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Mitogen-Activated Protein Kinase Pathway Activation by the CD6 Lymphocyte Surface Receptor

Anna Ibáñez, Maria-Rosa Sarrias, Montserrat Farnós, Idoia Gimferrer, Carles Serra-Pagès, Jordi Vives, and Francisco Lozano

CD6 is a cell surface receptor primarily expressed on immature thymocytes and mature T and B1a lymphocytes. Through its binding to activated leukocyte cell adhesion molecule (ALCAM/CD166), CD6 is considered to play an important role in lymphocyte development and activation. Accordingly, CD6 associates with the TCR/CD3 complex and colocalizes with it at the center of the mature immunological synapse on T lymphocytes. Moreover, the CD6-ALCAM interaction has been shown to be critical for proper immunological synapse maturation and T cell proliferative responses. However, the precise biological effects of CD6 ligation and its signaling pathway are still not well understood. The present study shows that CD6 ligation with three different specific Abs (161.8, SPV-L14.2, and MAE1-C10) induces time- and dose-dependent activation of ERK1/2 on normal and leukemic human T cells. This effect was also observed upon CD6 ligation with a chimerical ALCAM protein (ALCAM-Fc). The C-terminal cytoplasmic region of CD6, as well as Src tyrosine kinases, was critical for CD6-induced ERK1/2 activation. Synergistic effects were observed upon coligation of the TCR/CD3 complex with CD6. The ligation of CD6 induced the transcriptional activation of reporter genes under the control of the c-Fos serum responsive element and AP-1. Accordingly, CD6-mediated activation of p38 and JNK was also observed. These findings indicate that the CD6-ALCAM interaction results in activation of the three MAPK cascades, likely influencing the dynamic balance that determines whether resting or activated lymphocytes survive or undergo apoptosis. The Journal of Immunology, 2006, 177: 1152–1159.

The human CD6 receptor is a type I glycoprotein of 105–130 kDa expressed on immature thymocytes, mature T and B1a lymphocytes, chronic B cell lymphocytic leukemia, and various brain regions (1, 2). CD6 is a group B member of the scavenger receptor cysteine-rich (SRCR) superfamily of protein receptors based on the presence in its extracellular region of three 100- to 110-aa-long cysteine-rich domains characteristic of that family (3, 4). Through its most membrane-proximal SRCR domain, CD6 directly interacts with activated leukocyte cell adhesion molecule (ALCAM)/CD166 (5). ALCAM is a broadly expressed adhesion molecule of the Ig superfamily found on activated T and B lymphocytes, lymphomas, activated monocytes, thymic epithelium, keratinocytes, melanoma cells, mesenchymal stem cells, neurons, and brain cortex (6). Because CD6 and ALCAM are expressed on thymocytes and thymic epithelial cells, respectively, the CD6-ALCAM interaction has been implicated in thymocyte adhesion (6–8) and thymocyte development (9). Heterophilic CD6-ALCAM interactions have also been suggested to play a role in the development of hemopoietic stem cells and endothelial progenitors (10, 11). Recently a novel, still uncharacterized CD6 ligand has been found expressed on synovial and epithelial cells (12).

Available evidence indicates that CD6 is an accessory molecule involved in the modulation of the lymphocyte activation and differentiation processes. However, its ultimate function in both developing and mature lymphocytes is still unknown. During thymocyte development, CD6-dependent signals contribute to thymocyte survival and positive selection (9). Several reports show that ligation of CD6 with mAbs is capable of delivering costimulatory signals to mature peripheral T lymphocytes (13–18). However, the inhibition of Ag-specific responses of human T cell clones specific to tetanus toxoid by CD6 mAbs also has been reported (19).

On thymocytes and resting mature T cells, CD6 partially associates with the TCR/CD3 complex (20) and with CD5 (21), a close member of the SRCR superfamily. Moreover, CD6 accumulates at the central part of the mature immunological synapse (IS), where it colocalizes with the TCR/CD3 and CD5 (20). More importantly, CD6 has been implicated in early T cell-APC contacts influencing IS maturation (20, 22) and further T cell proliferative responses (20, 23). This finding suggests that CD6 is an important player in the early cell-to-cell events driving toward T cell activation. In B cells, CD6 ligation protects against anti-IgM-mediated apoptosis through bcl-2 induction (24).

The intracellular signals delivered by CD6 to influence T and B lymphocyte activation and maturation are currently mostly unknown. CD6 has a long cytoplasmic tail devoid of intrinsic catalytic activity, but it harbors several consensus motives related to signal transduction (25). CD6 is a constitutively phosphorylated molecule that becomes hyperphosphorylated by serum and protein...
kinase C (PKC) activators, causing a molecular mass shift from 105 to 130 kDa (15, 26). Upon CD3 stimulation, either alone or by co-cross-linking with CD2 or CD4, CD6 becomes transiently phosphorylated on the two most C-terminal tyrosine residues (Y629 and Y662) (27, 28). Accordingly, it has been shown that the CD6-mediated effects on T cell proliferation involve a tyrosine kinase activity, which is dependent on PKC activation (29). Recently it has been reported that CD6 associates with the scaffold- ing, PDZ-containing protein syntennin-1 and that they colocalize on the IS (30). This interaction represents the first reported protein-protein interaction involving the cytoplasmatic tail of CD6.

The aim of this study was to explore the intracellular signaling pathway(s) used by CD6 in human T lymphocytes, specifically focusing on the MAPK cascade. The MAPKs, which include the ERK, p38, and JNK kinases, are among the most ancient and evolutionarily conserved signaling pathways. They have been involved in many physiological processes, including cell proliferation, differentiation, and death. Consequently, they also participate in several aspects of the physiology of T cells, such as their development in the thymus, their activation and differentiation in the initiation of the peripheral immune system, and activation-induced cell death (31). In this study, we show that activation of MAPK (ERK1/2, p38, and JNK) is an integral component of the signaling pathway recruited by the CD6 lymphocyte surface receptor.

Materials and Methods

Cells

Human PBMCs were obtained from buffy coats of healthy volunteer blood donors from the Blood Bank of the Hospital Clinic of Barcelona (Barcelona, Spain) by standard density gradient centrifugation over Ficoll (density = 1.077 g/cm³) (Linfosep; Biomedics). COS-7 cells, the human leukemic T cell line HUT-78, the human T cell line Jurkat, and the Lck-deficient Jurkat JE6.1 cell line derivative (JCAM1) were obtained from the American Tissue Culture Collection. The CD5² and CD6² 2G5 Jurkat cell line derivative was obtained by FACS analysis and further cloning, as reported elsewhere (32). PBMCs and cell lines were grown in RPMI 1640 (BioWhittaker) supplemented with 10% FBS (BioWhittaker), 2 mM l-glutamine, 20 U/ml penicillin G, and 50 µg/ml streptomycin. Before stimulation cells were extensively washed with PBS and left in culture for 24 h in serum-free RPMI 1640 medium.

Abs and reagents

The mouse mAbs 161.8 (IgG1), 72-5D3 (IgG2a), and 33-2A3 (IgG2a) were produced in our laboratory by Dr. R. Villela (Hospital Clinic, Barcelona, Spain) and had been assigned to CD6, CD45, and CD3, respectively, in the successive International Workshops on Human Leukocyte Differentiation Antigens. The mouse anti-human CD6 mAbs MAE1-C10 (IgG1) and SPV-L14.2 (IgG1) were provided by Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain) (26) and Dr. J. Hildes (Biobred), respectively. The mAbs MAE1.8, MAE1-C10, SPV-L14.2, and 72-5D3 were purified from ascitic fluid by affinity chromatography using Hi-Trap protein G columns (Amersham Biosciences). The purified mouse anti-human CD6 mAb M-T005 (22) was from BD Biosciences. The anti-CD3 33-2A3 mAb was used as culture supernatant with an estimated Ab concentration of 6 mg/ml. The mouse mAb M-T605 (22) was from BD Biosciences. The anti-CD3 33-2A3 mAb was purchased from BioSource International, and the goat anti-p38 and anti-JNK1 antisera were purchased from Cell Signaling Technology. The Src family tyrosine kinase inhibitor PP1 was purchased from Biomol. PMA was purchased from Sigma-Aldrich. The human ALCAM-Fc and human CD6-Fc chimeras consisting of the extracellular domains of human ALCAM and human CD6, respectively, fused to the human IgG1 Fc region were purchased from R&D Systems.

DNA constructs

The generation of expression constructs coding for wild-type CD6 (pHB- CD6.wt) and the CD6 form devoid of its 141 C-terminal residues (pHB-CD6.P527stop) have been previously described elsewhere (21, 30). The serum responsive element (SRE):Luc plasmid containing the thymidine kinase promoter and the luciferase reporter gene under the control of two copies of the Fox-SRE consensus cDNA sequence (SRE-Luc) was provided by Dr. Y. Rosenstein (Instituto de Biologia Celular, Morelos, México) (33, 21), and the AP-1/2-O-tetradecanoylphorbol-13-acetate response element (TRE):Luc containing the luciferase reporter gene under the control of three copies of the AP-1 consensus sequence was provided by Dr. S. J. Cook (Onyx Pharmaceuticals) (34).

Cell stimulation

For Ab or PMA stimulation, serum-starved PBMCs, HUT-78 cells, or 2G5-CD6.P527stop cells (2 × 10⁶) were incubated in 300 µl of RPMI 1640 for 10 min at 37°C. The cells were then stimulated at 37°C for the indicated times with PMA (100 ng/ml) or by cross-linking with the indicated mAbs in solution. After stimulation, cells were washed once with cold PBS and lysed in Triton X-100 lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 1 mM Na3VO4, 0.1% SDS, Complete protease inhibitor mixture tablets (Roche), and 1 mM PMSF. The nuclei and cell debris were removed by centrifugation at 8000 × g for 15 min and protein concentration was measured with the BCA protein assay reagent kit (Pierce) following the manufacturer’s instructions.

For stimulation with Fc chimeras, these soluble proteins were immobilized onto the wells of tissue culture plates (Techno Plastics Products). Briefly, 6-well plates were coated with 1 µg of 4 µg/ml anti-human-IgG1 antiserum (Invitrogen Life Technologies) in TSM buffer (20 mM Tris, 150 mM NaCl, 1 mM CaCl2, and 2 mM MgCl2 (pH 8.0)) at 4°C overnight. To avoid nonspecific binding to the wells, the plates were blocked with 1% BSA (Merck) in TSM buffer for 30 min at 37°C and thereafter coated with 600 µl of human ALCAM-Fc chimera, human CD6-Fc chimera, or human IgG1 at 20 µg/ml in TSM buffer containing 1% BSA for 1 h at 37°C. A suspension of 2 × 10⁷ PBMCs in RPMI 1640 were then added and incubated for 40 min at 37°C, at which time point the cells were removed by pipetting and lysed with lysis buffer as described before. Alternatively, To avoid nonspecific binding to the wells, the plates were blocked with 1% BSA (Merck) in TSM buffer for 30 min at 37°C, and the cells were added, incubated, and lysed as described above.

Immunoprecipitation and Western blot analysis

Triton X-100 detergent solubilizes from 2G5 Jurkat cells were immuno- precipitated for 2 h at 4°C with 1 µg of anti-CD6 mAb (161.8) plus 20 µg of 5 µg/ml protein A-Sepharose CL-4B beads (Amersham Biosciences). Pre- cipitates and 50 µg of cell lysates were run in 10% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (Bio- Rad), and blocked with 5% nonfat milk powder in TBS-Tween 20. The membranes were incubated overnight at 4°C with the indicated Abs diluted in TBS-Tween 20 containing 5% nonfat milk powder. After three washes with TBS-Tween 20, the membranes were incubated with the appropriate HRP-coupled secondary antisera for 60 min at room temperature. After three more washes with TBS-Tween 20, bound Ab was detected by chemiluminescence with SuperSignal West Dura extended duration substrate (Pierce). The blots were analyzed by using a LAS3000 luminescent imager (Fuji). The blots were stripped for 5 min at room temperature with 200 mM NaOH. Densitometric analysis was performed by using the Scion Image program (integrated density was measured).

Flow cytometry analysis

Jurkat (2G5) cells (1 × 10⁶) expressing the wild-type CD6 or the cyto- plasmic tail-truncated CD6 were incubated with saturating amounts of anti-CD6 mAb (161.8) in PBS containing 1% FBS and 0.05% NaN₃ for 30 min at 4°C. After three washes with the same staining buffer, cells were incubated with FITC-conjugated goat anti-mouse IgG antiserum (Sigma-Aldrich) for 15 min at 4°C and then subjected to flow cytometry analysis with a FACSscan cyrometer (BD Biosciences) equipped with CellQuest software (BD Biosciences).

Cell transfections and luciferase assays

The 2G5 Jurkat cells were stably transfectected with the pH6-CD6.wt and pH6-CD6.P527stop constructs (21, 30) and further selected with genetic

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Effect of anti-CD6 mAbs on ERK1/2 activation and T cell proliferation. A, Serum-starved PBMCs (left) or HUT-78 cells (right) were stimulated with 10 μg/ml anti-CD6 (αCD6; 161.8) or 10 μg/ml anti-CD3 (αCD3; 33-2A3) mAb for the indicated times at 37°C. Where indicated, cells were pretreated with 10 μM MEK1/2 inhibitor U0126 in DMEM (+) or with DMEM alone (−) for 1 h at 37°C before stimulation with mAbs. B, Serum-starved PBMCs were stimulated with different concentrations of anti-CD6 (161.8) mAb for 40 min at 37°C (left) or with 10 μg/ml anti-CD6 (161.8) mAb for the indicated times (right). C, Serum-starved PBMCs were either unstimulated or stimulated with 10 μg/ml mAbs specific to CD6 (161.8, SPV-L14.2, and MAE1-C10) for the indicated times. In all cases, 50 μg of Triton X-100 cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to immunoblotting with anti-phospho-ERK1/2 (p-ERK1/2), and further reprobing with anti-ERK1/2 (ERK1/2) or anti-β tubulin. D, Monocyte-depleted PBLs (1 × 10⁶) were cultured for 5 days in the presence of immobilized anti-CD6 or anti-CD28 mAbs (1 μg/ml each) alone (−) or in combination (+) with suboptimal doses of immobilized anti-CD3 mAb (0.5 μg/ml). Proliferation was determined as the amount of [³H]thymidine incorporation during the last 16 h of the culture, measured in cpm. Representative data of one of three independent experiments are shown.
MEK1/2 activation (Fig. 1A, left). Similar results to those observed with PBMCs were obtained when serum-starved human leukemic HUT-78 T cells were studied (Fig. 1A, right). As illustrated in Fig. 1B, the effects induced by the 161.8 mAb on the phosphorylation status of ERK1/2 were dose- and time-dependent.

Next, we analyzed whether ERK1/2 phosphorylation was also observed when other anti-CD6 mAbs were used. For this purpose we used two additional anti-CD6 mAbs named SPV.L14.2 and MAE1-C10. As shown in Fig. 1C, all of the anti-CD6 mAbs used (161.8, SPV.L14.2, and MAE1-C10) shared the ability to induce ERK1/2 phosphorylation. Competition binding assays using unlabeled and biotin-labeled mAbs showed that the three anti-CD6 mAbs recognize a closely related epitope (data not shown). Furthermore, these anti-CD6 mAbs showed a similar behavior in cell proliferation assays. As shown in Fig. 1D, none of them was mitogenic by itself. When associated with suboptimal doses of immobilized anti-CD3 mAb, all anti-CD6 mAbs showed comitogenic effects that were comparable in magnitude to those induced by M-T605, a comitogenic anti-CD6 mAb (22), and by an anti-CD28 (CD82.2) mAb, both used as positive controls. Similar results were obtained when anti-CD6 and CD28 mAbs were used in combination with suboptimal doses of PHA (data not shown).

**Src kinases are critical for CD6 ligation-induced ERK1/2 activation**

The Src family of tyrosine kinases plays an important role in the signaling pathway of the TCR. Two Src tyrosine kinases, Lck and Fyn, are mainly expressed in T lymphocytes. To explore their involvement in ERK1/2 activation via CD6, serum-starved human PBMCs were preincubated with PP1 (10 μM), a specific inhibitor of the Src kinases, before CD6 ligation with specific mAbs. As shown in Fig. 2A, PP1 preincubation inhibited ERK1/2 phosphorylation induced not only by CD6 cross-linking (mAb 161.8), but also by CD3 (mAb 33-2A3). No significant inhibitory effects were observed when the PBMCs were preincubated with LY294002 (10 μM), a specific inhibitor of PI3K (Fig. 2A). This observation indicates the Src-dependence of CD6-mediated ERK1/2 activation.

To further explore the involvement of Src kinases in CD6-mediated ERK1/2 activation, we used JCAM1 cells, Lck-deficient derivatives of the Jurkat T cell line (36). Serum-starved Jurkat and JCAM1 cells were tested for positive surface expression of both CD3 and CD6 (data not shown) and then stimulated with 10 μg/ml anti-CD6 (161.8 or MAE1-C10) mAbs or 10 ng/ml anti-CD3 (33-2A3) mAbs. As shown in Fig. 2B (left), ERK1/2 phosphorylation could not be achieved in the JCAM1 cells following stimulation with any of the anti-CD6 and anti-CD3 mAbs used, indicating that CD6-mediated ERK1/2 activation is dependent on the activation of the Src family tyrosine kinases, likely Lck. Marked ERK1/2 phosphorylation was observed following JCAM1 cell stimulation with PMA (100 ng/ml), a potent PKC activator, which overcomes the need for Src kinase activation (Fig. 2B). The parental Jurkat cell line was able to phosphorylate ERK1/2 when the cells were stimulated with any of the anti-CD6 (SPV.L14.2 or MAE1-C10) and anti-CD3 (33-2A3) mAbs used (Fig. 2B, right).

**Coligation of CD6 and CD3 increases ERK1/2 activation**

It has been previously reported that a fraction of CD6 molecules physically associate with the TCR/CD3 complex in resting normal and leukemic T cells (20). Moreover, ligation of either CD3 or CD6 alone triggers ERK1/2 activation (see above). Therefore, we further studied whether simultaneous ligation of CD3 and CD6 by soluble mAbs had either synergistic or additive effects on ERK1/2 activation. As illustrated in Fig. 3, the simultaneous stimulation of human PBMCs with the 161.8 (anti-CD6) and 33-2A3 (anti-CD3) mAbs resulted in synergetic rather than additive effects on the phosphorylation level of ERK1/2, as deduced from densitometric analyses (see Fig. 3, upper panel). This result indicates that co-cross-linking of CD6 and CD3 enhances not only T cell proliferation (see Fig. 1D) but also ERK1/2 activation.

**The C-terminal cytoplasmic region of CD6 is required for ERK1/2 activation**

To assess the involvement of the cytoplasmic region of CD6 in ERK1/2 activation, we performed experiments on CD6-transfected 2G5 cells, a Jurkat cell line derivative negative for CD5 and CD6.
expression (32). Jurkat 2G5 cells were successfully transfected with expression constructs coding for either wild-type CD6 or a cytoplasmatic tail-truncated CD6 form, which is devoid of the 141 most C-terminal residues (Fig. 4A). The 2G5 transfecteds were either unstimulated or stimulated with 10 μg/ml anti-CD6 or 10 ng/ml anti-CD3 mAbs for 40 min at 37 °C and then assayed for ERK1/2 phosphorylation. As shown in Fig. 4B, ERK1/2 became phosphorylated in the Jurkat 2G5 transfecteds expressing wild-type CD6 following either CD6 or CD3 mAb ligation. By contrast, ERK1/2 phosphorylation could only be induced through CD3 ligation in transfecteds expressing cytoplasmatic tail-truncated CD6.P527stop (Fig. 4C). These results suggest that the C-terminal region of CD6, which includes the only two phosphorylatable tyrosine residues (Y629 and Y662) (27, 28), is essential for ERK1/2 activation via CD6.

Engagement of CD6 by ALCAM/CD166 induces ERK1/2 activation

CD6 specifically binds to ALCAM/CD166, whose interaction has been implicated in thymocyte adhesion and development (6–9). Because our data indicate that CD6 ligation by specific mAbs induces ERK1/2 activation, we questioned whether CD6 engagement with ALCAM/CD166 would yield the same results. To test this hypothesis, we used a human ALCAM-Fc chimera immobilized to a solid phase in two different ways. In a set of experiments, plastic plates were first coated with an anti-human IgG1 antiserum and subsequently incubated with ALCAM-Fc or CD6-Fc chimeric proteins, as well as with human IgG1. Then, serum-starved human PBMCs were added to the coated plates and incubated for 40 min. As shown in Fig. 5A, ERK1/2 phosphorylation was detected in cells incubated with ALCAM-Fc, but not with CD6-Fc or human IgG1. In another set of experiments, the ALCAM-Fc chimera was directly bound onto the plates at increasing concentrations. Then, serum-starved human PBMCs were added to the plates and incubated for 40 min before analysis. As shown in Fig. 5B, a dose-dependent effect on ERK1/2 phosphorylation was induced by the ALCAM-Fc chimera. These results indicate that ERK1/2 activation follows the ligation of CD6 not only by specific mAbs but also by its ligand ALCAM/CD166 and stress the importance of the CD6-ALCAM interaction in T cell signaling.

**CD6 ligation induces AP1 and SRE-dependent luciferase activity**

Activation of ERK1/2 is known to phosphorylate the Elk-1 transcription factor, which can then bind to the SRE at the c-fos promoter (37), thereby stimulating c-fos expression. Thus, we analyzed whether CD6 ligation could activate Elk-1 transcriptional activity by means of a luciferase reporter plasmid under the control of the SRE of c-Fos (SRE-Luc). Serum-starved COS-7 cells transiently cotransfected with the expression construct coding for wild-type CD6 (pH6-CD6.wt), together with the SRE-Luc reporter, were left either unstimulated or stimulated with the anti-CD6 161.8 mAb for 16 h at 37 °C. Anti-CD45 (72-5D3) mAb and 20% FBS were used as a negative and positive control, respectively.

**FIGURE 4.** Cross-linking of cytoplasmic tail-truncated CD6 does not induce ERK1/2 activation. A, Triton X-100 solubilates from 2G5 Jurkat cells expressing the wild-type CD6 (CD6.wt) or the cytoplasmatic tail-truncated CD6 (CD6.P527stop) were immunoprecipitated with anti-CD6 mAb (αCD6; 161.8) plus 20 μl of 50% protein A-Sepharose. Precipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting with a rabbit anti-CD6 polyclonal antiserum plus HRP-labeled goat anti-mouse Ig antiserum (left). Cell surface expression levels of CD6.wt and cytoplasmatic tail-truncated CD6.P527stop in the 2G5 Jurkat cell stable transfecteds were determined by flow cytometry using the anti-CD6 161.8 mAb plus FITC-labeled goat anti-mouse IgG antiserum (right). B and C, ERK1/2 activation analysis of serum-starved Jurkat 2G5 transfecteds expressing CD6.wt (B) or cytoplasmatic tail-truncated CD6.P527stop (C). In both cases, transfecteds were stimulated with 10 μg/ml anti-CD6 (αCD6; MAE1-C10 or 161.8) or 10 ng/ml anti-CD3 (αCD3; 33-2A3) mAb for 40 min at 37°C. After Triton X-100 lysis, 50 μg of cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to immunoblotting with anti-phospho-ERK1/2 (p-ERK1/2) and further reprobing with anti-ERK1/2 (ERK1/2) antisera. Representative data of one of two independent experiments are shown.

**FIGURE 5.** CD6 ligation with ALCAM/CD166 induces ERK1/2 activation. A, Six-well tissue culture plates were coated overnight at 4°C with 4 μg of anti-human-IgG1 (h IgG1). Plates were blocked with 1% BSA in TSM buffer at 37°C and then coated with 20 μg/ml ALCAM-Fc. B, Six-well tissue culture plates were directly coated overnight at 4°C with 1, 10, and 20 μg/ml ALCAM-Fc followed by blocking with 1% BSA in TSM buffer for 30 min at 37°C. Serum-starved PBMCs (2 × 10⁷ per well) were added and left for 40 min at 37°C. After Triton X-100 lysis, 50 μg of cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting with anti-phospho-ERK1/2 (p-ERK1/2) and further reprobing with anti-ERK1/2 (ERK1/2). Representative data of one of three independent experiments are shown.
positive controls, respectively (Fig. 6A). As expected, CD6 ligation induced a significant increase in SRE-dependent luciferase activity as compared with unstimulated cells or cells exposed to an anti-CD45 mAb (Fig. 6A).

The AP-1 transcription factor usually consists of heterodimers of the c-Fos and c-Jun proteins. Therefore, we next analyzed whether CD6 ligation could also induce AP-1 transcriptional activity. This was done by performing experiments similar to those mentioned above, except that the AP-1/TRE-Luc reporter plasmid was used to transiently cotransfect COS-7 cells with the pHβ-CD6.wt expression construct. As shown in Fig. 6B, CD6 ligation also induced significant AP-1-dependent luciferase activity as compared with the negative controls. Thus, our data indicate that the CD6 lymphocyte cell surface receptor can initiate a signaling cascade that involves the MAPK pathway and leads to AP-1-dependent gene expression.

**CD6 ligation induces p38 and JNK pathway activity**

AP-1 complexes are not only composed of c-Fos/c-Jun heterodimers but also of c-Jun homodimers and activating transcription factor-2 (ATF-2)/c-Jun heterodimers. Moreover, the transcription factors c-Jun and ATF-2, as well as Elk-1, are known substrates of the JNK and p38 MAPK (38). Therefore, we further investigated the possible involvement of the JNK and p38 kinases in the signal transduction pathway triggered upon CD6 ligation. This was performed by stimulating serum-starved human PBMCs with different amounts of anti-CD6 mAb (MAE1-C10) at different time points. A time- and dose-dependent phosphorylation of JNK was induced following CD6 ligation (Fig. 7A). Very similar results were also obtained when p38 phosphorylation was analyzed (Fig. 7B). Taken together, these results indicate that different MAPK cascades are concomitantly activated in response to CD6 ligation.

**Discussion**

The CD6 lymphocyte surface receptor is an accessory molecule that is likely involved in the modulation of certain immune cellular processes such as thymocyte maturation and peripheral T cell activation (9, 13, 14, 19, 23). This assumption is mainly based on the fact that CD6 specifically binds to ALCAM/CD166, an adhesion molecule constitutively expressed on thymic epithelial cells and APCs (macrophages and dendritic cells) and inductively expressed on activated T and B lymphocytes (6). Accordingly, the CD6-ALCAM/CD166 interaction is known to be important for thymocyte-thymic epithelial cell adhesion phenomena (6, 7), maturation of the IS formed between T and APC cells (20, 22), and proper T cell proliferative responses (20, 23). Nevertheless, little progress has been made on the knowledge of the precise biological effects following CD6-ligand interactions and the signaling pathway(s) downstream of CD6. Only very recently has the first protein-protein interaction involving the cytoplasmatic domain of CD6 been reported (30). In that report, syntenin-1, a protein containing PDZ domains, was shown to interact with the most C-terminal residues of CD6 and to colocalize with it at the IS. Here, we present the first evidence on the involvement of the MAPK cascades in the intracellular signaling pathway triggered by CD6. We demonstrate that CD6 ligation induces the time- and dose-dependent activation of the ERK1/2, but also of the JNK and p38 kinases.

To study CD6 signaling, we cross-linked the CD6 receptor with three different anti-CD6 mAbs (161.8, SPV.L14.2, and MAE1-C10) on normal and leukemic T cells. All of them shared the ability to induce MAPK activation. Accordingly, the three mAbs seem to recognize closely related epitopes on the CD6 molecule and exhibit similar behavior in T cell proliferation assays. Anti-CD6 mAbs can be either mitogenic or inhibitory to T lymphocyte proliferation, depending on the assay and on Ab tested (35). Our results showed that none of the anti-CD6 mAbs used was mitogenic by itself and that they do exhibit significant comitogenic effects when associated with suboptimal doses of immobilized anti-CD3 mAb.

Using pharmacological inhibitors and Jurkat cell line derivatives, we demonstrate that CD6-induced ERK1/2 activation is dependent on the integrity of the most C-terminal cytoplasmic domain of CD6, as well as on the activity of Src family tyrosine kinases, likely Lck. A comparison of CD6 from various species shows that the highest homology occurs in the cytoplasmic region. Although the CD6 cytoplasmic domain has no intrinsic protein tyrosine kinase (PTK) activity, it contains tyrosine residues (Y629 and Y662) that might serve as docking sites for Src homology (SH) 2 domains upon phosphorylation, as well as several proline-rich regions for binding to SH3 domains (28). These docking sites are absent in the cytoplasmatic tail-mutant CD6 molecule (CD6.P527stop), which was unable to induce ERK1/2 activation. The nonreceptor PTKs of the Src family Lck present both SH2 and SH3 domains, was shown to interact with the most C-terminal residues of CD6 and to colocalize with it at the IS. Here, we present the first evidence on the involvement of the MAPK cascades in the intracellular signaling pathway triggered by CD6. We demonstrate that CD6 ligation induces the time- and dose-dependent activation of the ERK1/2, but also of the JNK and p38 kinases.

**FIGURE 6.** CD6 ligation induces AP1- and SRE-dependent luciferase activity. COS-7 cells were transiently cotransfected with pHβ-CD6.wt plus either SRE:Luc (A) or AP1/TRE:Luc (B) reporter plasmids. Serum-starved transfectants were stimulated for 16 h with 10 μg/ml anti-CD6 (αCD6: 161.8) mAb, 10 μg/ml anti-CD45 (αCD45: 72-5D3) mAb, or 20% FBS, and then luciferase activity was determined on cell lysates samples. The data are the average of three different experiments. *, p < 0.05, compared with cells stimulated with anti-CD45 (72-5D3) mAb as a negative control. Representative data of one of two independent experiments are shown.
Other findings relevant to CD6 function are the specific and dose-dependent ERK1/2 activations induced upon ligation of CD6 by ALCAM/CD166, as well as the synergistic effects on ERK1/2 activation induced upon simultaneous ligation of CD3 and CD6. The later was in agreement with the observation of significant mitogenic effects by co-cross-linking CD3 and CD6 by immobilized mAbs (Fig. 1D). The results provide a molecular basis for the proposed role of CD6 on the modulation of cellular processes such as thymocyte maturation and peripheral T cell activation. ALCAM/CD166 is expressed on several cell types (thymic epithelial cells, macrophages, dendritic cells, and activated B cells) that interact with T cells during thymocyte development and immune responses. Moreover, CD6 physically associates with the TCR/CD3 complex on thymocytes and peripheral T cells (20). Thus, simultaneous ligation of CD6 and TCR/CD3 by ALCAM/CD166 and peptide/MHC complexes, respectively, either in the thymus or in the periphery, may influence the kinetics and duration of MAPK activation, which is central to multiple cellular immune responses ranging from activation and survival to anergy and apoptosis (42).

In accordance with the ERK1/2 activation data on normal and leukemic T cells, we could evidence Elk-1 and AP-1-dependent reporter gene expression in COS-7 cells transiently expressing the CD6 receptor. Because the transcription factors c-Jun and ATF-2, as well as Elk-1, are known substrates of the JNK and p38 MAPK, we studied whether CD6 cross-linking could also activate these two MAPK in PBMCs. Accordingly, we observed a dose- and time-dependent activation of JNK and p38 upon the binding of anti-CD6 mAbs. Based on these data, it can be speculated that CD6 would likely influence the dynamic balance between growth factor-activated ERK and stress-activated JNK-p38 pathways, determining whether a T (or B) cell survives or undergoes apoptosis. Indeed, the MAPKs are deeply involved in several aspects of T cell biology such as early thymocyte development, positive and negative selection and lineage commitment of thymocytes, and activation and differentiation of peripheral T cells (43). As an example, the ERK and p38 pathways are required for the differentiation of immature double negative thymocytes to the double positive stage (44, 45). Furthermore, the ERK pathway is important for positive selection of double positive thymocytes and lineage commitment (46–48), whereas JNK and p38 may be required for negative selection (49–51). Recent studies have shown that the expression of CD6 during thymocyte development correlates with positive selection and resistance to apoptosis (9), thus suggesting that CD6 sends anti-apoptotic signals to immature thymocytes. These studies also suggest that CD6-dependent signals increase the overall functional avidity of the interaction between thymic APCs and thymocytes to levels sufficient to enhance positive selection or negative selection, depending on the avidity of a specific TCR for peptide/MHC complexes. Our data indicate that coordinate activation of MAPK by the TCR and CD6, as well as other accessory molecules, may affect the balance between pro- and anti-apoptotic proteins in developing and mature lymphocytes.

In summary, the results reported here collectively suggest that CD6-mediated activation of the MAPK, alone or in combination with other signaling cascades, could be relevant to the fine tuning of TCR signaling during developmental and adaptive T cell responses.

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References


