphocyte migration to peripheral lymph nodes, mucosal lymphoid organs, synovium, and skin in an organ-specific manner (*8–10*). In inflammation, activation of the endothelial cell results in changes of its adhesion molecule status, which largely determines the magnitude and type of leukocyte influx into the affected tissue. Thus, endothelial cell molecules are a key element in controlling the characteristics of local immune response, and a detailed understanding of the mechanisms regulating lymphocyte traffic and leukocyte extravasation can provide new means to clinically manipulate the inflammatory response. Because in man the endothelial cell ligands mediating tissue-selective lymphocyte homing are largely unknown, we made an attempt to identify such molecules by producing monoclonal antibodies (MAbs) to human synovial vessels (*11*).

Immunohistological stainings revealed that one of the MAbs produced, 1B2, strongly stained HEV-like venules in inflamed synovial membranes (Fig. 1, A and B). No staining was observed in infiltrating leukocytes or in connective tissue components of the synovial stroma. The antigen recognized by MAb 1B2 was named VAP-1 (for vascular adhesion protein–1). In peripheral lymph node and tonsil, MAb 1B2 reacted with majority of HEV (Fig. 1C). VAP-1 was intensely expressed at the luminal side of the endothelial cells (Fig. 1D). A granular staining was seen in the endothelial cell cytoplasm, and also the abluminal surface was MAb 1B2 positive. Especially in tonsil, the staining intensity notably varied between different HEV, and few individual HEV with a typical plump morphology were 1B2-negative. In appendix (Fig. 1E) and in lamina propria of the gut, only faintly staining venules were detected. Weak expression of VAP-1 was also seen on dendritic-like cells in germinal centers and on smooth muscle cells of arteries, veins, and bowel wall. In contrast, VAP-1 was practically absent from the luminal surface of large vessels. Like leukocytes in tissue sections, peripheral blood lymphocytes, monocytes, natural killer (NK) cells, granulocytes, and isolated tonsillar leukocytes were all completely 1B2-negative in fluorescence-activated cell sorter (FACS) analyses. T lymphoblastoid (CCRF-CEM), B lymphoblastoid (KCA and IBW-4), monocytic (U937), and leukemic (KG-1, KG-1a, and K 562) cell lines all lacked VAP-1. VAP-1 was absent from human umbilical vein endothelial cells (HUVEC), and 4-hour or 20-hour treatments with interleukin-1 (IL-1) (20 or 100 U/ml), tumor necrosis factor (TNF) (200 U/ml), or lipopolysaccharide (LPS) (0.1 or 1.0 μg/ml) could not induce its expression. Primary cultures of smooth muscle cells, fibroblasts, and keratinocytes, and an epithelioid (HeLa) cell line did not express VAP-1.

To determine the molecular weight of VAP-1, affinity-isolated molecule from tonsillar stroma was subjected to SDS–polyacrylamide gel electrophoresis (PAGE). Silver staining of the gel revealed a major band of apparent molecular size 90 kD under reducing conditions (Fig. 2). Analyses of immunoprecipitates from iodinated stromal cells of tonsil confirmed the reactivity of MAb 1B2 with a 90-kD molecule (and a slightly smaller species; presumably a degradation product) (Fig. 2). VAP-1 migrated slightly slower under nonreducing conditions (M_r ~ 100 kD).

The tissue distribution of VAP-1 on endothelial cells in vivo suggested that it might function as a specific recognition element for leukocytes. Therefore, the functional role of VAP-1 in HEV-binding was studied with the modified Stamper-Woodruff in vitro assay (*12*). By first treating frozen sections with MAb 1B2, lymphocyte binding to HEV was inhibited (Fig. 3). The inhibitory effect was most pronounced in tonsil and peripheral lymph node, but binding to synovial HEV was also significantly reduced. Lymphocyte binding to appendix HEV and granulocyte binding to tonsil HEV were less affected (Fig. 3). Thus, VAP-1 either mediates or associates closely with endothelial cell elements mediating lymphocyte recognition of peripheral lymph node, tonsil, and synovial HEV. To directly evaluate the involvement of VAP-1 in lymphocyte-endothelial cell interaction, we analyzed binding of lymphocytes to affinity-isolated VAP-1 (Fig. 4). Lymphocytes adhered efficiently to plate-bound VAP-1. Lymphocyte binding to VAP-1 was specifically inhibited with MAb 1B2 but not with a control MAb 3G6. MAb 1B2 did not prevent lymphocyte binding to another unrelated endothelial cell molecule (Fig. 4).

The expression pattern, molecular weight, and function of VAP-1 indicate that it is not identical with any of the previously defined endothelial molecules

National Public Health Institute, and Department of Medical Microbiology, Turku University, Kiinamyllynkatu 13, 20520 Turku, Finland.

*To whom correspondence should be addressed.