Association of CD38 with Nonmuscle Myosin Heavy Chain IIA and Lck Is Essential for the Internalization and Activation of CD38*

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Activation of CD38 in lymphokine-activated killer (LAK) cells involves interleukin-8 (IL8)-mediated protein kinase G (PKG) activation and results in an increase in the sustained intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$), cADP-ribose, and LAK cell migration. However, direct phosphorylation or activation of CD38 by PKG has not been observed in vitro. In this study, we examined the molecular mechanism of PKG-mediated activation of CD38. Nonmuscle myosin heavy chain IIA (MHCIIA) was identified as a CD38-associated protein upon IL8 stimulation. The IL8-induced association of MHCIIA with CD38 was dependent on PKG-mediated phosphorylation of MHCIIA. Supporting these observations, IL8- or cell-permeable cGMP analog-induced formation of cADP-ribose, increase in [Ca$^{2+}]_i$, and migration of LAK cells were inhibited by treatment with the MHCIIA inhibitor blebbistatin. Binding studies using purified proteins revealed that the association of MHCIIA with CD38 occurred through Lck, a tyrosine kinase. Moreover, these three molecules co-immunoprecipitated upon IL8 stimulation of LAK cells. IL8 treatment of LAK cells resulted in internalization of CD38, which co-localized with MHCIIA and Lck, and blebbistatin blocked internalization of CD38. These findings demonstrate that the association of phospho-MHCIIA with Lck and CD38 is a critical step in the internalization and activation of CD38.

A type II transmembrane protein, CD38 possesses ADP-ribosyl cyclase and ADP-ribose hydrolase activities (1, 2). These two enzyme activities are involved in the conversion of β-NAD$^+$ first to cADP-ribose (cADPR)$^3$ and then to ADP-ribose (3–5). The metabolite cADPR is known to increase the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$) by release from intracellular Ca$^{2+}$ stores or by Ca$^{2+}$ influx through plasma membrane Ca$^{2+}$ channels in a variety of cells (6–10). Mounting evidence indicates that cADPR synthesis by ADP-ribosyl cyclases is stimulated through cell-surface heterotrimeric G-protein-coupled receptor signaling. The receptors involved in ADP-ribosyl cyclase activation include the β-adrenergic (11, 12), angiotensin II (13), and muscarinic (14) receptors. Activation of ADP-ribosyl cyclase/CD38 by cGMP has been reported (15, 16), and cAMP-dependent activation of the enzyme has also been observed in artery smooth muscle cells (12) and cardiomyocytes (17). However, the molecular mechanism of ADP-ribosyl cyclase/CD38 activation has not been completely elucidated.

The active site of CD38 is located in the extracellular domain, whereas the substrate β-NAD$^+$ and the targets of the metabolite cADPR are present inside cell (18). This topological paradox of CD38 has been addressed and explained by demonstrating 1) the ligand-induced, vesicle-mediated internalization of CD38, which is followed by an increase in the intracellular cADPR concentration ([cADPR]$_i$) (19), and 2) the β-NAD$^+$-transporting function of connexin-43 and the cADPR-transporting function of membrane-bound CD38 or an unidentified cADPR transporter (20). Thus, the exact molecular mechanism of CD38 activation remains to be clarified.

Recently, we reported that CD38 in lymphokine-activated killer (LAK) cells is activated by a sequential process involving ligation of the interleukin (IL)-8 receptor, inositol 1,4,5-trisphosphate-induced increase in [Ca$^{2+}]_i$, and activation of protein kinase G (PKG) (16). We also found that PKG does not directly activate or phosphorylate CD38 in vitro. In this study, we investigated the missing link between CD38 and PKG for activation of CD38 in LAK cells. In addition, we also examined whether CD38 is internalized during the activation process of CD38. The results indicate that PKG phosphorylates non-muscle myosin heavy chain IIA (MHCIIA) upon stimulation of the IL8 receptor and that the association of phosphorylated MHCIIA with CD38 through Lck induces the internalization and activation of CD38.

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The abbreviations used are: cADPR, cADP-ribose; [Ca$^{2+}]_i$, intracellular Ca$^{2+}$ concentration; [cADPR]$_i$, intracellular CD38-ribose concentration; LAK, lymphokine-activated killer; IL, interleukin; PKG, protein kinase G; MHCIIA, nonmuscle myosin heavy chain IIA; mAb, monoclonal antibody; pAb, polyclonal antibody; GST, glutathione S-transferase; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine 3′,5′-monophosphate; (R)$_{6}$-8-pCPT-cGMP-5′S, (R)$_{6}$-8-(4-chlorophenylthio)guanosine 3′:5′-monophosphorothioate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SH, Src homology.

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EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibodies were obtained as follows: anti-human CD38 monoclonal antibody (mAb) from BD Biosciences; anti-MHCIIA polyclonal antibody (pAb), anti-FLAG antibody, anti-actin mAb, and anti-FLAG antibody-agarose from Sigma; anti-Lck pAb from Upstate Biotechnology (Lake Placid, NY); anti-glutathione S-transferase (GST) pAb from Amersham Biosciences AB (Uppsala, Sweden); and horse-radish peroxidase-conjugated anti-mouse IgG, anti-goat IgG, and anti-rabbit IgG from Advanced Biochemicals Inc. (Jeonju, Korea). Ficoll-Hypaque and Percoll were obtained from Amersham Biosciences AB. Nylon wool was from Polyscience Inc. (Warrington, PA), and human recombinant IL2 was from Chiron B.V. (Amsterdam, The Netherlands). 8-pCPT-cGMP, (R)-8-pCPT-cGMP-S, and blebbistatin were purchased from Calbiochem. Human recombinant IL8, human AB serum, and all other reagents were obtained from Sigma. RPMI 1640 medium and antibiotics were from Invitrogen. Transwell were purchased from Corning Costar Corp. (Cambridge, MA). [32P]Orthophosphate was from PerkinElmer Life Sciences.

Preparation of LAK Cells—LAK cells were prepared as described previously (21, 22). Briefly, blood obtained from healthy volunteers was layered over Ficoll-Hypaque and centrifuged at 700 × g for 30 min to remove red blood cells. Cell preparations with red blood cells removed were incubated on a nylon-wool column at 37°C for 1 h in a 5% CO2 incubator to remove B lymphocytes and macrophages. Nylon-wool nonadherent cells were collected and further separated by Percoll density gradient centrifugation. Four layers of Percoll were used: 37, 44, 52, and 60%. After centrifugation at 700 × g for 20 min, cells from the 52% Percoll layer were collected, washed with serum-free RPMI 1640 medium, and incubated at a density of 2 × 10^6 cells/ml in culture medium (RPMI 1640 medium supplemented with 10% human AB serum, 0.25 μg/ml amphotericin B, 50 μg/ml gentamycin, 10 units/ml penicillin G, 100 μg/ml streptomycin, 1 mM l-glutamine, 1% nonessential amino acids, and 50 μM 2-mercaptoethanol) containing 3000 IU/ml IL2 in a 5% CO2 incubator at 37°C. After incubation for 24 h, the floating cells were removed, and the adherent cells were cultured in culture medium containing 1500 IU/ml IL2. LAK cells induced by IL2 for 10 days were used throughout this study.

Purification of MHCIIA—Jurkat T cells (~1-ml packed volume) were lysed in ice-cold lysis buffer containing 20 mM HEPES (pH 7.2), 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) Triton X-100, 100 mM NaCl, 1 mM Na2VO4, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprogin. Supernatants were obtained after centrifugation at 20,000 × g for 10 min. For immunoprecipitation, cell lysates (800 μg) precleared with protein G-agarose were incubated with anti-CD38 mAb or anti-MHCIIA pAb overnight at 4°C and then further incubated with protein G-agarose at 4°C for 1 h. The immunoprecipitates were washed four times with cell lysis buffer and boiled for 10 min. The immunoprecipitated proteins were subjected to SDS-PAGE on a 8 or 10% gel. The protein G-precleared lysate (10 μg/lane) was also subjected to immunoblotting to verify equal amounts of the proteins used for immunoprecipitation. After transfer to nitrocellulose membranes, the blots were incubated in blocking buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk for 2 h at room temperature and then with primary antibodies (CD38, 1:500 dilution; MHCIIA, 1:2000 dilution; actin, 1:5000 dilution; and Lck, 1:2000 dilution) in blocking buffer overnight at 4°C. The blots were rinsed four times with blocking buffer and incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:5000 dilution), anti-rabbit IgG (1:5000 dilution), or anti-goat IgG (1:5000 dilution) in blocking buffer at room temperature for 1 h. The immunoreactive proteins with the respective secondary antibodies were determined using an enhanced chemiluminescence kit (Amersham Biosciences AB) and exposed to an LAS-1000 ImageReader Lite (Fujifilm, Japan). Protein concentration was determined using a Bio-Rad protein assay kit, and known concentrations of bovine serum albumin (BSA) were used as the standard.

Measurement of [cADPR]—The [cADPR], was measured using a cyclic enzyme assay as described previously (25). Briefly, cells were treated with 0.5 ml of 0.6 M perchloric acid under sonication. Precipitates were removed by centrifugation at 20,000 × g for 10 min. perchloric acid was removed by mixing the aqueous sample with a solution containing 3 volumes of 1,1,2-trichlorotrifluoroethane to 1 volume of tri-n-octylamine.
After centrifugation for 10 min at 1500 × g, the aqueous layer was collected and neutralized with 20 mM sodium phosphate (pH 8.0). To remove all contaminating nucleotides, the samples were incubated with the following hydrolytic enzymes overnight at 37 °C: 0.44 unit/ml nucleotide pyrophosphatase, 12.5 units/ml alkaline phosphatase, 0.0625 unit/ml NAD glycohydrolase, and 2.5 mM MgCl₂ in 20 mM sodium phosphate buffer (pH 8.0). Enzymes were removed by filtration using a Centriflo filter (Amicon). To convert cADPR to β-NAD⁺, the samples (0.1 ml/tube) were incubated with 50 μl of reaction medium containing 1 mCi of [32P]orthophosphate at 37°C for 1 min. The cADPR-β-NAD⁺ was purified as described (26). The samples were further incubated with the cycling reagent (0.1 ml) containing 2% ethanol, 100 μg/ml alcohol dehydrogenase, 20 μM resazurin, 10 μg/ml diaphorase, 10 μM riboflavin 5'-phosphate, 10 mM nicotinamide, 0.1 mg/ml BSA, and 100 mM sodium phosphate (pH 8.0) at room temperature for 2 h. An increase in the resorufin fluorescence was measured at an excitation of 544 nm and an emission of 590 nm using a SpectraMax Gemini fluorescence plate reader (Molecular Devices Corp.). Various known concentrations of cADPR were also included in the cycling reaction to generate a standard curve.

**Measurement of [Ca²⁺]**—Cells were washed with Hanks’ balanced salt solution (2 mM CaCl₂, 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM D-glucose, and 20 mM HEPES (pH 7.3)) containing 1% BSA and incubated in the same solution containing 1% BSA for 6 h. Starved LAK cells were incubated with 5 μM fluo-3 acetoxyethyl ester (Molecular Probes, Eugene, OR) in Hanks’ balanced salt solution containing 1% BSA at 37 °C for 40 min. The cells were washed three times with Hanks’ balanced salt solution. Changes in [Ca²⁺] were measured at an excitation of 488 nm and an emission of 530 nm using an air-cooled argon laser system (27). The emitted fluorescence at 530 nm was collected using a photomultiplier. One image was scanned every 6 s for 10 min using a Nikon confocal microscope. For the calculation of [Ca²⁺], the method of Tsien et al. is used (450 nm for fluo-3 and F is the observed fluorescence level. Each tracing was calibrated for the maximal intensity (Fmax) by the addition of ionomycin (8 μM) and for the minimal intensity (Fmin) by the addition of EGTA (50 mM) at the end of each measurement.

**In Vivo Phosphorylation**—LAK cells were washed with phosphate-free medium and then incubated in phosphate-free medium containing 1 mCi of [³²P]orthophosphate at 37 °C for 6 h. The cells were incubated with various agents, washed twice with 10 ml PBS (pH 7.4), and then lysed with ice-cold cell lysis buffer. After centrifugation at 20,000 × g for 10 min, the supernatants were collected for immunoprecipitation. Immunoprecipitation was performed using anti-CD38 mAb as described above.

**Determination of Cell Migration**—Cell migration was determined as described previously (22). In brief, cells were washed with RPMI 1640 medium, scraped using a policeman, and washed with RPMI 1640 medium. Transwells with 8-μm pore size polycarbonate filters were used. Lower chambers contained 500 μl of RPMI 1640 medium and 1% BSA. LAK cells (4 × 10³) in 100 μl of RPMI 1640 medium containing various agents were placed in the upper chamber and then incubated in a 5% CO₂ incubator at 37 °C for 2 h. After removal of the unattached cells, the filters were removed, fixed with ice-cold 100% methanol, and stained with 15% Wright-Giemsa stain for 7 min. The cells were counted under a phase-contrast microscope.

**RESULTS**

**Treatment of LAK Cells with IL8 Induces Association of MHCIa with CD38**—Stimulation of the IL8 receptor induces CD38 activation via cGMP/PKG in LAK cells, but CD38 is neither directly phosphorylated nor activated by PKG in vitro (16). To identify the protein(s) located between CD38 and PKG, LAK cells metabolically labeled with [³²P]orthophosphate were treated with IL8 in the absence or presence of a PKG inhibitor, Rp-cAMPS (8-pCPT-cGMP-S), and the cell extracts were subjected to immunoprecipitation using anti-CD38 mAb. As shown in Fig. 1A, treatment with IL8 significantly increased the phosphorylation level of an ~200-kDa protein compared with the control. The intensity of the phosphoprotein was decreased in the presence of the PKG inhibitor, suggesting that the IL8-induced phosphorylation of the protein is mediated by PKG. The protein band was excised from the gel and subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis. The results revealed that the protein was MHCIa (Fig. 1B). To further confirm this observation, immunoprecipitation of MHCIa by anti-CD38 antibody was examined with and without IL8, the PKG inhibitor, or the cell-permeable cGMP analog 8-pCPT-cGMP. The level of MHCIa in the immunocomplex was increased upon treatment with IL8 compared with the control (Fig. 1C). The IL8-mediated association of MHCIa with CD38 was substantially reduced in the presence of the PKG inhibitor. Treatment of LAK cells with the cGMP analog also increased the co-immunoprecipitation of MHCIa compared with the control. Moreover, pretreatment of LAK cells with the specific MHCIa inhibitor blebbistatin completely inhibited the IL8-induced co-immunoprecipitation of MHCIa and CD38 (Fig. 1D). These results suggest that PKG
FIGURE 1. Association of CD38 with MHCIIA in LAK cells. A, IL8-induced CD38 co-immunoprecipitation of a phosphoprotein is inhibited by PKG inhibitor pretreatment in LAK cells. LAK cells were incubated with 1 mCi of $^{32}$P orthophosphate at 37 °C for 6 h. The labeled cells were treated with 10 pM IL8 or without (Control) for 90 s. LAK cells were preincubated with (Rp)-8-pCPT-cGMP-S (20 nM) for 30 min (Rp-8pCPTcGMP-S). LAK cell extract was immunoprecipitated with anti-CD38 mAb, and the immunoprecipitates were separated by SDS-PAGE, followed by autoradiography.

B, identification of MHCIIA as a protein associated with CD38 by MALDI-TOF analysis. Shown is a MALDI-TOF mass map of peptides obtained after in-gel digestion with trypsin. Matched peptides are shown in boldface and underlined in the protein sequence.

C, IL8 induces the association of MHCIIA with CD38 through cGMP/PKG in LAK cells. Cells were treated with 10 pM IL8 or 1 mM 8-pCPT-cGMP for 90 s. Cells pretreated with (Rp)-8-pCPT-cGMP-S (20 nM) for 30 min were incubated with IL8 for 90 s. The cells were extracted with lysis buffer and then subjected to immunoprecipitation using anti-CD38 mAb. The immunoprecipitated proteins were analyzed by immunoblotting with anti-CD38 mAb or anti-MHCIIA pAb.

D, IL8-induced association of MHCIIA with CD38 is inhibited by pretreatment of LAK cells with the MHCIIA inhibitor blebbistatin. LAK cells were incubated with blebbistatin (50 nM) for 30 min prior to treatment with IL8 for 90 s. Actin blotting was performed before immunoprecipitation to ensure that equal amounts of samples were subjected to immunoprecipitation. Three or four independent experiments were performed, and similar results were obtained.
phosphorylates MHCIIA in LAK cells upon IL8 stimulation and that phosphorylated MHCIIA is associated with CD38.

**MHCI A Is Involved in CD38 Activation**—Activation of CD38 by treatment of LAK cells with IL8 results in an increase in [Ca^{2+}][/sub], a production of a sustained Ca^{2+} signal, and an increase in cell migration (16). To evaluate the role of MHCI A in signaling involving CD38, we examined the effects of blebbistatin on these IL8/CD38-induced cellular responses. The increase in [Ca^{2+}][/sub], upon treatment with IL8 or the cGMP analog was significantly inhibited by pretreatment of LAK cells with blebbistatin (Fig. 2A). Consistent with these observations, the IL8- or cGMP analog-mediated sustained increase in [Ca^{2+}][/sub], and cell migration was also blocked by blebbistatin pretreatment (Fig. 2B and C). These data indicate that MHCI A is involved in IL8-induced CD38 activation.

**MHCI A Is Phosphorylated by PKG in IL8-induced LAK Cells**—Next, we examined whether MHCI A is directly phosphorylated by PKG. Indeed, purified MHCI A was shown to be a good substrate for PKG by in vitro phosphorylation (Fig. 3A). To determine which amino acid residues of MHCI A are phosphorylated upon IL8 stimulation, LAK cells were treated with IL8 in the absence and presence of the PKG inhibitor or cGMP analog. Cell extracts were subjected to immunoprecipitation using anti-MHCI A pAb, and phosphorylated amino acids in MHCI A were probed with anti-phosphoamino acid antibodies or anti-MHCI A pAb.

**PKG-phosphorylated MHCI A Is Associated with CD38 via the Lck SH3 Domain**—To examine whether phospho-MHCI A interacts directly with CD38, a binding study of phospho-MHCI A and CD38 was performed in vitro. However, the interaction of CD38 with MHCI A was not changed before or after the phosphorylation of MHCI A by PKG (Fig. 4A). We demonstrated previously that the tyrosine kinase Lck directly interacts with CD38 via its SH2 domain (24). The possibility that Lck is required for the interaction of CD38 with phospho-MHCI A was examined. In the presence of Lck only, the interaction of CD38 with phospho-MHCI A was significantly increased compared with that observed with non-phosphorylated MHCI A (Fig. 4A). To further confirm that Lck may lead to the association of phospho-MHCI A with CD38, various constructs of Lck domains were expressed, purified, and reconstituted with phospho-MHCI A and FLAG-CD38. Binding of phospho-MHCI A to CD38 was observed only in the presence...
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FIGURE 4. PKG-phosphorylated MHCIIA is associated with CD38 via the Lck SH3 domain. A, PKG-phosphorylated MHCIIA is associated with CD38 via Lck. FLAG-CD38 (2 μg) was incubated with or without GST-Lck (2 μg) and further incubated with phospho-MHCIIA (2 μg) or non-phosphorylated MHCIIA (2 μg) in a 500-μl volume overnight at 4 °C. Phospho-MHCIIA (10 μg) was obtained by incubation with PKG (3000 units) in the presence of 20 mM MgCl₂, 0.1 mM ATP, and 10 μM cGMP at 37 °C for 15 min. The proteins were immunoprecipitated (IP) with anti-FLAG antibody-agarose (20 μl of 1:1 suspension/tube). The immunoprecipitated proteins were subjected to immunoblotting using anti-MHCIIA pAb, anti-GST antibody, or anti-FLAG antibody. B, schematic presentation of GST-Lck domain fusion proteins. GST fusion proteins were expressed and purified as described under "Experimental Procedures." C, phosphorylated MHCIIA interacts with the SH3 domain of Lck. PKG-phosphorylated MHCIIA (2 μg) was incubated with FLAG-CD38 (2 μg) and GST-fused Lck domains (2 μg) in a 500-μl volume overnight at 4 °C, and the proteins were immunoprecipitated with anti-FLAG antibody-agarose. The immunoprecipitated proteins were subjected to immunoblotting using anti-MHCIIA pAb, anti-GST antibody, or anti-FLAG antibody. D, IL8-induced association of MHCIIA and Lck with CD38 in LAK cells. The cells were treated as described for Fig. 1C, extracted with lysis buffer, and then subjected to immunoprecipitation using anti-CD38 mAb. The immunoprecipitated proteins were analyzed by immunoblotting with anti-MHCIIA pAb, anti-Lck mAb, or anti-CD38 mAb. Three or four independent experiments were performed, and similar results were obtained.

of Lck constructs containing both the SH2 and SH3 domains (Fig. 4, B and C), suggesting that phospho-MHCIIA binds to the SH3 domain of Lck, the SH2 domain of which binds to CD38 (Fig. 4C, fourth lane). To confirm the findings, we examined whether stimulation of LAK cells with IL8 or the cGMP analog increases the association of these three molecules. Consistent with the above observations, the levels of MHCIIA were significantly increased in the immunoprecipitates of anti-CD38 mAb upon treatment of LAK cells with IL8 or the cGMP analog (Fig. 4D). Lck also co-immunoprecipitated with CD38 without changing the level under the various conditions applied. These results suggest that Lck is constitutively associated with CD38 and that phospho-MHCIIA is recruited to the CD38-Lck complex upon IL8 or cGMP stimulation.

MHCIIA Is Involved in CD38 Internalization—Because the above data indicate that complex formation of CD38 with MHCIIA and Lck activates CD38 and because MHCII is involved in protein trafficking in the process of vesicle budding (30, 31), localization of these three molecules in LAK cells was examined in the presence of IL8 with and without pretreatment with the MHCIIA inhibitor blebbistatin. A confocal microscope examination revealed that CD38 was internalized as early as 15 s after IL8 treatment and that MHCIIA and Lck co-localized with CD38 at this time point (Fig. 5A). After 60 s, CD38 returned to the initial position. In contrast, pretreatment of LAK cells with blebbistatin resulted in inhibition of CD38 internalization, and the location of MHCIIA and Lck was also unchanged (Fig. 5B). Fig. 5 (C and D) shows the percentage of cells showing CD38 internalization upon treatment with IL8 or with IL8 plus blebbistatin. These results suggest that MHCIIA and Lck are involved in the internalization and activation of CD38 induced by IL8.

DISCUSSION

We demonstrated previously that CD38 in LAK cells is activated through sequential signaling involving the IL8 receptor, inositol 1,4,5-trisphosphate-mediated increase in Ca²⁺, and activation of PKG (16). In this study, we have extended the molecular mechanism of the activation of CD38 in LAK cells. Our results reveal for the first time that MHCIIA is associated with CD38 through Lck and is involved in the internalization and activation of CD38. In addition, the results indicate that PKG phosphorylates the serine residue in MHCIIA upon stimulation of the IL8 receptor and that the phosphorylation of MHCIIA leads to binding to the CD38-Lck complex via the SH3 domain of Lck.

Previous studies demonstrated that CD38 internalization induced by external stimuli such as β-NAD⁺ and thiol compounds results in an increase in [cADPR], (19, 29). Our results reveal that stimulation of the IL8 receptor (a G-protein-coupled receptor) in LAK cells induces CD38 internalization and increases [cADPR]. These findings suggest that CD38 internalization leads to CD38 activation. Our previous data showing that the level of cADPR increases at ~30 s after IL8 treatment and reaches a maximal level at ~90 s (24) correlate well with the data of CD38 internalization, which occurred as early as 15 s and persisted for up to 30 s (Fig. 5A). However, NAD⁺ or glutathione-induced internalization of CD38 and increase in [Ca²⁺] occur relatively slowly and reach maximal levels at ~1 h (19). In our study, the CD38 internalization process was mediated by IL8 receptor signaling. Moreover, the intracellular cal-
Cium level was increased immediately after IL8 treatment and was sustained. Therefore, we suggest that the discrepancy is due to the different signaling mechanism of CD38 internalization: IL8 receptor signaling-mediated internalization of CD38 versus direct activation of CD38 with the substrate NAD$^+$. In addition, although it is unclear whether the internalization of CD38 is sufficient to target the substrate β-NAD$^+$, it appears, however, that the internalization of CD38 is the critical step for its activation because pretreatment of LAK cells with blebbistatin not only blocked IL8-induced internalization of CD38, but also inhibited IL8-stimulated cADPR production, sustained Ca$^{2+}$ increase, and migration of LAK cells.

Evidence for involvement of MHCIIA in the internalization of CD38 is that co-localization of CD38 with MHCIIA was found in LAK cells upon activation of the IL8 receptor, and the MHCIIA-specific inhibitor blebbistatin blocked the internalization of CD38. Moreover, our results indicate that the phosphorylation of MHCIIA by PKG is critical for the interaction with Lck: non-phosphorylated MHCIIA did not bind to the CD38-Lck complex, and the PKG inhibitor blocked the IL8-induced phosphorylation of the seine residue in MHCIIA. In eukaryotic cells, there are at least two nonmuscle MHCII genes that encode separate isoforms of the heavy chain, MHCIIA and MHCIIIB (32). T cells express MHCIIA only (33). They are involved in diverse processes, including cytokinesis, cell motility, and protein trafficking in the process of vesicle budding (34). MHCIIA have been shown to play a role in protein sorting at the plasma membrane level (31) and in the production of vesicles and association with membrane vesicles (35). In addition, an association of MHCIIA with the chemokine receptor CXCR4 in T cell lysates has been reported (36). Muñoz et al. (37) reported that CD38 signaling in T cells is initiated within a subset of membrane rafts containing Lck and the CD3ζ subunit of the T cell receptor. Clustering of T cell plasma membrane proteins into lipid raft microdomains has been suggested to play an important role in signal transduction (38).

Lck is one of eight members of the Src family of tyrosine kinases, which are activated by T cell stimulation and required for T cell proliferation, IL2 production, and cytotoxicity of LAK cells (39). Lck consists of four domains, viz. the N-terminal, SH2, SH3, and catalytic domains. Our study on the complex formation of CD38 with Lck and MHCIIA has revealed that Lck acts as a linker between CD38 and MHCIIA. Moreover, SH3 domain-containing fusion proteins of Lck are able to interact

**FIGURE 5.** IL8-induced internalization and co-localization of CD38 with MHCIIA and Lck are blocked by blebbistatin. A, IL8-induced internalization and co-localization of CD38 with MHCIIA and Lck. B, pretreatment with blebbistatin blocks the internalization and co-localization of CD38 with MHCIIA and Lck. Serum-starved LAK cells were preincubated in the absence and presence of blebbistatin (50 μM) at 37 °C for 30 min, followed by stimulation with IL8 (10 pM) for the indicated times. The cells were fixed, stained, and examined with a confocal microscope as described “Experimental Procedures.” Approximately 60% of the cells showed good capping and comparable staining of the three fluorochromes. C, percentages of cells exhibiting internal localization of CD38 with and without IL8 treatment. D, percentages of cells exhibiting internal localization of CD38 with and without treatment with IL8 plus blebbistatin. n indicates the total number of cells examined under a confocal microscope.
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with purified phosphorylated MHCIIA, but the SH2 domain
does not. Several studies have demonstrated that SH3 domain-
ligand interaction is critical for activation of members of the Src
kinase family, including Lck (40, 41). However, our data suggest
that Lck kinase activity is not involved in CD38 activation
and/or internalization because no phosphorylation of tyrosine
residues in CD38 or MHCIIA upon IL8 stimulation was
observed. It appears that Lck is tightly associated with CD38 in
LAK cells, forming a complex, because Lck was immunoprecip-
itated by anti-CD38 mAb without stimulation of the IL8
receptor and because the level of Lck in the immunoprecipi-
tates was not changed by the receptor stimulation. Because
these findings are unexpected, further detailed studies are
required.

In conclusion, we have demonstrated that recruitment of
phospho-MHCIIA in the CD38-Lck complex is the critical step
for the internalization and activation of CD38 and that IL8-
activated PKG phosphorylates MHCIIA directly. In addition,
the results suggest that Lck acts as an adaptor for the associa-
tion of CD38 with MHCIIA in IL8/CD38 signaling in LAK cells.

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