THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 25, Issue of June 21, pp. 22959–22965, 2002

Printed in U.S.A.

Active Site Residues of Cyclophilin A Are Crucial for Its Signaling Activity via CD147*

Received for publication, February 15, 2002
Published, JBC Papers in Press, April 9, 2002, DOI 10.1074/jbc.M201593200

Vyacheslav Yurchenko‡, Gabriele Zybarth‡§, Matthew O'Connor‡, Wei Wei Dai‡, Giovanni Franchin‡, Tang Hao‡, Huiming Guo¶, Hsiu-Cheng Hung‖, Bryan Toole¶, Philippe Gallay‖, Barbara Sherry‡, and Michael Bukrinsky‡ ‡‡§§**

From the ‡Picower Institute for Medical Research, Manhasset, New York 11030, the ¶Department of Anatomy and Cellular Biology, Tufts Medical School, Boston, Massachusetts 02111, the ‡‡Department of Microbiology and Tropical Medicine, George Washington University Medical Center, Washington, D. C. 20037, and the ‖Department of Immunology, the Scripps Research Institute, La Jolla, California 92037

Cyclophilin A (CyPA), a ubiquitously distributed intracellular protein, is a peptidylprolyl *cis-trans*-isomerase and the major target of the potent immunosuppressive drug cyclosporin A. Although expressed predominantly as an intracellular molecule, CyPA is secreted by cells in response to inflammatory stimuli and is a potent neutrophil and eosinophil chemoattractant *in vitro* and *in vivo*. The mechanisms underlying CyPA-mediated signaling and chemotaxis are unknown. Here, we identified CD147 as a cell surface receptor for CyPA and demonstrated that CD147 is an essential component in the CyPA-initiated signaling cascade that culminates in ERK activation. Both signaling and chemotactic activities of CyPA depended also on the presence of heparans, which served as primary binding sites for CyPA on target cells. The proline 180 and glycine 181 residues in the extracellular domain of CD147 were critical for signaling and chemotactic activities mediated by CD147. Also crucial were active site residues of CyPA, because rotamase-defective CyPA mutants failed to initiate signaling events. These results establish cyclophilins as natural ligands for CD147 and suggest an unusual, rotamase-dependent mechanism of signaling.

Cyclophilin A (CyPA)[1] is a ubiquitously distributed intracellular protein belonging to the immunophilin family (1) and is recognized as the host cell receptor for the potent immunosuppressive drug cyclosporin A (2, 3). CyPA has also been shown to possess peptidylprolyl *cis-trans*-isomerase activity and is thought to play an important role in protein folding (1, 4). Although CyPA was initially believed to exist solely as an intracellular protein, later studies have revealed that it can be secreted by cells in response to inflammatory stimuli (5, 7). Secreted CyPA can initiate signaling response in target cells (6) and is a potent neutrophil (5), eosinophil (7), and T cell (8) chemoattractant *in vitro*. It also elicits an inflammatory response characterized by a rapid influx of neutrophils when injected *in vivo* (7).

The intercellular effector activities of CyPA imply the presence of a binding protein (receptor) to transduce the signal to the target cell. Binding sites have been described for other members of the cyclophilin family, cyclophilins B (CyPB) and C (CyPC) (9, 10). For CyPB, two types of binding sites have been identified. The type I sites correspond to specific functional receptors that mediate internalization of bound CyPB, and the type II sites are represented by sulfated glycosaminoglycans (9). Both sites appear to be specific for CyPB because affinity of CyPC for the type I sites was 6-fold lower than that of CyPB, and CyPA did not interact with these sites at all (9, 11). We reported recently that CyPB can signal through CD147, a type 1 integral membrane protein also known as extracellular matrix metalloproteinase inducer (EMMPRIN) (12). It is unlikely, however, that CD147 is the type I binding site described by Denys and colleagues (9) because unlike type I receptor, CD147 was shown to interact with CyPA (13). In that case, interaction was observed between CD147 and CyPA associated with HIV-1 virions and was found to enhance HIV-1 infection at a step of entry or uncoating (13, 14).

Neither CyPA nor CyPC was able to inhibit CyPB binding to type II sites (glycosaminoglycan) (9). Nevertheless, a recent report demonstrated binding of CyPA to heparan sulfates, a subtype of glycosaminoglycans (15). It appears, therefore, that CyPA and CyPB use different classes of glycosaminoglycan molecules for binding or that the affinity of CyPA for glycosaminoglycan is much lower than that of CyPB.

In the case of CyPB, binding to the type II site (glycosaminoglycan) is believed to present the immunophilin for interaction with the type I site (11). Anticipating a similar arrangement for CyPA, we searched for a signaling receptor that mediated intercellular effector activities of CyPA. These studies identified CD147 as a protein responsible for CyPA-induced signaling and chemotactic activities. These signaling events also required the presence of heparans on the cell surface and depended on active site residues of CyPA.

MATERIALS AND METHODS

Yeast Two-hybrid Screening—An expressed sequence tag identified as human CyPA (16) was obtained from ATCC (ATCC 78809D). *In vitro* translation and sequencing confirmed that this sequence represented a

* This work was supported in part by National Institutes of Health Grants R01 AI 38245 (to M. B.) and R01 AI 29110 (to B. S.) and by the funds from the Picower Institute for Medical Research. 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.

§ Present address: Biodiscovery, Central Research Division, Pfizer Inc., Groton, CT 06340.

** Supported by Grant SFP-1363 from Novartis.

§§ To whom correspondence should be addressed: Dept. of Microbiology and Tropical Medicine, George Washington University, Ross Hall, Rm. 734, 2300 Eye St., N.W., Washington, D. C. 20037. E-mail: mtmmib@gwumc.edu.

[1] The abbreviations used are: CyPA, CyPB, and CyPC, cyclophilin A, B, and C, respectively; BSA, bovine serum albumin; CHO, Chinese hamster ovary; EMMPRIN, extracellular matrix metalloproteinase inducer; ERK, extracellular signal-regulated kinase; fMLP, formyl-methionyl-leucyl-phenylalanine; HIV-1, human immunodeficiency virus type 1; JNK, c-Jun NH_2-terminal kinase; mAb, monoclonal antibody; MAP, mitogen-activated protein; PBS, phosphate-buffered saline; RANTES, regulated on activation normal T cell expressed and secreted; SAPK, stress-activated protein kinase; CA, capsid antigen.

full-length CyPA cDNA (essentially homologous to GenBank Y00052). The sequence was cloned into the vector pAS2-1 (CLONTECH) to generate plasmid pAS2-1-CyPA encoding CyPA-GAL4 DNA binding domain fusion protein. This plasmid was used as a bait to screen a human B cell cDNA library (from S. Fields, SUNY at Stony Brook, NY) constructed in pSE1107 encoding the GAL4 activation domain (library complexity $\approx 10^8$). Screening was performed using Matchmaker Two-hybrid System 2 (CLONTECH) essentially as suggested by the manufacturer. In control experiments, pAS2-1-CyPA did not activate transcription from the GAL4 promoter (measured as β-galactosidase activity) when introduced alone into yeast reporter host strains (Y187 or Y190) or when cotransformed with an empty GAL4 activation domain vector (pACT2, CLONTECH) (negative controls). A strong positive signal was detected when pAS2-1-CyPA was cotransformed with a pGAD424-glycosaminoglycan construct (expressing a fusion between the full-length HIV-1 *gag* protein and GAL4 activation domain (17)) (positive control). The actual screening was performed by cotransforming pAS2-1-CyPA together with pSE1107-cell library into yeast reporter host strain Y190. To select for cotransformants in which the two proteins interact, the culture was plated on triple dropout plates (synthetic dropout/-His/-Trp/-Leu), and colonies were tested for expression of the *lacZ* reporter gene by a filter assay protocol. All blue colonies were screened further by cycloheximide counterselection according to the supplied (CLONTECH) protocol to identify false positives. Plasmids from true positive colonies were isolated and their inserts sequenced.

Cells and Reagents—The wild-type CHO-K1 and heparan-deficient CHOpgsB-618 cell lines were purchased from ATCC. The antibodies to p42/44 MAP kinase, p38 MAP kinase, and SAPK/JNK were from New England Biolabs (Beverly, MA); anti-CD147 and anti-CD8 mAbs were purchased from Ancell (Bayport, MN), and anti-heparan sulfate mAb was purchased from Seikagaku America (Falmouth, MA). Heparitinase I (cleaves heparan sulfate at α-*N*-acetylglucosaminide-D-glucuronic acid linkage), heparitinase II (cleaves heparin sulfate and heparin), and heparitinase III (cleaves heparin at α-*N*-acetylglucosaminide-L-iduronic acid linkage) were purchased from Seikagaku America.

Plasmids—Human CyPA was cloned into the pET14b vector (Novagen, Madison, WI) and purified using His-Bind methodology (Novagen) as described (14). Expression vectors for CyPA mutants CyPAm4, F60A, F113A, W121A, and H126A were described earlier (15, 18). For transient and stable expression in CHO cells, a DNA fragment encoding the full-length CD147 was cloned into pcDNA3.1 vector (Invitrogen). For solution binding studies, a DNA fragment encoding full-length CD147 without the signal peptide was cloned into the pT7Blue2 vector (Novagen) and expressed using the troponin T (TnT) Coupled Reticulocyte Lysate System (Promega, Madison, WI).

Cell Binding Assays—For fluorescent labeling of recombinant human CyPA the Alexa™488 Protein Labeling Kit (Molecular Probes, Eugene, OR) was used according to the manufacturer's instructions. In brief, 50 μl of 1 M bicarbonate was added to 0.5 ml of a 2 mg/ml solution of recombinant human CyPA, and the entire mixture was then added to a vial of reactive Alexa dye (Alexa 488 carboxylic acid, succinimidyl ester, dilithium salt). Labeling was allowed to proceed for 1 h at room temperature, and then the reaction was terminated by adding hydroxylamine and stirring for additional 30 min. Fluorescently labeled CyPA was purified away from unreacted dye by gel filtration. The final molar ratio of dye to protein was 0.97. For binding assays with Alexa 488-labeled CyPA, cells were harvested with 0.5 mM EDTA in PBS, washed with PBS, and the pellet was resuspended in binding buffer at 6×10^6 cells/ml. 3.5×10^5 cells were aliquoted into 6-ml Falcon tubes, mixed with appropriate volumes of labeled CyPA, and incubated on ice for 45 min. At that time 4 ml of PBS was added to each tube, and the cells were centrifuged at 1,200 rpm for 5 min. The supernatant was aspirated, and the pellets were resuspended in 0.5 ml of PBS and analyzed immediately for cell-associated fluorescence by flow cytometry.

Iodination of CyPA and CyPAm4 was performed using *N*-succinimidyl-3-(4-hydroxy-3,5-[^{125}I]diiodophenyl)propionate (diiodinated Bolton-Hunter reagent, PerkinElmer Life Sciences) according to the manufacturer's instructions. The specific activities of wild-type and mutant CyPA used in all binding experiments were 280 and 300 Ci/mmol, respectively. For binding experiments with iodinated CyPA, cells were plated in 24- (2×10^5 cells/well) or 48-well plates (1×10^5 cells/well) and allowed to adhere firmly for 18 h before performing the binding experiment. Cells were incubated with 130 nM iodinated cyclophilin in a binding buffer (Dulbecco's modified Eagle's medium supplemented with 10% BSA and 0.09% azide) for 3 h at 4 °C. Cells were washed twice with cold PBS, lysed in radioimmune precipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, and 1% SDS), and then counted in a gamma counter (Gamma Track 1193, TM Analytic, Brandon, FL). Nonspecific background binding was determined by counting radioactivity bound in the presence of a 100-fold excess of unlabeled CyPA.

Cross-linking Assay—CyPA or BSA (150 μM each) was activated by incubating with a biotin-containing trifunctional cross-linking reagent (Sulfo-SBED, Pierce) for 30 min at room temperature in the dark. After removal of unincorporated cross-linker by gel filtration on D-Salt Dextran Desaltic column (Pierce), activated CyPA or BSA was affinity purified on a monomeric avidin column (Pierce) to remove unreacted protein molecules. Activated purified CyPA or BSA was then incubated with CHO.pcDNA (CHO) or CHO.CD147 cells at 4 °C for 45 min in the dark. After UV cross-linking (15 min, 365 nm), cells were washed twice with PBS at room temperature, lysed, and biotin-containing complexes were immunoprecipitated with streptavidin-agarose (30 min, room temperature). Immunoprecipitates were washed three times with PBS and analyzed by Western blotting on nonreducing or reducing (β-mercaptoethanol-containing) SDS-gels using anti-CD147 monoclonal antibody (RDI, Flanders, NJ) followed by peroxidase-conjugated anti-mouse antibody.

Chemotaxis Assays—Neutrophils were obtained from heparinized venous blood by Ficoll-Paque gradient centrifugation, as described (5). Neutrophil chemotaxis was assessed in a 48-well modified Boyden chamber (19) with the two compartments separated by a polyvinylpyrrolidone-free polycarbonate filter with a 5-μm pore size (Whatman Nuclepore, Tewksbury, MA). Neutrophils (1.5×10^6 cells/ml) in Gey's balanced salt solution supplemented with 2% BSA and 20 mM HEPES (GBSA) were added to the compartment above the filter, and test samples of possible chemoattractants diluted in chemotaxis medium were present below the filters. Chambers were incubated at 37 °C and 5% CO_2 for 30 min; then the filters were recovered, fixed, and stained with Giemsa reagent. The number of cells appearing on the lower face of the filter was recorded in four high power fields for each well, and each experimental condition was assayed in triplicate wells. 10^{-7} M formyl-methionyl-leucyl-phenylalanine (fMLP) was used as a positive control. To control for chemokinetic activity, CyPA was added to both chambers.

CHO.pcDNA and CHO.CD147 cells were assayed for chemotaxis in a manner identical to that described for neutrophil chemotaxis except that the two wells were separated by a 10-μm pore size filter (Neuro Probe, Inc., Gaithersburg, MD) precoated with fibronectin (10 μg/ml fibronectin for 2 h at 37 °C and 5% CO_2), and the chambers were incubated at 37 °C and 5% CO_2 for 4.5 h.

Calcium Mobilization Assay—Mobilization of intracellular calcium in CyPA-stimulated cells was assessed as described by Sherry *et al.* (14). Briefly, 0.6 ml of Fura-2/AM-loaded cells (5×10^6 cells/ml) were stimulated with 100–500 nM human CyPA per sample, and fluorescence emission at 340 and 380 nm was measured on a PerkinElmer Luminescence Spectrometer LS50B.

Signaling Analysis by Western Blotting—Serum-starved CHO cells transiently or stably transfected with pcDNA or CD147 constructs were treated with 50–500 nM CyPA. Cell lysates were separated on 10% SDS-PAGE and subjected to Western blotting analysis using antibodies specific for the nonphosphorylated and phosphorylated forms of ERK1/2, p38, and SAPK/JNK MAP kinases following the protocol recommended by the manufacturer (New England Biolabs).

RESULTS

Previous studies demonstrated a chemotactic activity of CyPA for neutrophils, eosinophils, and T lymphocytes (5, 7, 8), suggesting the presence of a CyPA receptor on target cells. To identify potential binding partners of CyPA, we performed a yeast two-hybrid screen with a B cell cDNA library. Preliminary screening yielded 1×10^6 positive cDNA clones, of which 10 clones were identified as true positives after several rounds of screening (see "Materials and Methods") and sequenced. Two of the clones carried an insert, which was 97% identical in its sequenced 5′ 186 nucleotides to human EMMPRIN cDNA (20). We selected EMMPRIN for further analysis because it was the only transmembrane protein among CyPA-interacting clones, which made it a good candidate for the role of CyPA receptor. Consistent with this hypothesis, our recent report demonstrated that EMMPRIN stimulates HIV-1 infection by mediating activity of virus-incorporated CyPA (13).

EMMPRIN is a type I integral membrane glycoprotein of 50–60 kDa expressed on a wide variety of cells including he-

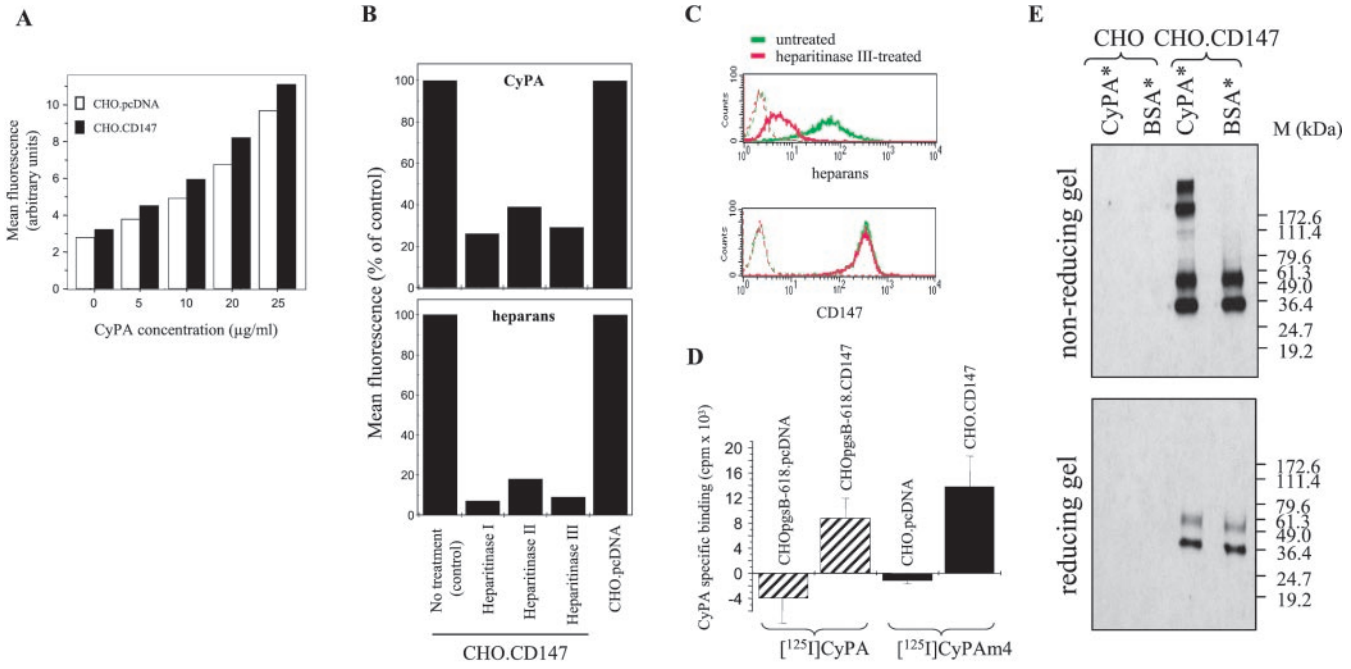

FIG. 1. **CyPA binds to glycosaminoglycans and CD147 on target cells.** *A*, binding of fluorescently labeled CyPA to CHO cells is determined primarily by heparans. The indicated concentrations of fluorescently labeled CyPA were incubated with CHO.pcDNA or CHO.CD147 cells at 4 °C. Cells were pelleted by centrifugation and analyzed by flow cytometry. *B*, CyPA binds to heparans. Fluorescently labeled CyPA was incubated with CHO.pcDNA cells or with CHO.CD147 cells pretreated with the indicated heparitinase. Binding of CyPA was analyzed as in *A* (*upper panel*), and heparan sulfate expression was assessed by flow cytometry using fluorescein isothiocyanate-labeled mAb to heparans (*bottom panel*). Results are presented relative to mean fluorescence detected with untreated CHO.CD147 cells and are shown for one representative experiment of three performed. *C*, pretreatment with heparitinase does not affect CD147 expression. CHO.CD147 cells were treated with heparitinase III and analyzed by flow cytometry for expression of heparan sulfate and CD147 using fluorescein isothiocyanate-labeled mAbs (*solid lines*) against heparans or CD147, respectively, or isotype control (*dashed lines*). *D*, specific binding of CyPA to CD147. Iodinated CyPA or mutant CyPAm4 defective in binding to heparans was used to analyze binding to CHO (*black bars*) or heparan-negative CHOpgsB-618 cells (*hatched bars*) stably transfected with CD147 or pcDNA. Cells were plated in 24- (CHOpgsB-618 cells) or 48-well plates (CHO cells). Results are presented after subtraction of binding in the presence of a 100-fold excess of cold CyPA (nonspecific binding). Results are the mean ± S.D. and are shown for a representative experiment of three performed. *E*, cross-linking of CyPA and CD147. CyPA or BSA was activated by incubating with a biotin-containing trifunctional cross-linking reagent (Sulfo-SBED). Activated CyPA (*CyPA**) or BSA (*BSA**) was affinity purified on a monomeric avidin column to remove unreacted CyPA or BSA. Activated purified CyPA or BSA was then incubated with CHO.pcDNA (*CHO*) or CHO.CD147 cells. After UV cross-linking (15 min, 365 nm), cells were washed, lysed, and biotin-containing complexes were immunoprecipitated with streptavidin-agarose. Immunoprecipitates were analyzed by Western blotting on nonreducing or reducing (β-mercaptoethanol-containing) SDS-gels using anti-CD147 mAb as a probe. High molecular mass bands (~200-kDa protein) observed in the CyPA (CHO.CD147) *lane* of the nonreducing gel correspond to complexes containing CyPA and CD147, whereas the two *lower bands* correspond to CD147 (glycosylated and unglycosylated forms). Note: SBED is a reversible cross-linker; therefore, reducing conditions release free CD147.

mopoietic, microglial, endothelial, and peripheral blood cells (21–25). At the Sixth International Workshop and Conference of Human Leukocyte Antigens it was designated as CD147 antigen (26) and will be referred to as such in this report.

CyPA Binds to CD147 and Heparans on CHO Cells—Because CD147 is expressed naturally as an integral membrane protein, we used whole cell binding assay to demonstrate specific binding interactions between CyPA and CD147. Because CD147 is expressed on most cell types, we employed CHO cells transfected with a vector expressing human CD147 as target cells. We first compared binding of fluorescently labeled CyPA to CHO.CD147 cells and to control CHO cells transfected with the empty vector (CHO.pcDNA). To our surprise, CyPA bound almost equally to both cell types (Fig. 1*A*). This result can be explained by a recent report that identified glycosaminoglycans as the major CyPA-binding molecules on CHO and other cells (15). Sulfated glycosaminoglycans also serve as low affinity binding sites for CyPB (11). Indeed, treatment of CHO.CD147 cells with three different heparitinases, which significantly eliminate surface heparans (Fig. 1*B*, *bottom panel*, and 1*C*), also significantly diminished binding of fluorescently labeled CyPA (Fig. 1*B*, *upper panel*) without affecting expression of CD147 (Fig. 1*C*). Therefore, heparans, but not CD147, appear to determine principally CyPA binding to the cell surface.

Because heparans are extremely abundant on the cell surface, their high capacity interaction with CyPA can mask CyPA binding to CD147. Three approaches were used to demonstrate specific CyPA-CD147 interaction. We first transfected a heparan-deficient clone of CHO cells, CHOpgsB-618, with a CD147-expressing construct (or an empty pcDNA vector) and produced cells stably expressing CD147. Using iodinated CyPA, we demonstrated CD147-specific (cold competable) binding of CyPA to these cells (Fig. 1*D*). In another approach, we used mutant CyPAm4, which does not bind heparans because four basic residues in its COOH-terminal region (Arg^{148}, Lys^{151}, Lys^{154}, and Lys^{155}) responsible for heparan binding had been replaced with alanines (15). When iodinated, CyPAm4 specifically bound to CHO.CD147 but not to CHO.pcDNA cells (Fig. 1*D*). Our attempts to measure the K_d of CyPA-CD147 binding were unsuccessful, likely because this binding is of relatively low affinity and requires a large excess of cold CyPA for saturation. This result suggests that interaction between CyPA and CD147 is different from classical ligand-receptor interactions and might be of transitory nature, similar to enzyme-substrate interactions (see below) and consistent with the enzymatic activity of CyPA. Heparans might serve as primary binding sites for CyPA and thus stimulate its interaction with CD147, consistent with their requirement for CyPA-induced signaling and chemotaxis (see below).

To demonstrate directly the interaction between CyPA and

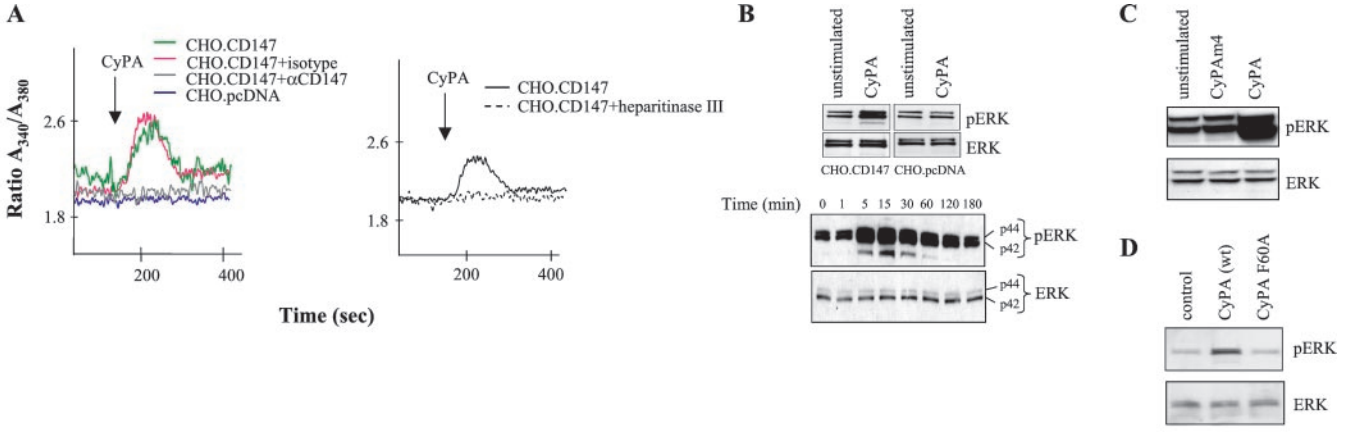

FIG. 2. **CD147 mediates CyPA-specific signaling events.** *A*, CyPA induces Ca^{2+} flux in CD147-expressing cells. CHO.pcDNA or CHO.CD147 cells (treated with anti-CD147 mAb or isotype control in the *left panel* or with heparitinase III in the *right panel*) were loaded with Fura-2/AM, stimulated with 500 ng/ml native human CyPA, and analyzed on a luminometer. Results are shown for one representative experiment of three performed. *B*, CyPA stimulates ERK phosphorylation. Serum-starved CHO.CD147 or CHO.pcDNA cells were stimulated or not with 500 ng/ml CyPA, lysed, and analyzed by Western blotting using anti-ERK (*bottom panels*) or anti-phospho-ERK (*pERK*) monoclonal antibodies (*upper panels*). For the time course analysis, CHO.CD147 cells were lysed at the indicated intervals after addition of CyPA and analyzed as above. Results are for one representative experiment of four performed. *C*, mutant CyPA defective in heparan binding does not induce signaling response. Phosphorylation of ERKs was analyzed as in *B* 5 min after stimulation of CHO.CD147 cells with 100 ng/ml wild-type CyPA or mutant CyPAm4 that does not bind heparans (15). *D*, effect of active site mutations on CyPA-induced signaling. CHO.CD147 cells were stimulated or not with 100 ng/ml wild-type (*wt*) CyPA or CyPA carrying the indicated mutation. Phosphorylation of ERKs was analyzed as in *B*.

CD147 expressed on CHO cells we employed a cross-linking approach. CyPA (and BSA used as a negative control) was activated by incubating with a biotin-containing trifunctional cross-linking reagent, Sulfo-SBED. This reagent has amine group-specific reactivity, a nonspecific photoreactivity, and a biotin handle. The cross-linking can be reversed by thiol cleavage. Activated purified CyPA or BSA was incubated with CHO.pcDNA or CHO.CD147 cells, and cross-linking was induced by UV irradiation. After cell lysis, biotin-containing complexes were immunoprecipitated with streptavidin-agarose and analyzed by Western blotting for the presence of human CD147. As shown in Fig. 1*E*, high molecular mass bands (~200 kDa) were observed in the lysate of CHO.CD147 (but not CHO.pcDNA) cells incubated with CyPA (but not with BSA) and analyzed under nonreducing conditions. Under reducing conditions that reverse cross-linking, these bands disappeared. Two faster migrating bands (obviously corresponding to non-glycosylated and glycosylated forms of CD147 with a molecular mass of 35 and 55 kDa, respectively) revealed in the lysates from CHO.CD147 cells under both reducing and nonreducing conditions likely represent CD147 nonspecifically labeled by contaminating Sulfo-SBED. This conclusion was supported by a control experiment with mock treated CHO.CD147 cells in which these bands were still observed (not shown).

Taken together, these results demonstrate that CyPA specifically associates with CD147.

CyPA Induces Intracellular Signaling in CD147-transfected CHO Cells—To determine whether binding of CyPA to CD147 initiates intracellular signaling events, we measured Ca^{2+} flux in CHO.CD147 and CHO.pcDNA cells stimulated with CyPA. A characteristic flux of intracellular Ca^{2+} was observed in CHO.CD147, but not in CHO.pcDNA cells (Fig. 2*A*, *left panel*). This signal was eliminated by anti-CD147 antibody that recognizes an epitope in the extracellular domain of CD147, but not by isotype control (Fig. 2*A*, *left panel*). Consistent with the essential role of heparans in CyPA binding to cells, pretreatment of cells with heparitinase also eliminated CyPA-specific Ca^{2+} flux (Fig. 2*A*, *right panel*).

To investigate other signaling pathways that could be initiated by CyPA through CD147, we analyzed the phosphorylation status of ERK, JNK, and p38 MAP kinases in CyPA-treated CHO.CD147 and CHO.pcDNA cells. A characteristic increase in the level of phosphorylated ERKs was observed after the addition of CyPA to CHO.CD147, but not to CHO.pcDNA cells (Fig. 2*B*, *upper panels*). A time course analysis demonstrated that ERK activation reached maximum at 5 min after stimulation and gradually decreased afterward (Fig. 2*B*, *bottom panels*). No such increase in phosphorylation was detected for JNK or p38 MAP kinases (not shown). The observed CyPA-induced CD147-mediated signaling is similar to signaling induced in vesicular smooth muscle cells by CyPA secreted in response to oxidative stress (6), suggesting that CD147 might be involved in the pathogenesis of vascular diseases. Consistent with the requirement of heparans for CyPA signaling, mutant CyPAm4, which does not bind heparans, did not induce (at a 100 ng/ml concentration) signaling events in CHO.CD147 cells (Fig. 2*C*). Some signaling was seen at higher concentrations (1 μg/ml) of mutant CyPA (not shown), conforming the role of heparan in stimulating physiological activity of low concentrations of ligands (27).

To determine whether integrity of the CyPA active center was essential for signaling, we made use of a previously described panel of active site mutants of CyPA. The F60A and H126A mutants have been shown to retain less than 1% of the wild-type catalytic efficiency in a tetrapeptide assay, whereas the F113A mutant retains about 10%, and the rotamase activity of the W121A mutant is reduced only by about 2-fold (28, 49). The W121A mutation, despite its relatively moderate effect on catalytic activity, caused a 75-fold decrease in sensitivity to cyclosporin A (49). This mutation, therefore, separates peptidylprolyl isomerase activity from cyclosporin A binding and provides a control to demonstrate that the observed effects on ERK activation are not the result of a general alteration of the protein caused by active site mutations. The mutants were expressed in *Escherichia coli* in parallel with the wild-type CyPA and used to stimulate CHO.CD147 cells. No ERK activation was detected in cells stimulated with the F60A and H126A mutants, whereas F113A and W121A mutants induced ERK phosphorylation at a level slightly lower than ERK activation induced by the wild-type CyPA (Fig. 2*D*). Taken together, these results suggest that CyPA signals through CD147 by a mechanism involving rotamase activity of cyclophilin.

Pro^{180}/Gly^{181} of the CD147 Extracellular Domain Are Critical for CyPA-specific Signaling—Our finding that rotamase

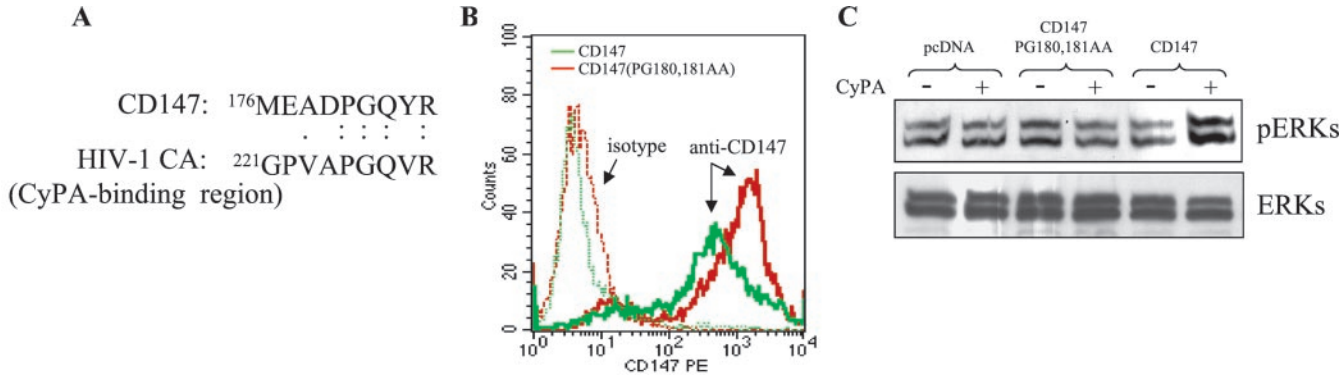

FIG. 3. **Pro[180]-Gly[181] residues are critical for CD147-mediated signaling response to CyPA.** *A*, partial alignment of CD147 with the CyPA binding region of HIV-1 CA. *B*, CD147(P180A,G181A) mutant is expressed at wild-type level. CD147 expression was measured by flow cytometry using phycoerythrin-labeled anti-CD147 mAb. *C*, CD147(P180A,G181A) mutant does not mediate signaling response to CyPA. Pools of CHO cells stably transfected with the wild-type or mutant CD147 or empty pcDNA vector were stimulated or not with 1 μg/ml CyPA. Phosphorylated and total ERKs were measured as in Fig. 2*B*.

activity is essential for CyPA signaling suggested involvement of proline residues in CD147 in signaling mechanism. CyPA binds specifically to a proline-rich region of HIV-1 CA (29). Alignment of the CD147 extracellular domain and CA revealed a 9-amino acid long region of 44.4% identity between these sequences; importantly, this region overlapped with the CyPA binding sequence in CA (Fig. 3*A*). Both sequences contained adjacent proline and glycine residues believed to be critical for CyPA binding (30). To determine whether Pro[180]-Gly[181] of CD147 were critical for CyPA-induced signaling, we replaced these residues with alanines and produced stable pools of CHO cells expressing the wild-type and mutant CD147 constructs. Both constructs expressed similar levels of CD147, as evidenced by flow cytometry (Fig. 3*B*). However, although cells expressing the wild-type CD147 responded to CyPA stimulation with ERK activation, no such response was detected with cells expressing the mutant CD147 (Fig. 3*C*).

Taken together, these results demonstrate that CyPA induces signaling events through interaction with the proline residue in the extracellular region of CD147, suggesting that peptidylprolyl isomerization might be involved in the mechanism of signaling. This interpretation is supported by analysis of CyPA mutants (previous section) and explains the transient nature of CyPA-CD147 interaction, which precluded Scatchard analysis. It appears that CyPA is accumulated and presented for interaction with CD147 by heparans.

CyPA-induced Chemotaxis Is Mediated by CD147—CyPA is a potent chemoattractant for inflammatory cells (5, 7, 8), but the binding interactions and signaling events involved have not been identified. Our finding that CyPA-CD147 interaction initiates a cascade of intracellular signaling events prompted us to investigate whether this interaction is required for CyPA-mediated chemotaxis. Using modified Boyden chambers, we first analyzed chemotaxis of CHO cells engineered to express CD147 (CHO.CD147). Results presented in Fig. 4*A* demonstrate that CyPA is chemotactic for CHO.CD147 but not for control CHO.pcDNA cells. No chemotaxis was detected with CHO.CD147(P180A,G181A) cells that expressed CD147 mutant defective in signaling. Chemotaxis did not occur in the presence of anti-CD147 mAb, supporting the notion that CD147 is necessary for CyPA-induced chemotactic activity.

To extend our observations to primary cells, we used an antibody neutralization strategy to assess the role of CD147 in CyPA-mediated neutrophil chemotaxis. Similar to results obtained with CHO.CD147 cells, CyPA-induced chemotaxis of primary neutrophils was blocked by the addition of anti-CD147 mAb but not by isotypic control antibody (Fig. 4*B*). Therefore, CD147 is required for CyPA-dependent neutrophil chemotaxis. Dose-response analysis of neutrophil chemotaxis produced a characteristic bell-shaped response (Fig. 4*C*), typical for many chemotactic agents.

Because both CyPA binding to cells and CyPA-induced signaling depended on the presence of heparans (Figs. 1*B* and 2*A*), we tested whether heparans were involved in the CyPA-induced chemotaxis of neutrophils. Pretreatment of neutrophils with heparitinase III eliminated CyPA-specific chemotaxis but did not affect chemotaxis induced by fMLP (Fig. 4*D*). We conclude that the presence of heparans on target cells is required for chemotactic activity of CyPA.

DISCUSSION

The experiments presented in this report demonstrate an important role for CD147 in the cellular responses to exogenous CyPA. These results provide a potential mechanism for intercellular effector activities of CyPA, such as its role as a chemoattractant for monocytes, T lymphocytes, eosinophils, and neutrophils (5, 7, 8). Despite its critical role in transducing CyPA-mediated signaling, CD147 does not function as a major binding site for CyPA; this role is taken by cell surface heparans. The finding that two distinct binding interactions, one with heparans (15) and another with CD147 (this report), are required for transcellular CyPA activities is not unprecedented. A similar situation has been described for CyPB (9, 11) and for chemokines RANTES and MIP-1β (31, 32), whose binding to sulfated glycosaminoglycans was hypothesized to present them for a more efficient interaction with their respective signaling receptors. We believe that a similar mechanism applies to CyPA (see below).

High expression of heparans on the cell surface masks interaction between CyPA and CD147. When CyPA binding to heparans is eliminated (either by using heparan-negative cells or mutant CyPA), specific binding to CD147 can be detected. However, this binding is of low affinity and obviously of transitory character. It appears plausible that heparans might facilitate CyPA-CD147 interaction by first binding CyPA and then presenting it to CD147, thus stimulating activity of low CyPA concentrations. This interpretation is supported by our finding that much higher ligand concentrations are required for signaling events initiated by mutant CyPA deficient in heparan binding than by the wild-type CyPA. Such activity of heparans has been described for many chemotactic agents, such as RANTES or fibroblast growth factor (27).

Sequence analysis of the extracellular domain of CD147 identified a short region with 44.4% identity to the CyPA binding region of HIV-1 CA (29, 30). Mutagenesis of Pro[180]-Gly[181] in CD147 eliminated signaling and chemotactic activity of this

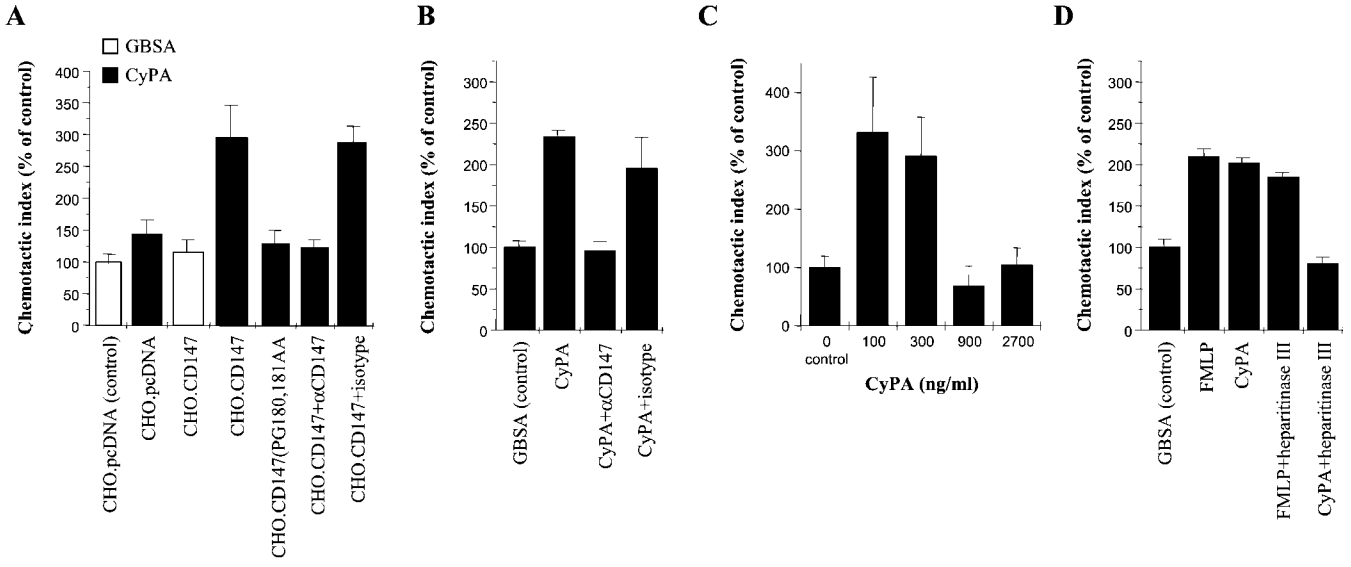

FIG. 4. **CD147 and heparan sulfate are required for chemotactic response to CyPA.** *A*, CyPA induces chemotaxis of CHO.CD147 cells. 100 ng/ml CyPA or BSA in Gey's solution (GBSA) was added to the bottom chamber of the Boyden chamber assembly; the upper chamber contained CHO.pcDNA, CHO.CD147, or CHO.CD147(P180A,G181A) cells. Anti-CD147 mAb or isotype control antibody was added to both chambers where indicated. Chemotaxis was measured as the number of cells migrating to the bottom side of the filter separating the chambers and is presented relative to GBSA-specific chemotaxis of CHO.pcDNA. Data are presented as mean of three independent wells ± S.D. One representative experiment of three performed is shown. *B*, CD147 is required for neutrophil chemotaxis. The experiment was performed as in *A*, except that primary human neutrophils isolated from peripheral blood were used. Results are for one representative experiment of three performed. *C*, neutrophil chemotaxis shows a bell-shaped dose-response curve. The assay was performed as described in *B*, except that the indicated concentrations of CyPA were used. Results are representative of three experiments. *D*, neutrophil chemotaxis depends on heparan sulfate. Chemotactic assay was performed with CyPA or fMLP and primary neutrophils treated or not with heparitinase III. Three experiments with similar results were performed.

molecule. Therefore, these two residues are critical for functional activity of CD147, suggesting that peptidylprolyl isomerization is involved in CyPA-induced signaling. This interpretation is supported by our observation that mutations in the CyPA active site disrupting its rotamase activity also abrogate signaling by CyPA. It is also consistent with the previously demonstrated sensitivity of CyPA-induced chemotaxis to cyclosporin A (5) and explains low affinity and transitory character of the CyPA-CD147 interaction detected in the present study.

Because of ubiquitous expression of CD147 on human cells, we performed our analysis on CHO cells transfected with human CD147. CyPA is among the most conserved proteins known, and the human and Chinese hamster CyPA are 96% identical, whereas extracellular domains of human CD147 and Chinese hamster basigin are 55% identical. Importantly, the Pro^{180}-Gly^{181} motif found critical for CD147 activity is conserved in Chinese hamster basigin. Therefore, human CyPA may also interact with hamster basigin, if CHO cells express this protein. Unfortunately, because of a lack of specific antibodies we could not test expression of hamster basigin. However, the fact that CHO cells do not signal or chemotax in response to human CyPA suggests either that basigin expression is low on CHO cells or that basigin is an inefficient receptor for human CyPA.

The molecular mechanism(s) by which CyPA interaction with CD147 is converted into intracellular signaling events requires additional studies. Truncation of the cytoplasmic tail of CD147 abrogated signaling and chemotactic response,[2] suggesting involvement of the cytoplasmic domain of CD147 in assembly of the signal transducing complex. Given the reported association of CD147 with α_3b_1 and $\alpha_6\beta_1$ integrins (33), it is also possible that integrins are involved in the transduction of the signal from CD147.

Identification of CD147 as a signaling partner for cyclophilins introduces a new twist into the long studied activities of these proteins. Indeed, CD147 is a highly glycosylated cell surface protein of the immunoglobulin superfamily that includes a plethora of different molecules involved in cell surface interactions and immunological recognition (33, 34). This protein is also known as EMMPRIN, and early studies by Ellis and colleagues (35) and Biswas and colleagues (36) demonstrated that EMMPRIN expressed by cultured tumor cells stimulates fibroblasts to produce very high levels of collagenase activity. In view of the results presented in this report, it would be important to determine the role of cyclophilins in metalloproteinase induction.

Another previously described activity of CD147 (EMMPRIN) is to regulate intercellular adhesion pathways through an intracellular signaling-mediated mechanism (23). Such processes are important for the directed movement of immune cells to the site of inflammation, and results reported herein and in our other report (12) raise the possibility that cyclophilins might be involved. Indeed, cyclophilins might be the missing natural ligands of CD147 which regulate adhesion and chemotactic activity of peripheral blood cells involved in immune functions. Mice lacking CD147 (basigin) developed normally during the preimplantation period, but the majority of embryos died around the time of implantation, suggesting that basigin is involved in intercellular recognition during implantation (37). Because this process critically depends on chemotactic activities of participating cells, it would be interesting to determine whether extracellular cyclophilins also regulate implantation and embryogenesis through interaction with CD147.

Results described in this report also suggest that CyPA may contribute to pathology in certain diseases in which elevated levels of this immunophilin have been reported. The most striking example is rheumatoid arthritis, where levels of CyPA in synovial fluids and CD147 on reactive neutrophils have been shown to correlate with disease severity (38–40). Our finding

[2] V. Yurchenko, unpublished observation.

that CyPA-mediated neutrophil chemotaxis involves CD147 suggests a role for CyPA-CD147 interaction in the pathogenesis of rheumatoid arthritis. CyPA might induce chemoattraction of reactive neutrophils to the synovial space. Once within the joint, neutrophils may be activated, most likely by phagocytosing cellular debris and immune complexes, resulting in the release of proteinases, reactive oxidants, and cytokines, which contribute to the pathogenesis of the disease (41–45). Indeed, blocking neutrophil-secreted elastase with specific inhibitors (*e.g.* MDL 101,146 and ONO-5046) results in striking decreases in cartilage destruction in animal models of arthritis (46, 47). We propose that a similar effect may be achieved by blocking CyPA-CD147 interactions using, for example, neutralizing antibody to CD147.

In summary, this report demonstrates that CD147 acts as a signaling receptor for CyPA, providing a mechanism for previously observed extracellular activities of CyPA (5, 6, 13). Our results are consistent with a model in which CyPA binds to heparans, which then present it to CD147 for signaling interactions. This arrangement resembles the situation with toll-like receptors, which bind lipopolysaccharide with low affinity and require an additional lipopolysaccharide-binding molecule, CD14, to initiate signaling response to lipopolysaccharide (48). Future studies will determine the physiological and pathophysiological role of CyPA-CD147 interaction.

Acknowledgments—We thank our colleagues at the Picower Institute for Medical Research for helpful discussions and Kirk Manogue for critical reading of the manuscript. We are especially grateful to Anthony Cerami for valuable suggestions during the course of this work.

REFERENCES

1. Galat, A. (1993) *Eur. J. Biochem.* **216,** 689–707
2. Fruman, D. A., Burakoff, S. J., and Bierer, B. E. (1994) *FASEB J.* **8,** 391–400
3. Liu, J. (1993) *Immunol. Today* **14,** 290–295
4. Kofron, J. L., Kuzmic, P., Kishore, V., Colon-Bonilla, E., and Rich, D. H. (1991) *Biochemistry* **30,** 6127–6134
5. Sherry, B., Yarlett, N., Strupp, A., and Cerami, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3511–3515
6. Jin, Z. G., Melaragno, M. G., Liao, D. F., Yan, C., Haendeler, J., Suh, Y. A., Lambeth, J. D., and Berk, B. C. (2000) *Circ. Res.* **87,** 789–796
7. Xu, Q., Leiva, M. C., Fischkoff, S. A., Handschumacher, R. E., and Lyttle, C. R. (1992) *J. Biol. Chem.* **267,** 11968–11971
8. Allain, F., Vanpouille, C., Carpentier, M., Slomianny, M. C., Durieux, S., and Spik, G. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 2714–2719
9. Denys, A., Allain, F., Carpentier, M., and Spik, G. (1998) *Biochem. J.* **336,** 689–697
10. Friedman, J., Trahey, M., and Weissman, I. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6815–6819
11. Carpentier, M., Allain, F., Haendler, B., Denys, A., Mariller, C., Benaissa, M., and Spik, G. (1999) *J. Biol. Chem.* **274,** 10990–10998
12. Yurchenko, V., O'Connor, M., Dai, W. W., Guo, H., Toole, B., Sherry, B., and Bukrinsky, M. (2001) *Biochem. Biophys. Res. Commun.* **288,** 786–788
13. Pushkarsky, T., Zybarth, G., Dubrovsky, L., Yurchenko, V., Tang, H., Guo, H., Toole, B., Sherry, B., and Bukrinsky, M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 6360–6365
14. Sherry, B., Zybarth, G., Alfano, M., Dubrovsky, L., Mitchell, R., Rich, D., Ulrich, P., Bucala, R., Cerami, A., and Bukrinsky, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1758–1763
15. Saphire, A. C., Bobardt, M. D., and Gallay, P. A. (1999) *EMBO J.* **18,** 6771–6785
16. Adams, M. D., Dubnick, M., Kerlavage, A. R., Moreno, R., Kelley, J. M., Utterback, T. R., Nagle, J. W., Fields, C., and Venter, J. C. (1992) *Nature* **355,** 632–634
17. Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993) *Cell* **73,** 1067–1078
18. Braaten, D., Ansari, H., and Luban, J. (1997) *J. Virol.* **71,** 2107–2113
19. Boyden, S. (1962) *J. Exp. Med.* **115,** 453–466
20. DeCastro, R., Zhang, Y., Guo, H., Kataoka, H., Gordon, M. K., Toole, B., and Biswas, G. (1996) *J. Invest. Dermatol.* **106,** 1260–1265
21. Wimazal, F., Ghannadan, M., Muller, M. R., End, A., Willheim, M., Meidlinger, P., Schernthaner, G. H., Jordan, J. H., Hagen, W., Agis, H., Sperr, W. R., Czerwenka, K., Lechner, K., and Valent, P. (1999) *Tissue Antigens* **54,** 499–507
22. Inoue, H., Sawada, M., Ryo, A., Tanahashi, H., Wakatsuki, T., Hada, A., Kondoh, N., Nakagaki, K., Takahashi, K., Suzumura, A., Yamamoto, M., and Tabira, T. (1999) *Glia* **28,** 265–271
23. Kasinrerk, W., Tokrasinwit, N., and Phunpae, P. (1999) *Immunology* **96,** 184–192
24. Kirsch, A. H., Diaz, L. A. J., Bonish, B., Antony, P. A., and Fox, D. A. (1997) *Tissue Antigens* **50,** 147–152
25. Mutin, M., Dignat-George, F., and Sampol, J. (1997) *Tissue Antigens* **50,** 449–458
26. Stockinger, H., Ebel, T., Hansmann, C., Koch, C., Majdic, O., Prager, E., Patel, D. D., Fox, D. A., Horejsi, V., Sagawa, K., and Shen, D.-C. (1997) in *Leucocyte Typing VI* (Kishimoto, T., Kikutani, H., Borne, A. E. G., Goyert, S. M., Mason, D., Miyasaka, M., Moretta, L., Okumura, K., Shaw, S., Springer, T., Sugamura, K., and Zola, H., eds) pp. 760–766, Garland Publishing, Inc., New York
27. Schonherr, E., and Hausser, H. J. (2000) *Dev. Immunol.* **7,** 89–101
28. Zydowsky, L. D., Etzkorn, F. A., Chang, H. Y., Ferguson, S. B., Stolz, L. A., Ho, S. I., and Walsh, C. T. (1992) *Protein Sci.* **1,** 1092–1099
29. Gamble, T. R., Vajdos, F. F., Yoo, S., Worthylake, D. K., Houseweart, M., Sundquist, W. I., and Hill, C. P. (1996) *Cell* **87,** 1285–1294
30. Yoo, S., Myszka, D. G., Yeh, C., McMurray, M., Hill, C. P., and Sundquist, W. I. (1997) *J. Mol. Biol.* **269,** 780–795
31. Tanaka, Y., Adams, D. H., Hubscher, S., Hirano, H., Siebenlist, U., and Shaw, S. (1993) *Nature* **361,** 79–82
32. Gilat, D., Hershkoviz, R., Mekori, Y. A., Vlodavsky, I., and Lider, O. (1994) *J. Immunol.* **153,** 4899–4906
33. Berditchevski, F., Chang, S., Bodorova, J., and Hemler, M. E. (1997) *J. Biol. Chem.* **272,** 29174–29180
34. Berditchevski, F., Tolias, K. F., Wong, K., Carpenter, C. L., and Hemler, M. E. (1997) *J. Biol. Chem.* **272,** 2595–2598
35. Ellis, S. M., Nabeshima, K., and Biswas, C. (1989) *Cancer Res.* **49,** 3385–3391
36. Biswas, C., Zhang, Y., DeCastro, R., Guo, H., Nakamura, T., Kataoka, H., and Nabeshima, K. (1995) *Cancer Res.* **55,** 434–439
37. Igakura, T., Kadomatsu, K., Kaname, T., Muramatsu, H., Fan, Q. W., Miyauchi, T., Toyama, Y., Kuno, N., Yuasa, S., Takahashi, M., Senda, T., Taguchi, O., Yamamura, K., Arimura, K., and Muramatsu, T. (1998) *Dev. Biol.* **194,** 152–165
38. Billich, A., Winkler, G., Aschauer, H., Rot, A., and Peichl, P. (1997) *J. Exp. Med.* **185,** 975–980
39. Konttinen, Y. T., Li, T. F., Mandelin, J., Liljestrom, M., Sorsa, T., Santavirta, S., and Virtanen, I. (2000) *Arthritis Rheum.* **43,** 275–280
40. Zucker, S., Hymowitz, M., Conner, C., Zarrabi, H. M., Hurewitz, A. N., Matrisian, L., Boyd, D., Nicolson, G., and Montana, S. (1999) *Ann. N. Y. Acad. Sci.* **878,** 212–227
41. McCachren, S. S. (1991) *Arthritis Rheum.* **34,** 1085–1093
42. Firestein, G. S. (1996) *Arthritis Rheum.* **39,** 1781–1790
43. Sewell, K. L., and Trentham, D. E. (1993) *Lancet* **341,** 283–286
44. Edwards, S. W., and Hallett, M. B. (1997) *Immunol. Today* **18,** 320–324
45. Harris, E. D. J. (1990) *N. Engl. J. Med.* **322,** 1277–1289
46. Kakimoto, K., Matsukawa, A., Yoshinaga, M., and Nakamura, H. (1995) *Cell. Immunol.* **165,** 26–32
47. Janusz, M. J., and Durham, S. L. (1997) *Inflamm. Res.* **46,** 503–508
48. Ingalls, R. R., Heine, H., Lien, E., Yoshimura, A., and Golenbock, D. (1999) *Infect. Dis. Clin. North Am.* **13,** 341–353, vii
49. Liu, J., Chen, C. M., and Walsh, C. T. (1991) *Biochemistry* **30,** 2306–2310