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Ligation of HLA Class I Molecules on Endothelial Cells Induces Phosphorylation of Src, Paxillin, and Focal Adhesion Kinase in an Actin-Dependent Manner

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The development of chronic rejection is the major limitation to long-term allograft survival. HLA class I Ags have been implicated to play a role in this process because ligation of class I molecules by anti-HLA Abs stimulates smooth muscle cell and endothelial cell proliferation. In this study, we show that ligation of HLA class I molecules on the surface of human aortic endothelial cells stimulates phosphorylation of Src, focal adhesion kinase, and paxillin. Signaling through class I stimulated Src phosphorylation and mediated fibroblast growth factor receptor (FGFR) translocation to the nucleus. In contrast, Src kinase activity was not involved in class I-mediated transfer of FGFR from cytoplasmic stores to the cell surface. Inhibition of Src protein kinase activity blocked HLA class I-stimulated tyrosine phosphorylation of paxillin and focal adhesion kinase. Furthermore, HLA class I-mediated phosphorylation of the focal adhesion proteins and FGFR expression was inhibited by cytochalasin D and latrunculin A, suggesting a role for the actin cytoskeleton in the signaling process. These findings indicate that anti-HLA Abs have the capacity to transduce activation signals in endothelial cells that may promote the development of chronic rejection. The Journal of Immunology, 2002, 168: 5415–5423.

A form of chronic rejection, termed accelerated transplant arteriosclerosis, is the major cause of solid organ graft failure after the first year posttransplantation (1, 2). Transplant arteriosclerosis is characterized by the proliferation of intimal smooth muscle cells (SMC) and endothelial cells (EC) in the walls of the arteries of the transplanted organ resulting in occlusion of the vessels and fibrosis of the graft. Anti-HLA Abs have long been implicated in the process of chronic rejection, as several studies have shown that patients developing anti-donor HLA Abs following transplantation are at increased risk of developing chronic rejection and graft loss (3–10). A consistent finding in graft arteriosclerotic lesions is Ig deposits in affected vessel walls and within the media (2, 11, 12). In addition, allografts transplanted to Ig-deficient mice fail to develop fibrotic arteriopathic lesions, whereas prominent fibrotic lesions occur in recipients developing humoral immunity to the allograft (13).

Although anti-HLA Abs have been implicated in chronic rejection, their precise role in the disease process is not well understood. In previous studies we have shown that ligation of class I molecules with human anti-HLA Abs recognizing polymorphic residues located on the class I H chain transduces activation signals in EC and SMC and initiates cell proliferation in a model relevant to the development of transplantation-associated vasculopathies (14–18). Thus, engagement of class I molecules by anti-HLA Abs stimulated tyrosine phosphorylation of intracellular proteins, increased fibroblast growth factor (FGF) receptor cell surface expression, and enhanced proliferative responses to basic FGF (bFGF). These results have led us to conclude that anti-HLA Abs can contribute to the process of chronic rejection by binding to the surface of the endothelium and smooth muscle of the allograft and transducing signals that ultimately result in cell proliferation.

Numerous studies have shown that HLA class I molecules can transduce signals that regulate various aspects of cell metabolism, including activation and cell growth, or cell cycle arrest and apoptosis (19–33). For example, in both resting and activated T and B cells, Src and Syk kinases become activated following class I ligation, leading to phosphorylation of phospholipase Cγ-1, generation of inositol triphosphate and diacylglycerol, and regulation of intracellular free ionized calcium (19, 32, 34). It has also been reported that phosphoinositide-3 kinase and threonine/serine kinases are involved in MHC class I-induced signal transduction in T and B cells (35). Furthermore, MHC class I ligation activates the Janus tyrosine kinase-2, leading to phosphorylation of the transcription factor STAT-3 (19, 20, 23, 36, 37). Cross-linking of class I molecules has also been reported to induce apoptosis of T and B lymphocytes through the activation of the Src kinase p56lck and c-Jun N-terminal kinase activity (20). Although MHC class I molecules expressed by other cell types such as macrophages, mast cells, fibroblasts, and ECs have been shown to transduce proliferative signals, the intracellular signaling pathways remain to be elucidated (38).

In the present study, we have investigated the intracellular signal transduction pathway triggered by the binding of anti-HLA class I Abs to human ECs. This study is the first to show that binding
of anti-HLA Abs to class I molecules expressed on ECs induces tyrosine phosphorylation of Src kinases, including Src and Fyn, and the focal adhesion proteins focal adhesion kinase (FAK) and paxillin. Anti-HLA Abs mediate increased FGFR cell surface expression through a FAK-dependent pathway that requires the integrity of the actin cytoskeleton. Src kinase activity was not required for class I-induced FGFR cell surface expression; however, it was necessary for class I-mediated redistribution of FGFRs to the nucleus.

Materials and Methods

Abs and chemicals

Cytochalasin D and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO). Latrunculin A was obtained from Molecular Probes (Eugene, OR). The Src kinase inhibitor PP2 and inactive analog PP3 were obtained from Calbiochem (La Jolla, CA). Anti-human bFGF neutralizing Ab and recombinant human bFGF were purchased from R&D Systems (Minneapolis, MN). The anti-phosphotyrosine mAb 4G10 and polyclonal rabbit anti-FGFR Ab were obtained from Upstate Biotechnology (Lake Placid, NY). The following Abs were used for immunoprecipitation: anti-paxillin (Ab 1; Transduction Laboratories, Lexington, KY); anti-FAK (2A7; Upstate Biotechnology); anti-v-Src ( Oncogene Research Products, Boston, MA); and anti-Lck (3A5), rabbit polyclonal Ab against FAK (C-20), c-Src (N-16), Fyn (Fyn3), anti-Lyn (44), anti-extracellular signal-regulated kinase (ERK; K-23), and Flg (C-15) all from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p42/44 mitogen-activated protein (MAP) kinase (Thr202 /Tyr 204 ) was purchased from Cell Signaling Technology (Santa Cruz, CA). Anti-phospho-ERK1/2 (antiphospho-ERK1/2, 77-15A2) primary Ab (1/500 dilution in 4% goat serum in PBS; Vector Laboratories) for overnight. The cells were washed three times with 0.5% Triton X-100 in PBS followed by staining with a biotinylated anti-rabbit secondary Ab (1/200 dilution in 4% goat serum in PBS; Vector Laboratories) at room temperature for 30 min. The cells were washed three times and incubated with avidin-fluorescein (1/200 dilution in PBS; Vector Laboratories) for 30 min followed by staining with propidium iodide (1 μg/ml; Sigma-Aldrich) for 10 min at room temperature. Slides were mounted with fluorescent mounting medium (Vector Laboratories), and fluorescent staining was analyzed using a FACScan flow cytometer using CellQuest Software (both from BD Biosciences, Mountain View, CA). Gates for forward and side scatter measurements were set on EC, and a minimum of 10,000 events was acquired. Instrument calibration was performed using CalBRITE beads and FACScomp software (BD Biosciences).

Flow cytometry analysis

ECs were grown in 60-mm culture dishes and incubated with 10 μg/ml mAb W6/32 for up to 2 h. Where indicated, the cells were pretreated with inhibitors for 1 h before exposure to mAb W6/32. The cells were washed three times with HBSS and detached with 0.125% trypsin/0.05% EDTA. Expression of FGFR was determined by indirect immunofluorescence on a FACScan flow cytometer as previously described (15). Briefly, ECs (0.5 × 10^6) were incubated with a rabbit anti-FGFR polyclonal Ab (Upstate Biotechnology) for 30 min at 4°C. The cells were washed three times and stained with a FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Following a 30-min incubation at 4°C, the cells were washed and fluorescent staining was analyzed on a FACScan flow cytometer using CellQuest Software (both from BD Biosciences, Mountain View, CA). Gates for forward and side scatter measurements were set on EC, and a minimum of 10,000 events was acquired. Instrument calibration was performed using CalBRITE beads and FACScomp software (BD Biosciences).

Immunohistochemical staining and confocal microscopy analysis

ECs were grown in four-well chamber slides (BD Biosciences) in EC growth medium until they were 80% confluent. The cell monolayers were rinsed with PBS, fixed, and permeabilized with methanol for 20 min at −20°C. Cells were rehydrated with PBS for 10 min, incubated with 4% goat serum for 1 h at room temperature to prevent nonspecific staining, and incubated with the rabbit anti-FGFR-1 (anti-flg, C15) primary Ab (1/500 in 4% goat serum in PBS) at 4°C for overnight. The cells were washed three times with 0.5% Triton X-100 in PBS followed by staining with a biotinylated anti-rabbit secondary Ab (1/200 dilution in 4% goat serum in PBS; Vector Laboratories) at room temperature for 30 min. The cells were washed three times and incubated with avidin-fluorescein (1/200 dilution in PBS; Vector Laboratories) for 30 min followed by staining with propidium iodide (1 μg/ml; Sigma-Aldrich) for 10 min at room temperature. Slides were mounted with fluorescent mounting medium (Vector Laboratories) and immunofluorescence staining was analyzed using a OLYMPUS FLUOVIEW confocal laser scanning biological microscope (Olympus, Melville, NY). Quantitation of the pixel intensities of FGFR-1 and propidium iodide in the nuclei of EC were quantified by measuring the average pixel intensity in each section using FLUOVIEW software version 2.1. Overall differences in fluorescent intensities between treated EC and controls was performed using ANOVA and Scheffe’s post hoc test for multiple pairwise comparisons (STATA Statistical Software, Release 7.0, Stata, College Station, TX).

Results

Ligation of HLA class I molecules by anti-HLA Abs induces tyrosine phosphorylation of EC proteins

To determine whether ligation of HLA class I molecules on EC with anti-HLA Abs induces tyrosine phosphorylation of intracellular proteins, confluent ECs were incubated with mAb W6/32 for various periods of time and the cell lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with the anti-phosphotyrosine mAb 4G10. Lysates prepared from EC treated with mouse isotype IgG were used as a negative control. As shown in Fig. 1, treatment with W6/32 induced rapid tyrosine phosphorylation of a protein at an approximate molecular mass of 60 kDa. Tyrosine phosphorylation of the 60-kDa protein occurred as early as 1 min, peaked at 30 min, and remained at high levels at 60 min. In contrast, tyrosine phosphorylation was not observed in isotype control-treated ECs (Fig. 1, A, lane 1, and B).

Previous studies from our laboratory have shown that ligation of class I molecules on the surface of ECs with anti-HLA class I Abs induces FGFR expression, increased bFGF ligand binding, and subsequent cell proliferation (14, 16–18). HLA class I-mediated cell proliferation could be prevented by the addition of neutralizing
Abs to bFGF, suggesting that the FGFR is required for the generation of class I-mediated signals. In view of these results, experiments were performed to determine whether FGFR signaling contributes to class I-mediated tyrosine phosphorylation of intracellular proteins. For this, ECs were pretreated with anti-bFGF neutralizing Ab for 2 h before exposure to anti-HLA Abs. The addition of neutralizing anti-bFGF Abs had no effect on class I-mediated tyrosine phosphorylation of the 60-kDa protein (Fig. 1A, lanes 7–10). Furthermore, treatment of ECs directly with bFGF induced tyrosine phosphorylation of proteins with an approximate molecular mass of 42–44 kDa (Fig. 1A, lanes 11–14). Increased tyrosine phosphorylation of the p42/44 proteins was detected at 10 min and declined to almost baseline levels by 60 min. These results demonstrate that binding of anti-HLA Abs to class I Ags expressed by EC transduces signals resulting in a heavily tyrosine-phosphorylated 60-kDa protein. The results also indicate that the proteins that become phosphorylated following exposure to anti-HLA Abs are distinct from the proteins phosphorylated following activation of the FGFR by bFGF.

**Anti-HLA class I Abs stimulate tyrosine phosphorylation of Src kinases**

Because we observed that anti-HLA Abs stimulate tyrosine phosphorylation of a major protein band of an approximate molecular mass of 60 kDa, we considered the possibility that the candidate protein may be a member of the Src family of tyrosine kinases. To investigate this possibility, cells were treated with mAb W6/32 for various time points and the cell lysates were immunoprecipitated with a panel of anti-Src Abs. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine mAb 4G10. As shown in Fig. 2A, anti-HLA class I Abs induced a marked increase in tyrosine phosphorylation of Src. Tyrosine phosphorylation was increased at 1 min following treatment with W6/32, peaked at 30 min, and remained at high levels thereafter. Densitometric scanning showed that anti-class I Abs (10 μg/ml) induced a 4-fold increase in phosphorylation of Src at 30 min compared with EC treated with isotype control IgG (Fig. 2A). Confirmation that similar amounts of Src were recovered from lysates of anti-HLA Ab-treated and nontreated cells was obtained by blotting the immunoprecipitated proteins with the anti-Src Ab (Fig. 2A, lower panel). Ab ligation of class I molecules also stimulated a time-dependent phosphorylation of p59 Fyn. As shown in Fig. 2B, an increase in tyrosine phosphorylation of Fyn was detected at 1 min after the addition of 10 μg/ml W6/32, reaching a peak at 30 min. Immunoblotting with anti-Fyn Ab of Fyn immunoprecipitates verified that similar amounts of Fyn were recovered from lysates after anti-class I stimulation (Fig. 2B, lower panel). In contrast, no increase in tyrosine phosphorylation of p56/ p53 Lyn was observed when EC were treated with mAb W6/32 (Fig. 2C). Lck appeared to be constitutively phosphorylated in EC, with no observed increase in tyrosine phosphorylation above the baseline level when ECs were treated with anti-class I mAb (Fig. 2D). Immunoblotting with anti-Lyn and anti-Lck Abs confirmed that similar amounts of protein were recovered from cell lysates (Fig. 2, C and D, lower panels).

To exclude the possibility that FGFR signaling contributes to class I-mediated tyrosine phosphorylation of Src, ECs were pre-treated with anti-human bFGF neutralizing Ab for 2 h before exposure to anti-HLA Abs. As presented in Fig. 2, neutralizing anti-bFGF Abs had no apparent effect on class I-mediated tyrosine phosphorylation of Src or Fyn. Furthermore, treatment of ECs with recombinant human bFGF failed to stimulate tyrosine phosphorylation of Src or Fyn.

During these investigations we found that anti-class I Abs stimulated increased phosphorylation of a 70-kDa protein that coprecipitated with anti-Src and anti-Fyn Abs (Fig. 2, A and B). To determine whether Src kinase mediates phosphorylation of the p70 protein, cells were preincubated in the presence of PP2, a specific inhibitor of Src kinase activity, before class I stimulation. Following treatment with anti-class I Abs for various periods of time, cell lysates were immunoprecipitated with anti-Src Abs and the immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine Abs. As shown in Fig. 2E, pretreatment of EC with PP2 completely prevented HLA class I-mediated phosphorylation of the p70 protein. In contrast, increased tyrosine phosphorylation of the p70 protein was unaffected by pretreatment of the cells with PP3, an inactive control analog of PP2. These results demonstrate that binding of anti-HLA Abs to class I Ags on ECs transduces signals, resulting in phosphorylation of several members of the Src family including Src and Fyn. Ligation of class I molecules also induced the phosphorylation of a p70 protein that

![FIGURE 1](http://www.jimmunol.org/)

Ligation of class I molecules by anti-HLA Abs induces tyrosine phosphorylation of EC proteins. A, Quiescent ECs were incubated with 10 μg/ml isotype control IgG (lane 1), 10 μg/ml mAb W6/32 (lanes 2–5), or 1 ng/ml bFGF (lanes 11–14) for the times indicated. EC (lanes 6–10) were pretreated for 2 h at 37°C with 1 μg/ml anti-human bFGF neutralizing Ab followed by stimulation with mAb W6/32. B, Quiescent ECs were incubated with 10 μg/ml isotype control IgG for the times indicated (lanes 2–5) or without isotype control (lane 1). Proteins in the cell lysates were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with anti-phosphotyrosine mAb 4G10. Arrows indicate the protein bands with increased density in W6/32-treated ECs compared with untreated cells. The band sizes are indicated in kilodaltons. The results are representative of four independent experiments.
coprecipitates with Src and is of unknown identity. Phosphorylation of the p70 protein was completely blocked by the specific Src kinase inhibitor PP2, suggesting that phosphorylation of this protein results from the action of Src kinases.

Anti-HLA class I Abs stimulate tyrosine phosphorylation of FAK and paxillin in EC

Growth factor receptor and integrin stimulation of ECs and SMCs is known to promote the recruitment of Src family protein tyrosine kinases (PTKs) into a signaling complex with focal adhesion proteins (39). Therefore, we investigated the possibility that the major substrates for Src tyrosine phosphorylation reside within focal adhesion proteins FAK and paxillin. To determine whether anti-HLA Abs stimulate the phosphorylation of focal adhesion proteins, quiescent cells were stimulated with different concentrations of W6/32 for 10 min and lysed, and the extracts were immunoprecipitated with anti-paxillin or anti-FAK Abs. The immunoprecipitates were subsequently analyzed by immunoblotting with the anti-phosphotyrosine Ab 4G10. As shown in Fig. 3A, treatment with mAb W6/32 stimulated an increase in tyrosine phosphorylation of paxillin in a dose-dependent manner. Stimulation of phosphorylation was observed at concentrations of anti-HLA Ab ranging from 0.625 to 10 μg/ml with maximal effects (86% increase) at 10 μg/ml. To determine the kinetics of class I-induced phosphorylation of paxillin, cells were treated for different periods of time with anti-HLA Abs. As shown in Fig. 3B, increased paxillin phosphorylation was detected as early as 1 min after the addition of anti-class I Abs, reaching a maximum after 10 min. Densitometric scanning showed
that anti-class I Abs (10 μg/ml) induced a 3-fold increase in phosphorylation of paxillin at 10 min compared with EC treated with isotype control IgG (Fig. 4A). Class I-mediated induction of paxillin phosphorylation was not altered by pretreatment of the cells with anti-bFGF neutralizing Ab. As shown in Fig. 3C, lane 3, pretreatment of EC with anti-bFGF neutralizing Ab followed by class I ligation resulted in a 2.6-fold increase in paxillin phosphorylation at 10 min compared with EC treated with isotype control. Moreover, treatment of ECs directly with bFGF did not induce significant differences in tyrosine phosphorylation of paxillin in five independent experiments (Fig. 3C).

Anti-HLA class I Abs also stimulated tyrosine phosphorylation of FAK (Fig. 4B). Densitometric scanning showed a 40% increase in FAK phosphorylation in cells treated with mAb W6/32 compared with control cells (Fig. 4B). Immunoblotting with anti-FAK Abs of FAK immunoprecipitates verified that similar amounts of FAK were recovered from cell lysates after HLA class I Ab treatment. Thus, class I ligation induces a rapid and parallel increase in the tyrosine phosphorylation of the focal adhesion proteins FAK and paxillin.

To determine whether Src tyrosine kinase activity is required for class I-induced phosphorylation of paxillin and FAK, ECs were treated in the presence or absence of PP2 followed by stimulation with mAb W6/32. As shown in Fig. 5B, pretreatment with PP2 blocked class I-induced phosphorylation of paxillin. Exposure of EC to PP2 also abrogated class I-induced phosphorylation of FAK (Fig. 5A). In contrast, class I-mediated phosphorylation of paxillin or FAK was not affected by pretreatment with the inactive analog PP3 (data not shown).

Because tyrosine-phosphorylated FAK and paxillin are found in focal contacts associated with cell membrane ruffles and stress fibers (40), experiments were designed to evaluate whether disruption of the actin cytoskeleton could prevent tyrosine phosphorylation of these focal adhesion proteins in response to anti-class I Abs. For this, cells were pretreated with 2.5 μM cytochalasin D or 1 μM latrunculin A for 1 h and then stimulated with mAb W6/32 for 10 min. As shown in Fig. 5, both latrunculin A and cytochalasin D completely blocked W6/32-induced tyrosine phosphorylation of FAK and paxillin. These results indicate that the integrity of the actin cytoskeleton is required for class I-induced tyrosine phosphorylation of FAK and paxillin.

**BFGF induces phosphorylation of ERK in ECs**

As indicated in Fig. 1, exposure of EC to bFGF resulted in the phosphorylation of heavily tyrosine-phosphorylated proteins at the approximate molecular mass of 42–44 kDa. To determine whether the candidate proteins are the p44/p42 ERK, whole cell lysates were immunoblotted with an anti-phospho-ERK Ab. Treatment with recombinant human bFGF induced rapid and transient activation of p44ERK1/p42ERK2 in ECs (Fig. 6, upper panel). BFGF-induced ERK phosphorylation reached maximum levels at 10 min and declined by 30 min. W6/32 failed to induce phosphorylation of ERK alone or augment phosphorylation of ERK when added to the cells in combination with bFGF. Confirmation that similar
FIGURE 5. Class I-mediated phosphorylation of FAK and paxillin is Src dependent and requires an intact cytoskeleton. ECs were pretreated for 1 h at 37°C in presence (+) or absence (−) of 25 μM PP2, 2.5 μM cytochalasin D, or 1 μM latrunculin A, and stimulated with 2.5 μg/ml mAb W6/32 for 10 min. Precleared cell lysates were immunoprecipitated with anti-FAK (2A7, A), or anti-paxillin Ab (349, B). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine Ab 4G10. One of three representative experiments is presented.

FIGURE 6. FGFR ligation stimulates phosphorylation of ERK. ECs were treated with isotype control IgG (C), FGF (1 ng), 10 μg/ml mAb W6/32 (W), FGF (10 ng) plus W (10 μg/ml mAb W6/32), or FGF (10 ng) for the time points indicated. Cell lysates were fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and incubated with anti-phospho-ERK polyclonal Ab. Upper panel, The blots were incubated in HRP-conjugated secondary Ab, developed with an ECL, and exposed to x-ray film to visualize the proteins. Lower panel, Immunoblotting with anti-ERK was performed to confirm equal loading of proteins. The results are representative of three independent experiments.

FIGURE 4. HLA class I induced tyrosine phosphorylation of FAK and paxillin in EC. ECs were incubated in the presence (+) or absence (−) of 10 μg/ml W6/32 for 10 min and the cell lysates were prepared and precleared for 2 h with protein A/G plus agarose at 4°C. The precleared cell lysates were immunoprecipitated with anti-paxillin Ab (349, A) or anti-FAK (2A7, B) overnight at 4°C. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with the anti-phosphotyrosine Ab 4G10. Quantification of paxillin and FAK phosphorylation was performed by scanning densitometry. Values shown are expressed as the percentage of the maximal increase in paxillin and FAK tyrosine phosphorylation value above control (nonstimulated) values. The results of one of five independent experiments are presented.

amounts of ERK were recovered from lysates of anti-HLA Ab-treated and nontreated cells was obtained by blotting the immunoprecipitated proteins with anti-ERK Ab (Fig. 6, lower panel).

Role of Src and focal adhesion proteins in class I-mediated FGFR translocation in ECs

In previous studies we have shown that ligation of class I molecules on the surface of EC by anti-HLA Abs stimulates FGFR-1 redistribution from cytoplasmic stores to the nucleus and cell surface (41). To determine whether Src tyrosine kinase is involved in HLA class I-mediated redistribution of FGFR-1 to the nucleus, EC were treated for 2 h with PP2 and the cells were labeled with anti-FGFR-1 Abs and optically sectioned and analyzed by confocal immunofluorescence microscopy. Quiescent EC show diffuse cytoplasmic FGFR-1 staining and low FGFR-1 nuclear staining (Fig. 7A). However, after treatment with W6/32 for 30 min, the majority of cells show intense nuclear FGFR-1 fluorescence (Fig. 7B). Similarly, class I-stimulated EC pretreated with PP3 show a marked increase in nuclear FGFR-1 labeling (Fig. 7F). In contrast, the addition of PP2 inhibited class I-mediated induction of FGFR-1 nuclear labeling (Fig. 7D). Quantitative analysis of the confocal nuclear immunofluorescence data was performed by measuring the levels of FGFR fluorescence (Fig. 7, green) and propidium iodide nuclear fluorescence (Fig. 7, red) in each optical section. The average nuclear fluorescence intensity of these two values is presented in Fig. 7G. The average nuclear fluorescence intensity of propidium iodide was similar in both treated and nontreated EC (Fig. 7, A–F). The average nuclear fluorescence intensity of FGFR was 18 in cells treated with mAb W6/32 alone or PP3 and mAb W6/32. In contrast, the average fluorescence intensity of the nuclei of cells treated with isotype control, PP2, or PP3 was 8. These differences were highly significant (p < 0.0001), indicating that class I signaling stimulates the translocation of FGFR-1 to the nucleus. Moreover, these results suggest that HLA class I-mediated redistribution of FGFR-1 to the nucleus is mediated by Src kinases.

Because these studies clearly support a role of Src tyrosine kinases in the redistribution of FGFRs within the cell, it was of interest to determine whether Src mediates class I-induced accumulation of FGFRs on the cell surface. For these experiments, ECs were pretreated with PP2 for 2 h before the addition of anti-class I Abs, and cell surface expression of FGFRs was determined by FACS analysis (Fig. 8A). Treatment with mAb W6/32 for 10 min stimulated a 4-fold increase in FGFR surface expression (Fig. 8A) compared with cells treated with isotype control IgG. Pretreatment of cells with PP2 followed by stimulation with anti-class I Abs had no appreciable effect on class I-induced FGFR surface expression. These results indicate that Src family PTKs mediate HLA class I-induced FGFR-1 translocation to the nucleus. However, Src tyrosine kinase activity does not appear to be required for the redistribution of FGFR to the cell surface.

The results presented above suggest that different signaling pathways are responsible for the redistribution of FGFR to distinct subcellular compartments. We next examined whether the focal adhesion proteins FAK and paxillin participate in class I-induced FGFR translocation to the cell surface. For this, ECs were pretreated for 1 h with 1 μM latrunculin A or 2.5 μM cytochalasin D, concentrations that disrupt the actin cytoskeleton and the assembly of focal adhesions in EC, and then stimulated with 10 μg/ml mAb W6/32 for another 2 h. The cells were analyzed by flow cytometry for FGFR surface expression using a polyclonal anti-FGFR Ab. Treatment of ECs with either latrunculin A (Fig. 8B) or cytochalasin D (Fig. 8C) inhibited FGFR expression in response to class I Abs. Thus, our results indicate that the actin cytoskeleton is involved in class I-mediated translocation of FGFRs to the cell surface.
Discussion

HLA class I signaling pathways have been implicated in the proliferation of vascular ECs, SMCs, T cells, and B cells, as well as apoptosis of activated T and B cells (19–33). Although the mechanisms and molecules involved in the HLA-mediated apoptosis have been recently suggested (20, 23), information on HLA class I-mediated cell proliferation is sparse. The studies described in this report were aimed at elucidating the signal transduction machinery initiated following ligation of class I molecules by anti-HLA Abs on human ECs. As described in our earlier investigations, Ab ligation of class I molecules expressed on the surface of ECs and SMCs induces cell proliferation by up-regulating FGFR expression and, as a result, enhances the binding of bFGF to the cell (14–18). Therefore, our attention was focused on determining the signaling proteins mediating class I-induced FGFR expression in human ECs. Our studies show for the first time that anti-HLA Ab binding to class I molecules on ECs results in the phosphorylation of Src, paxillin, and FAK.

FIGURE 7. Ligation of class I molecules by anti-HLA Abs increases FGFR-1 association with the nucleus. Quiescent ECs were treated with 10 μg/ml mouse IgG for 30 min (A); 10 μg/ml anti-HLA class I mAb W6/32 for 30 min (B); 12.5 μM PP2 for 30 min (C); 12.5 μM PP2 for 30 min followed by stimulation with 10 μg/ml W6/32 for 30 min (D); 12.5 μM PP3 for 30 min (E); and 12.5 μM PP3 for 30 min followed by stimulation with 10 μg/ml W6/32 for 30 min (F). The monolayers were fixed, permeabilized, blocked, and labeled with the anti-FGFR-1 Ab. The cells were incubated with biotinylated anti-rabbit secondary Ab followed by staining with avidin-fluorescein and propidium iodide. FGFR-1 fluorescence was analyzed using an OLYMPUS FLUOVIEW confocal laser scanning biological microscope. The fluorescence intensities of FGFR (green) and propidium iodide (red) in EC were quantified by measuring the average pixel intensity in each section using FLUOVIEW software and the data was plotted (G). A minimum of 40 cells was analyzed in each section. In F, the bar = 5 μm. The results are representative of three independent experiments.

The Src family of PTKs consists of at least nine members, including Src p60, Yes p62, Lck p56, Lyn p56/p53, Fyn p59, Fgr p55, Hck p56/p59, Blk p55, and Rak (42, 43). Src family members have been shown to play important roles in regulating complex signal transduction pathways and are differentially expressed in a wide variety of tissues. Consequently, we examined whether class I stimulation induces tyrosine phosphorylation of distinct members of the Src PTK family. Our studies revealed that Fyn and Src become immediately phosphorylated on tyrosine residues in response to class I ligation. These results are consistent with previous studies documenting the ability of class I molecules to phosphorylate members of the Src family in T and B cells (36, 44).
human B cells, class I ligation results in the phosphorylation of p53/S6K, whereas p56Lck tyrosine kinase is activated in human T cells following class I ligation (36). Our studies showed that treatment of EC with anti-class I Abs induced the phosphorylation of a 70-kDa protein that coimmunoprecipitated with anti-Src and anti-Fyn Abs. Pretreatment of cells with the Src tyrosine kinase inhibitor PP2 specifically blocked class I-induced tyrosine phosphorylation of the p70 protein. Thus, our results suggest a Src PTK-dependent pathway in the tyrosine phosphorylation of this unidentified 70-kDa protein in response to class I stimulation.

The results presented in this report also demonstrate that Ab ligation of HLA class I molecules rapidly induces phosphorylation of paxillin and FAK. Paxillin serves as an adapter protein that provides multiple docking sites at the plasma membrane for an array of signaling and structural proteins, including the tyrosine kinases Src and FAK (45). FAK is a nonreceptor tyrosine kinase that plays a key role mediating integrin signaling and cell adhesion (46). FAK, in association with Src, have been shown to stimulate the phosphorylation of paxillin at two main sites, tyrosine 31 and tyrosine 18 (47–52). Together, paxillin, Src, and FAK form a signaling complex that transduces signals to downstream molecules, thereby stimulating gene expression (reviewed in Refs. 45, 53, and 54). This suggests that class I ligation stimulates Src phosphorylation and binding to FAK, resulting in the formation of a FAK-Src complex, enhanced FAK PTK activity, and subsequent phosphorylation of paxillin. Consistent with this interpretation, we found that treatment of EC with PP2 blocked class I-mediated phosphorylation of paxillin and FAK, indicating a Src PTK-dependent pathway in the tyrosine phosphorylation of these focal adhesion proteins in response to class I stimulation. Our data also show that treatment of EC with either cytochalasin D or latrunculin A, at concentrations known to disrupt the actin cytoskeleton, abrogated class I-mediated increases in tyrosine phosphorylation of paxillin and FAK. These results indicate that an intact cytoskeleton is required for class I-induced tyrosine phosphorylation of FAK and paxillin and suggest that actin-dependent clustering of molecules is required to trigger class I signals.

The mechanism whereby class I molecules transduce signals is unknown. Although the H chain of class I contains serine, threonine, and tyrosine residues that can be phosphorylated, studies characterizing class I signal transduction using constructs lacking most of the cytoplasmic tail have shown that signal transduction does not require this portion of the molecule (55). This suggests that there is no direct interaction between class I molecules and Src, paxillin, and FAK. Thus, class I molecules probably associate with other molecules that have the capacity to transduce signals or generate intracellular messengers. In this respect, class I molecules have been shown to interact with various peptide hormone receptors that function as tyrosine kinases, such as the insulin receptor and the epidermal growth factor receptor (56–58). The data presented in this paper support a model in which class I molecules associate with a coreceptor to form a signaling complex initiated by Src-dependent binding of Src family PTKs to FAK and paxillin. Elucidation of the coreceptor requires further studies; however, it is tempting to speculate that this molecule may be a member of the integrin family, because it is well established that Src, paxillin, and FAK are activated following engagement of integrins with the extracellular matrix (39, 46, 59).

FGFRs are generally known as plasma membrane proteins that send signals to the nucleus principally via the MAP kinase and the Janus kinase-STAT pathways (60, 61). However, over the past few years, data has accumulated to suggest that nuclear targeting and action of FGFs and FGFRs could occur as well. This alternative or complementary nuclear signaling pathway also appears to be involved in the induction of cell proliferation (62–64). In previous studies, we reported that anti-HLA Abs transduce signals that result in the redistribution of FGFRs to the cell surface and nucleus (15–18, 41). These studies have clearly demonstrated that HLA class I-induced redistribution to the cell surface results in increased ligand binding and initiation of cell proliferation through the MAP kinase pathway. However, the function of the class I-targeted nuclear FGFR-1 is still unknown. In the current study, we observed that class I initiates different signaling cascades that target the FGFR to distinct intracellular locations. We found that Src kinase inhibitor PP2 had no effect on class I-mediated induction of FGFR expression on the surface of EC, whereas this inhibitor completely blocked class I-induced FGFR translocation to the nucleus. These results indicate a Src-dependent pathway for class I-induced nuclear translocation of FGFR-1 and a Src-independent pathway for class I-induced FGFR plasma membrane expression. FACS studies suggest that the integrity of the actin cytoskeleton is required for productive class I signaling and FGFR-1 translocation. Thus, treatment of EC with either latrunculin A or cytochalasin D blocked FAK and paxillin phosphorylation, as well as FGFR-1 translocation to the cell surface. Our data show that inhibition of Src kinase, which is important for the phosphorylation of paxillin, inhibits the nuclear translocation but not the plasma membrane translocation of FGFR, whereas the disruption of the actin cytoskeleton prevented class I-mediated plasma membrane FGFR translocation. The actin cytoskeleton is regulated by a variety of pathways, including Rho, Rac, Cdc42, Rho-associated kinase, and profilin, as well as the tyrosine phosphorylation of FAK, CAS, and paxillin. We hypothesize that the role of paxillin in actin cytoskeleton organization is likely to be subtle (it is one of multiple pathways), whereas cytochalasin D disrupts the whole actin cytoskeleton network. Thus, cytochalasin D leads to more severe consequences than inhibition of paxillin phosphorylation.

Together, our previous results (14–18, 41, 65) and current findings are consistent with a model in which anti-HLA Ab-mediated clustering of class I molecules stimulates the organization of paxillin, Src, and FAK into cell matrix adhesions, which act in concert as signaling units. These protein phosphorylation events activated by class I signaling stimulate the translocation of FGFR from cytoplasmic stores to the nucleus and cell surface. Class I-induced up-regulation of FGFR augments FGFR binding and triggers a series of downstream events including activation of the ERK/MAP kinase pathway, cyclin E-dependent kinase activity, and Rb inactivation, causing the EC to proliferate (65). Class I-dependent translocation of FGFR-1 to the nucleus of EC may also play a direct role in regulating gene transcription and cell proliferation.

Further studies are required to determine the effect of class I signaling in an in vivo allograft model. However, our in vitro studies support the hypothesis that anti-HLA Abs may contribute to the process of chronic rejection by binding to the endothelium and smooth muscle of the graft and initiating protein tyrosine phosphorylation events that stimulate FGFR translocation and subsequent cell proliferation. Because signaling through class I molecules can induce cell proliferation in vitro, agents that block this process may be useful in the prevention and treatment of chronic allograft rejection.

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