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Control of the Interferon-Induced 68-Kilodalton Protein Kinase by the HIV-1 *tat* Gene Product

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The *tat*-responsive region (TAR) of the human immunodeficiency virus–1 (HIV-1) exhibits a trans-inhibitory effect on translation in vitro by activating the interferon-induced 68-kilodalton protein kinase (p68 kinase). Productive infection by HIV-1 was shown to result in a significant decrease in the amount of cellular p68 kinase. The steady-state amount of p68 kinase was also reduced in interferon-treated HeLa cell lines stably expressing *tat*, as compared to the amount of the kinase in interferon-treated control HeLa cells. Thus, the potential translational inhibitory effects of the TAR RNA region mediated by activation of p68 kinase may be downregulated by *tat* during productive HIV-1 infection.

THE HIV-1 *tat* GENE PRODUCT trans-activates viral gene expression (*1*) and is essential for HIV-1 replication (*2*). Its mode of action has been proposed to be either transcriptional, post-transcriptional, or a combination of both (*3*). TAR is present at the 5′ end of all HIV-1 mRNAs (*4*) and assumes a stable stem and loop structure in vitro, as determined by RNA nuclease mapping (*5*). When fused to a heterologous mRNA, TAR exhibits a strong inhibitory effect on translation in cell-free extracts and *Xenopus* oocytes (*6*). Translational inhibition is partly due to a trans-inhibitory effect mediated by the activation of the interferon-induced, double-stranded (ds) RNA-dependent protein kinase (*7*), also termed dsI, DAI, or p68 kinase. This results in autophosphorylation of the kinase (*8*), which then catalyzes the phosphorylation of the α subunit of eukaryotic initiation factor–2 (eIF-2), with subsequent inhibition of protein synthesis (*9*). The p68 kinase is one of many proteins induced by interferon; its activation has been suggested to be important in the establishment of the antiviral state mediated by interferon (*10*).

Activation of p68 kinase and subsequent inhibition of protein synthesis can be outcomes of viral infection (*11, 12*). However, a number of eukaryotic viruses have developed strategies to escape the inhibitory effects caused by activation of p68 kinase (*12, 13*). Consequently, we examined the possibility that HIV-1 regulates the expression of p68 kinase. The human T-lymphoid cell line CEM was infected with the LAV isolate (*14*) of HIV-1 under conditions in which >90% of cells express viral protein 3 days after infection, as determined by indirect immunofluorescence. One to 5 days after infection, cell extracts were prepared and analyzed by protein immunoblotting for viral proteins, p68 kinase, and actin (Fig. 1). Viral proteins p24, p55, p66, gp120, and gp41 were prominent as early as 2 days after infection, as determined with the use of serum from an individual with acquired immunodeficiency syndrome (AIDS) (Fig. 1A). The amount of p68 kinase in infected cells, measured with a monoclonal antibody to the kinase (*15*), did not significantly differ from that in mock-infected cells during the first 2 days after infection (an increase in the amounts of p68 kinase was observed on day 2 in both mock- and HIV-1–infected cells, possibly because the medium was changed) but was reduced by day 3 and virtually undetectable at 4 and 5 days after infection (Fig. 1B). The decline in the amount of p68 kinase occurred before the appearance of virus-induced cytopathic effects, which were generally apparent 7 days after infection. The decrease in p68 kinase is unlikely to reflect a general proteolysis, because the amount of actin remained relatively stable over the time course of infection, with only a slight decrease detected after 3 days (Fig. 1B). This difference cannot be explained by different half-lives of the proteins, as the half-lives of p68 kinase and actin are 6 to 7 hours and 12 to 16 hours, respectively, as determined by immunoblotting of CEM cells treated with the protein synthesis inhibitor anisomycin (*16*). Thus, productive HIV-1 replication is associated with a decrease in the amount of cellular p68 kinase.

It is conceivable that one of the viral trans-acting proteins is responsible for the

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downregulation of p68 kinase. As the 5′ noncoding region of HIV-1 that activates p68 kinase in vitro is the cis-acting element responsive to *tat* (*4*), immunoprecipitation and immunoblot analyses of p68 kinase levels were performed in *tat*-expressing cell lines. We used a HeLa S3 cell line that expresses *tat* under the control of the long terminal repeat (LTR) of murine leukemia virus (MuLV) (*17*) and that was established by retroviral gene transfer (*18*). Because HeLa cells express low basal amounts of p68 kinase, cells were treated for 16 hours with lymphoblastoid interferon to induce p68 kinase. Metabolic labeling was performed for 7 hours and therefore reflects the steady-state amount of the protein. In the absence of interferon, levels of p68 kinase were low in *tat*-expressing (+*tat*) and non-expressing (−*tat*) HeLa cells, as determined after immunoprecipitation of ^{35}S-labeled extracts with a monoclonal antibody to p68 kinase (Fig. 2A, lanes 1 and 3). Although not evident in this figure, other experiments showed that the constitutive level of p68 kinase in HeLa (−*tat*) was approximately two times as great as that in HeLa (+*tat*) cells (*19*). Interferon treatment significantly induced (fourfold) p68 kinase levels in HeLa (−*tat*) cells (Fig. 2A, lanes 1 and 2), but had little effect in HeLa (+*tat*) cells (Fig. 2A, lanes 3 and 4). A similar pattern was observed when p68 kinase was immunoprecipitated from ^{32}P-labeled cell extracts (Fig. 2B), suggesting that kinase activity parallels physical levels of the enzyme. To further substantiate our results, extracts from HeLa (+*tat* or −*tat*) cells were analyzed by immunoblotting (Fig. 2C). The constitutive level of p68 kinase in *tat*-expressing cells was half that of control cells (Fig. 2C, lanes 1 and 3). Treatment with interferon resulted in a large increase (sixfold) in the amount of p68 kinase in control cells (Fig. 2C, lanes 1 and 2), but a small increase (1.5-fold) in *tat*-expressing cells (Fig. 2C, lanes 3 and 4). To ensure that similar protein amounts were analyzed in each sample, we determined the actin levels by immunoblotting and found no significant variation between samples (Fig. 2C). These studies were repeated with similar results (*19*) in a HeLa R19 cell line in which *tat* is expressed under the control of the HIV-1 promoter-enhancer element (*20*). In a further control experiment, we found that downregulation of p68 kinase was not observed in a HeLa cell line stably expressing a gene conferring neomycin resistance (*19*).

To provide additional evidence that downregulation of p68 kinase is dependent on the expression of *tat*, we performed immunoprecipitation analysis of p68 kinase in HeLa cell lines stably transfected with *tat* expression vectors encoding wild-type or mutant *tat* protein (C37) (*21*) (Fig. 3). The mutation substitutes Ser for Cys^{37} and results in dramatic reduction in *tat* gene product trans-activation activity (*19, 21*). Although the interferon-induced amount of p68 kinase was reduced in cells expressing a functional *tat* (Fig. 3A, lane 4), an amount comparable to that in HeLa (−*tat*) cells was expressed in the C37 mutant cell (Fig. 3A, lanes 2 and 6). These results could not be accounted for by differences in *tat* expression, as similar amounts were detected in the

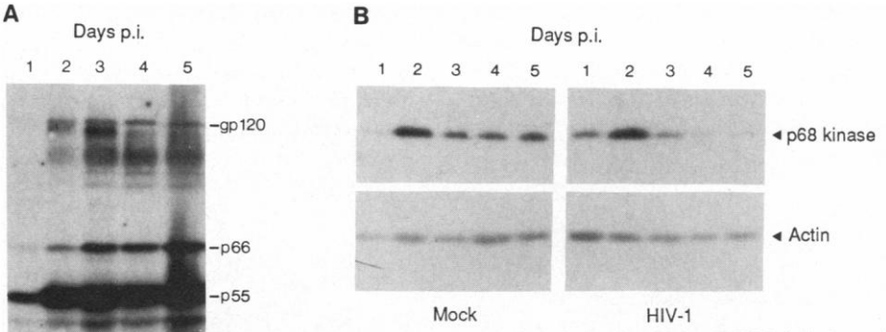

Fig. 1. (**A**) Detection of viral proteins during HIV-1 infection of CEM cells. Cell extracts were analyzed by immunoblotting with serum from an individual with AIDS. (**B**) Amount of p68 kinase and actin measured during HIV-1 infection of CEM cells. Cell extracts were analyzed by immunoblotting with a monoclonal antibody to p68 kinase (*15*) (upper panels) or a monoclonal antibody to actin (ICN) (lower panels). Cells (2×10^7) were infected with 0.1 $TCID_{50}$ (median tissue culture infectious dose, estimated by infecting cell cultures with serial dilutions of virus stock and monitoring for syncitia formation and cytopathic effects after 7 to 14 days) of the LAV isolate (*14*) of HIV-1 per cell, which was the minimal dose that resulted in >90% of the cells expressing viral proteins 3 days after infection (as determined by indirect immunofluorescence). A mock infection was carried out at the same time. After 1 hour of adsorption, cells were centrifuged and resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS). At the indicated times of infection, cells were washed with ice-cold Hanks balanced salt solution and disrupted in lysis buffer [10 m*M* tris-HCl (*p*H 7.5), 50 m*M* KCl, 1 m*M* dithiothreitol (DTT), 2 m*M* $MgCl_2$, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 0.2% SDS, and 0.5% deoxycholate]. Three detergents were used in order to ensure that the p68 kinase was completely solubilized. Cell extracts were centrifuged at 10,000*g* for 5 min, and the protein content was determined by the Bio-Rad protein assay. Immunoblot analysis on 100 μg of total protein was performed as described (*26*), except that the second antibody was ^{125}I-labeled rabbit antibody to mouse immunoglobulin G; p.i., post-infection.

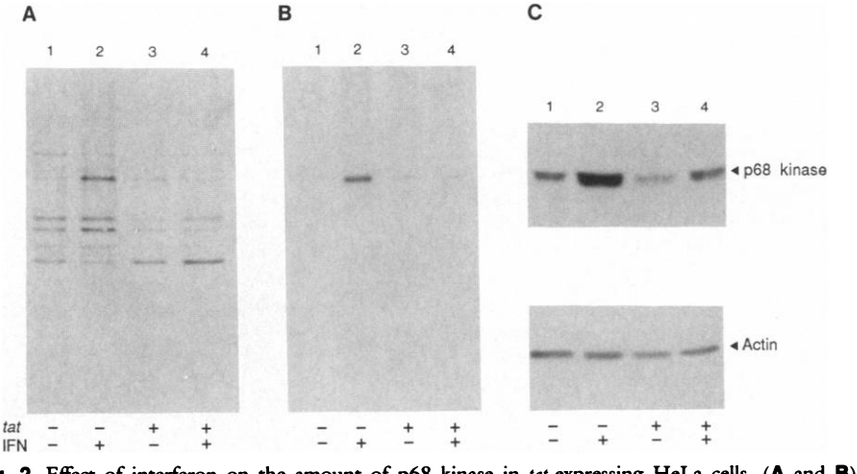

Fig. 2. Effect of interferon on the amount of p68 kinase in *tat*-expressing HeLa cells. (**A** and **B**) immunoprecipitation analysis. (**C**) Immunoblotting. HeLa S3 cells expressing *tat* were established by retroviral gene transfer (*17, 18*) and maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS. Cells were incubated in the presence of lymphoblastoid interferon (IFN) (1000 IU/ml; Hayashibara Biochemical Laboratories, Okayama, Japan) for 16 hours. For immunoprecipitation, cells were labeled for 7 hours with either (A) [^{35}S]methionine (0.5 mCi; trans ^{35}S-label, (ICN) in α-MEM lacking methionine or (B) ^{32}P-labeled inorganic phosphate ([^{32}P]P_i) (1 mCi; ICN) in DMEM lacking phosphate. Cell extracts were prepared as described in Fig. 1. Incorporation of radiolabel into proteins was measured by trichloroacetic acid (TCA) preparation, and 50×10^6 counts (^{35}S-labeled samples) or 10×10^6 counts (^{32}P-labeled samples) were subjected to immunoprecipitation analysis, as described by Katze *et al.*, 1987 (*13*). For immunoblot analysis, cells were lysed immediately after interferon treatment and treated as described in Fig. 1.

wild-type and C37 mutant *tat*-expressing cell lines by immunoprecipitation with a polyclonal antibody to *tat* (*22*) (Fig. 3B, lanes 2 and 3).

Further evidence for *tat*-mediated modulation of p68 kinase, independent of the antibody used for the immunoprecipitation and immunoblot analyses, was obtained by assaying for kinase activity in cytoplasmic extracts (S10) from HeLa cells treated with interferon or without it. Extracts were incubated with [γ-^{32}P]ATP (adenosine triphosphate) in the presence of two concentrations of reovirus ds RNA and then analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Fig. 4). Incubation of control extract with [γ-^{32}P]ATP in the presence of reovirus ds RNA (1 μg/ml) led to the specific phosphorylation of p68 kinase and the α subunit of eIF-2 (Fig. 4, lanes 1 and 2). When an extract from interferon-treated cells was incubated with reovirus ds RNA (1 μg/ml), the extent of phosphorylation of p68 kinase was three times as great as that seen in control cells (Fig. 4, lanes 1 and 3), whereas the extent of eIF-2α phosphorylation was 1.5 times greater. Incubation of extracts from *tat*-expressing cells with [γ-^{32}P]ATP and reovirus ds RNA (1 μg/ml) resulted in reduced phosphorylation levels relative to HeLa (−*tat*) cells. In extracts from nontreated cells expressing *tat*, phosphorylation of p68 kinase was one half that seen in HeLa (−*tat*) cells (Fig. 4, lanes 1 and 5). In extracts from interferon-treated *tat*-expressing cells, no phosphorylation of p68 kinase and eIF-2α was detectable (Fig. 4, lane 7). Thus, the kinase activity paralleled physical amounts of p68 kinase measured by immunoprecipitation and immunoblotting.

It is possible that the *tat*-mediated reduction in the concentration of p68 kinase reflects a global malfunction of the interferon response in *tat*-expressing cells. To examine this possibility, we assayed for the transcriptional induction of 2-5A synthetase in

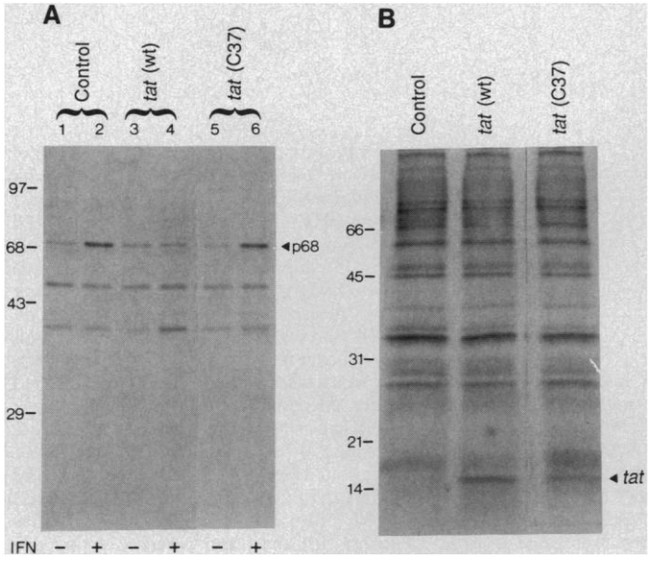

Fig. 3. (**A**) Immunoprecipitation analysis of p68 kinase in HeLa cells expressing wild-type (wt) or mutant (C37) *tat*. (**B**) Immunoprecipitation analysis of *tat* in HeLa cells expressing wild-type (wt) or mutant (C37) *tat*. *Tat* expression vectors (*21*) were cotransfected with pSV2neo (*27*) into HeLa S3 cells by the calcium-phosphate precipitation method (*28*). Clones resistant to G418 (450 μg/ml) were screened for *tat* mRNA expression. Interferon treatment and immunoprecipitation analysis of p68 kinase were performed as described in Fig. 2. For immunoprecipitation analysis of *tat*, cells were labeled with [^{35}S]cysteine (150 μCi; ICN) in α-MEM lacking cysteine. After 3 hours, cells were lysed in RIPA buffer [10 m*M* tris-HCl (*p*H 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 0.25 m*M* PMSF, 1% Triton X-100, 0.1% SDS, and 1% deoxycholate]. The extracts were incubated with a rabbit polyclonal antibody to the *tat* gene product [generated against amino acids 1 to 62 (*22*)] and protein A–Sepharose CL-4B (Pharmacia) for 2 hours at room temperature. After washing five times in RIPA buffer, the immunoprecipitates were boiled in 3× electrophoresis disruption buffer. They were then subjected to electrophoresis on an SDS–15% polyacrylamide gel and autoradiography.

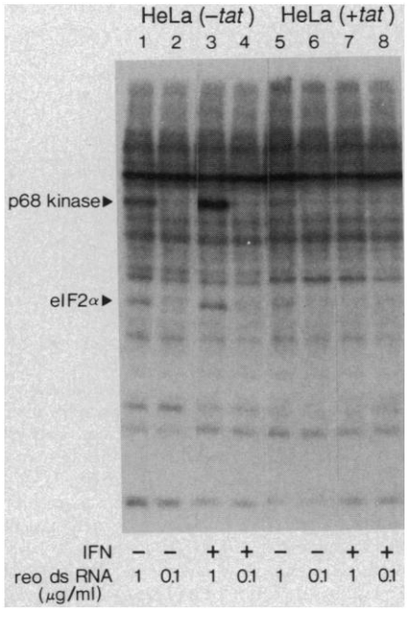

Fig. 4. Effect of interferon on in vitro p68 kinase activity in extracts from *tat*-expressing HeLa cells. Subconfluent cell monolayers were incubated in the presence of interferon, as described in Fig. 2, and harvested with a rubber policeman. After washing in phosphate-buffered saline (PBS), cells were suspended in 1.5 times the packed cell volume of hypotonic buffer containing 10 m*M* Hepes (*p*H 7.5), 10 m*M* potassium acetate, 1.5 m*M* magnesium acetate, and 2 m*M* DTT. Cells were incubated on ice for 5 min and lysed by rapidly pushing them several times through a 25-gauge hypodermic needle. Cell lysates were centrifuged at 10,000*g* for 5 min, and the protein content of the supernatant (S10) was determined with the Bio-Rad protein assay. The in vitro p68 kinase reaction was performed by mixing 20 μg of S10 extract with the indicated amounts of reovirus ds RNA in a buffer containing 20 m*M* Hepes (*p*H 7.5), 50 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* β-mercaptoethanol, 2 μ*M* ATP, and [γ-^{32}P]ATP (10 μCi; Du Pont Biotechnology Systems) at 30°C for 20 min. The reaction was stopped by addition of 3× electrophoresis buffer and boiling. The samples were subjected to electrophoresis on an SDS–12.5% polyacrylamide gel and autoradiography.

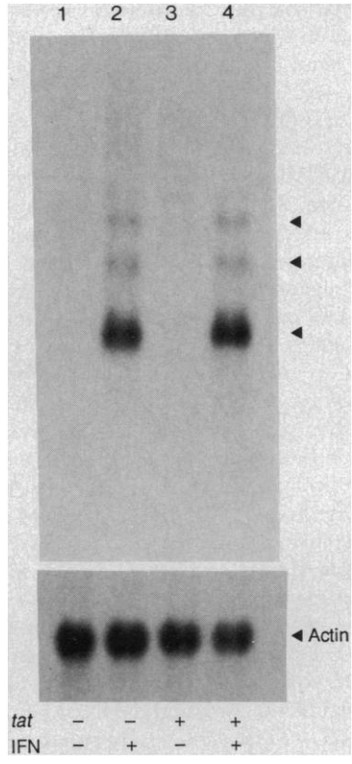

Fig. 5. Northern blot analysis of 2-5A synthetase and actin mRNAs in interferon-treated, *tat*-expressing HeLa cells. Total RNA was isolated by guanidine-hydrochloride precipitation and phenol-chloroform extraction (*29*) from cells either untreated or treated with interferon as described in Fig. 2. Polyadenylated RNAs were purified by oligo(dT)-cellulose (Pharmacia) chromatography (*30*). RNAs were electrophoresed on a 6% formaldehyde–1% agarose gel, blotted on nitrocellulose paper, and hybridized with ^{32}P-labeled 2-5A synthetase (upper panel) or actin (lower panel) DNA probes prepared by random priming (*31*). Unlabeled arrowheads indicate the 1.5/1.7-, 2.5-, and 3.5-kb 2-5A synthetase mRNAs.

response to interferon. On activation by ds RNA, 2-5A synthetase synthesizes 2′-5′ oligoadenylates, which in turn activate ribonuclease L [which is involved in mRNA and ribosomal RNA degradation (*23*)]. The 2-5A synthetase activity may also play an important role in the establishment of the antiviral state mediated by interferon (*10*). Northern (RNA) blot analysis was performed with a probe derived from a 2-5A synthetase cDNA clone (*24*) on polyadenylated RNA that had been isolated from HeLa cells (+*tat* or −*tat*) after a 16-hour incubation with lymphoblastoid interferon. This cDNA clone hybridizes to 1.5-, 1.7-, 2.5-, and 3.5-kb mRNAs in HeLa cells (*24, 25*). HeLa (−*tat*) cells expressed increased amounts of mRNA for 2-5A synthetase in response to interferon (Fig. 5, lanes 1 and 2). The interferon-mediated induction of these mRNAs in HeLa (+*tat*) cells (Fig. 5, lanes 3 and 4) was similar to that observed in HeLa (−*tat*) cells. As an internal control, actin mRNA was measured and found to be constant (Fig. 5). These results suggest that the interferon receptors have not undergone qualitative or quantitative changes in *tat*-expressing cells and that a general inhibition of the interferon response did not occur in these cells.

Our results show that in HIV-1–infected cells p68 kinase is downregulated. We propose that *tat* is responsible for mediating this downregulation, as interferon-induced p68 kinase levels were decreased in HeLa cells expressing a functional *tat*, as compared to control cells in which *tat* is absent or mutated. More mutant *tat* proteins need to be examined in order to establish a correlation between *tat*-mediated trans-activation and p68 kinase downregulation. The mechanism by which *tat* decreases p68 kinase levels may occur transcriptionally or posttranscriptionally.

The observation that *tat*-expressing cells are deficient in their ability to upregulate the expression of p68 kinase in response to interferon may have physiological implications for the replication cycle of HIV-1. Expression of *tat* is essential for productive viral infection. This may be due, in part, to the role of the gene product in countering the antiviral state that is mediated by the activation of interferon-inducible proteins. Downregulation of p68 kinase could provide a means for the virus to switch from latency to active replication.

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PECAM-1 (CD31) Cloning and Relation to Adhesion Molecules of the Immunoglobulin Gene Superfamily

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An antibody to a platelet integral membrane glycoprotein was found to cross-react with the previously identified CD31 myelomonocytic differentiation antigen and with hec7, an endothelial cell protein that is enriched at intercellular junctions. This antibody identified a complementary DNA clone from an endothelial cell library. The 130-kilodalton translated sequence contained six extracellular immunoglobulin (Ig)-like domains and was most similar to the cell adhesion molecule (CAM) subgroup of the Ig superfamily. This is the only known member of the CAM family on platelets. Its cell surface distribution suggests participation in cellular recognition events.

HUMAN PLATELETS PARTICIPATE IN the normal hemostatic process and after vascular injury change from unreactive disks to adherent, pseudopod-containing spheres. Many platelet functions, including adhesion to extracellular matrix components, self-association (aggregation), and spreading (*1*), are reproduced by other cell types. The realization that several membrane glycoproteins thought to be "platelet-specific" are also on other cells (*2*) has allowed a number of molecular mechanisms for mediating cell surface interactions during hemostasis, differentiation and development, wound healing, and oncogenesis to be redefined.

Platelets and endothelial cells share a number of common membrane components (*3*), including several members of the integrin family of cytoadhesive receptors (*4*). To identify additional surface glycoproteins that might participate in platelet and endothelial cell function, we prepared a polyclonal antibody to human platelet integral membrane proteins (*5*) and used this antibody to screen an endothelial cell λgt11 expression library. Antibody-positive clones

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