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Human Leukocytes Contain a Large Pool of Free Forms of CD18

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Monoclonal antibodies to CD18, the common chain of leukocyte integrins, recognize in various types of human lymphoid and myeloid cells under the conditions of nonreducing Western blotting three species of CD18 of mol. wt. 96, 87, and 78 kDa, respectively. Using a unique monoclonal antibody MEM-148 reacts exclusively with free CD18 molecules, but not with leukocyte integrin heterodimers. We demonstrate that only the upper one (96 kDa) is present on the cell surface within the CD11/CD18 integrin heterodimers, while the lower ones (87 and 78 kDa) are found intracellularly as free molecules unassociated with CD11 chains or other molecules. These intracellular free CD18 chains may in part represent biosynthetic precursors; alternatively, these species may represent an intracellular source of the recently observed free, proteolytically truncated CD18 chains expressed on the surface of activated myeloid cells.

Key Words: **CD18; leukocyte integrins; LFA-1; native electrophoresis; MALDI.**

Leukocyte (β_2) integrins are major adhesion molecules involved in interactions between different leukocytes and endothelia, T cells and antigen presenting cells or effector T cells and their targets. At least two of four known leukocyte integrins function also as complement receptors; additional interactions with several other ligands have been described (for review see Refs. 1–3). Leukocyte integrins are heterodimers of noncovalently associated transmembrane chains CD18 (β_2) and CD11 (α); four different α chains are known as α_L (CD11a), α_M (CD11b), α_X (CD11c) and α_D. The CD11a/CD18 heterodimer is the major leukocyte adhesion molecule LFA-1, CD11b/CD18 is complement receptor type 3 (CR3) expressed mainly on myeloid cells, CD11c/CD18 is complement receptor type 4 of myeloid cells (CR4). The fourth member of the family ($\alpha_D\beta_2$) is a recently discovered molecule of less well characterized functions. Major cellular ligands of β_2 integrins are ICAM-1, -2, and -3 (CD54, CD102, and CD50, respectively) molecules of the immunoglobulin superfamily (for review see: (4, 5)). Interestingly, the common β_2 chain (CD18) can be readily expressed on the surface of cells transfected with CD18 cDNA without the need for coexpression of any of the CD11 chains. On the other hand, CD11 chains can be expressed only within the CD11/CD18 heterodimers. Recently we observed occurrence of proteolytically truncated CD18 chains, apparently unassociated with CD11 chains, on the surface of myeloid cells (peripheral blood monocytes and neutrophils) activated by various stimuli (Drbal *et al.*, submitted). Therefore, in the present study we looked systematically for cell surface and intracellular expression of free CD18 chains in various types of lymphoid and myeloid cell lines, employing a unique mAb MEM-148. This mAbs recognizes CD18 chains either free or "partially" dissociated from CD11 chains but under standard conditions does not bind to CD11/CD18 heterodimers in which the MEM-148 epitope is inaccessible.

MATERIALS AND METHODS

Cells, reagents, and antibodies. T cell line HPB-ALL was obtained from Dr. J. L. Strominger (Harvard University, Cambridge, MA), T cell line Jurkat and myeloid cell line THP-1 from American Type Culture Collection (ATCC; Rockville, MD). COS-7 cells were transiently transfected with a pCDM8-based expression plasmid encoding full length cDNA of human CD18 (kindly provided by Dr. H. Stockinger, University of Vienna, Austria) using a modified DEAE-dextran method (6) and harvested 48 h later by vigorous pipetting after 10 min incubation with 5 mM EDTA in Hanks' balanced salt solution (HBSS) at 37°C. RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 20 μg/ml gentamycin, 50 μg/ml streptomycin and 10^4 U/ml penicillin was used for cell culture. Low pH-treated cells were prepared by brief (2 min) incubation in

Abbreviations used: BNE, blue native electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; mAb, monoclonal antibody.

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ice-cold isotonic citrate/phosphate buffer to give the final pH 3.5 followed by immediate neutralization with 20-fold excess of ice-cold HBSS containing 0.2% gelatin and 0.1% NaN_3 without Ca^{2+} and Mg^{2+} (HBSS-GA) and final washing.

CNBr-activated Sepharose 4B and molecular mass standards were purchased from Pharmacia (Uppsala, Sweden), pre-stained standards and anti-Ig-peroxidase conjugates from Bio-Rad (Hercules, CA), chemicals for buffers and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as well as detergents, biotinamidocaproic acid 3-sulfo-*N*-hydroxysuccinimide ester, somatostatin and protease inhibitors from Sigma (St. Louis, MO), enhanced chemiluminescence Western blotting kit from Amersham Pharmacia Biotech (Little Chalfont, UK), BioTrace PVDF membrane (used for electroblotting of native gels and of biotinylated proteins) from PALL Gelman Sciences (Ann Arbor, MI), nitrocellulose (used for other Western blotting experiments) from Schleicher&Schuell (Dassel, Germany). Anti-CD18 mAbs MEM-48 (IgG1) (7) and MEM-148 (IgG1) were obtained by standard techniques from mice immunized with peripheral blood mononuclear cells; MEM-148 was characterized and clustered within the 6th HLDA Workshop (8). mAbs MEM-83 (IgG1; anti-CD11a), MEM-144 (IgG1; anti-CD11a) and MEM-28 (IgG1; anti-CD45), were also produced and characterized in our laboratory; specificities of most of them were confirmed in previous HLDA Workshops. mAb 6.7 (IgG1; anti-CD18) which performed optimally in immunoprecipitation experiments was kindly provided by Dr. A. Bensussan (INSERM 448, Creteil, France).

Flow cytometry. Cells were pretreated for 10 min with 20% human AB serum in HBSS-GA which was used throughout the washing steps. Cells were incubated on ice with solutions of fluorescein-labeled mAbs (20 μg/ml) for 30 min and washed twice. Prior to measurement on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA), propidium iodide (PI; Sigma; 0.1 μg/ml) was added and at least 10,000 viable cells were collected for each sample.

For intracellular staining, cells were permeabilized with 40 μg/ml digitonin (Fluka; Buchs, Switzerland) in HBSS-GA (HBSS-GAD) for 15 min on ice prior to staining with mAbs. After three washes in HBSS-GAD digitonin was omitted from the last wash. Performance (sensitivity, linearity, and stability) of the instrument in a standard three-color setup was checked on a daily basis using Rainbow beads (Spherotech; Libertyville, IL). Finally, all list mode data files were analyzed using WinMDI 2.8 software (J. Trotter, The Scripps Research Institute, La Jolla, CA).

Biochemical methods. Preparation of detergent cell lysates, preparation and solubilization of cell membranes, immunoprecipitation (by the solid phase immunoisolation technique using antibody-coated plastic wells), SDS–PAGE and Western blotting (employing luminographic detection) were all performed as described in detail elsewhere (9, 10). Briefly, the cells ($10–50 \times 10^6$) were solubilized in isotonic lysis buffer (0.15 M NaCl, 0.1 M Tris/HCl buffer pH 8.2, 1% NP40 and protease inhibitors 5 mM iodoacetamide and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride). After 30 min of incubation on ice the insoluble nuclei and cytoskeleton were removed by centrifugation (20,000*g* for 3 min) and the supernatant was used for further work.

For immunoprecipitation, the cells (2×10^7) were washed in ice-cold PBS containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+}, suspended in 1 ml of the same solution and 1 mg of biotinamidocaproic acid 3-sulfo-*N*-hydroxysuccinimide ester was added. The suspension was incubated 1 h on ice, then the cells were washed and the reaction was quenched by 40 mM Tris buffer pH 8.2. The wells of flexible polyvinylchloride microtitration plates were incubated with a solution of affinity purified goat anti-mouse Ig (0.1 mg/ml) and then with solutions of various mouse mAbs (approx. 0.1 mg/ml). Such mAb-coated wells then served as immunosorbents: 50 μl volumes of cell detergent lysates were incubated in these immunosorbent wells (at 0°C, 4–10 h). After washing, these immunoprecipitates were eluted by sample buffer for SDS–PAGE and after electrophoretic separation followed by electroblotting onto PVDF membrane the biotinylated proteins were detected by streptavidin-peroxidase conjugate.

Immunoaffinity chromatography. MAbs were covalently bound to CNBr-activated Sepharose according to the manufacturer's instructions (5 mg mAb per 1 ml of the gel). Immunoaffinity chromatography was performed at 4°C on minicolumns of the immunosorbents (42 mm long, total volume 300 μl) washed thoroughly with the lysis buffer. 50 μl of detergent lysate of Jurkat cells (5×10^7 per ml of the lysis buffer) was applied at the top of the column, which was then eluted with the lysis buffer (at the rate of 15 μl/min), and the unbound fraction was collected.

300 μl of detergent lysate from low pH-treated Jurkat cells applied onto 0.5 ml column was used for immunoisolation of CD18 species which were subsequently subjected to MALDI-TOF analysis. After washing with 10 column volumes of the lysis buffer, the adsorbed proteins were eluted with alkaline buffer (0.1 M glycine-NaOH, pH 11.5, containing 0.1% NP40), precipitated with 10% trichloroacetic acid and separated by nonreducing SDS–PAGE followed by Coomassie brilliant blue R staining.

MALDI-TOF analysis. Three Coomassie brilliant blue R-stained zones (identified as CD18 by a separate Western blotting analysis) were digested directly in the gel by endoproteinase Lys-C (Promega; Madison, WI) using a modification of the previously described method (11). The resulting peptides mixtures were analysed on a Bruker BIFLEX (Bruker-Franzen; Bremen, Germany) MALDI-TOF mass spectrometer equipped with a nitrogen laser (337 nm) and a delayed extraction ion source. Positive-ion mass spectra of peptide maps were measured in the reflectron mode. A saturated solution of α-cyano-4-hydroxycinnamic acid in aqueous 30% acetonitrile and 0.1% TFA was used as a MALDI matrix. 1 μl of the sample and 1 μl of the matrix solution were mixed on the target and allowed to dry at the ambient temperature. Spectra were internally calibrated by employing the monoisotopic $[M + H]^+$ ion of peptide standard (somatostatin).

Native electrophoresis. Native non-SDS–PAGE (blue native electrophoresis, BNE) was performed essentially as described elsewhere (12). Briefly, 5×10^6 cells were solubilized in 200 μl of the native lysis buffer (1% *n*-dodecyl β-D-maltoside, 5 mM iodoacetamide and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride in 750 mM aminocaproic acid, 50 mM Bis–Tris, pH 7.0) and centrifuged for 3 min at 20,000*g;* Coomassie brilliant blue G was mixed with the supernatant to yield the final concentration of 0.25%. 5 μl samples were run on water-cooled gradient gels (6–15%). After the electrophoresis the separated proteins were electroblotted using native cathode buffer (15 mM Bis–Tris, 50 mM Tricine, pH 7.0) onto PVDF membrane and visualized by immunoperoxidase staining. The mol. wt. standards used were monomers and oligomers (obtained by chemical cross-linking) of bovine serum albumin or mouse IgG1 mAb.

RESULTS

mAb MEM-148 Recognizes an Epitope Present in Free CD18 Chains Unassociated with CD11

mAb MEM-148 binds very weakly to resting peripheral blood leukocytes and all lymphoid cell lines tested (not shown). It binds strongly to COS-7 cells transfected with CD18 cDNA (Fig. 1). On the surface of activated monocytes and neutrophils it recognizes a proteolytically truncated form of free CD18 (Drbal *et al.*, submitted). Leukocytes became strongly MEM-148 positive after low pH-treatment or after detergent-induced membrane permeabilization (Fig. 1). MEM-148 stained a triplet of zones (78–96 kDa) on nonreduced Western blots of detergent lysates of various leukocytes that were essentially surface-negative in cytofluorometry; the pattern of staining was indistin-

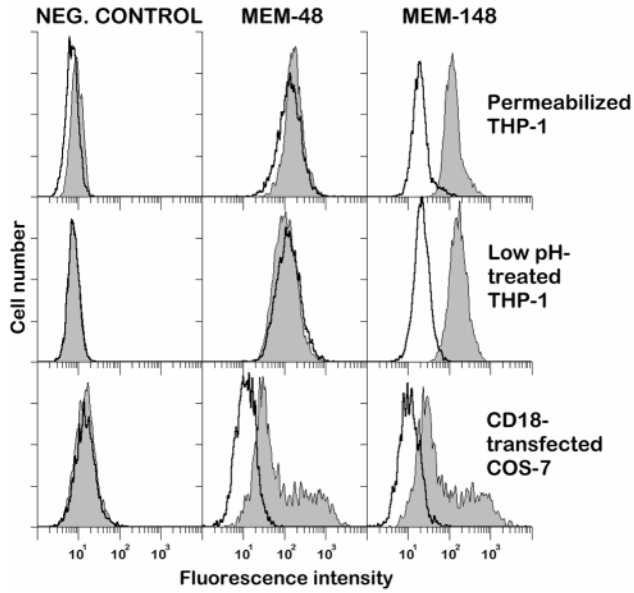

FIG. 1. Flow cytometric detection of the MEM-148 CD18 epitope on CD18-transfected COS-7 cells and after low pH-treatment or permeabilization in the THP-1 myeloid cell line (shaded peaks). Solid bold lines correspond to the surface staining of intact THP-1 cells or mock-transfected COS-7 cells with either standard isotype-matched mAb, anti-CD18 mAb MEM-48 or MEM-148, as indicated at the top.

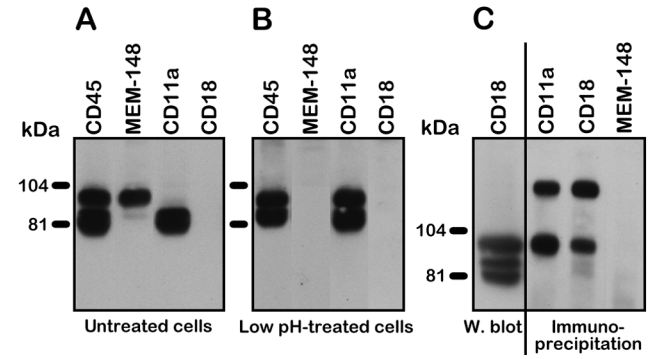

FIG. 3. Immunoisolation of various forms of CD18. (A) Jurkat cell were solubilized by 1% NP40 and subjected to immunoafinity chromatography on a minicolumn made of immobilized mAbs indicated at the top: MEM-28 (CD45; negative control), MEM-148, MEM-83 (anti-CD11a) and MEM-48 (standard anti-CD18). The unbound materials passed through the columns were analyzed by SDS–PAGE and Western blotting (immunostained by anti-CD18 mAb MEM-48). (B) As in (A) but using low pH-treated Jurkat cells. (C) Immunoprecipitation from detergent lysates of surface biotinylated Jurkat cells; note that mAbs to CD11a (MEM-83) and CD18 (a standard mAb 6.7) both immunoprecipitate the CD11a/CD18 heterodimers while containing exclusively the 96 kDa CD18 chains. The left lane represents Western blotting of the same cell lysate immunostained for CD18; note that the surface biotinylated CD18 matches only the upper CD18 zone in the blot.

guishable from that yielded by a standard CD18 mAb (Fig. 2). All these results indicate that MEM-148 is able to bind to free forms of CD18 chains (arising after dissociation of the integrin heterodimers by SDS or low pH-treatment, expressed on the surface of transfectants or (in a truncated form) on activated myeloid cells) but not with CD18 present in intact leukocyte integrins where the epitope is obviously inaccessible for the mAb.

Leukocytes Contain a Large Pool of Intracellular Free CD18

Intense intracellular immunostaining of various types of leukocytes with MEM-148 mAb following detergent permeabilization of the membrane indicated the existence of free intracellular CD18 molecules. As shown in Fig. 2, three species of CD18 could be demonstrated in various types of leukocytes by Western blotting of nonreduced samples. When cells were solubilized in a nonionic detergent and the lysates subjected to immunoisolation, only the lower two zones (corresponding to mol. wt. of 78 kDa and 87 kDa, respectively) but not the upper one (96 kDa) readily bound to the MEM-148 immunosorbent. In contrast, only the 96 kDa form was bound to an immunosorbent made of anti-CD11a mAb. All three forms were bound to an immunosorbent made of a standard anti-CD18 mAb (Fig. 3A).

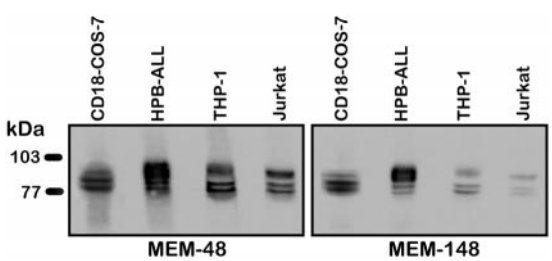

FIG. 2. Western blotting of nonreduced cell lysates. Different cell membranes were detergent solubilized (1% NP-40) and nonreduced nonboiled samples subjected to SDS–PAGE, electroblotting and immunostaining with mAbs MEM-48 (an anti-CD48 standard) and MEM-148, as indicated at the bottom.

After low pH-treatment of Jurkat cells (presumably dissociating the CD11/CD18 heterodimers), all forms were bound to the MEM-148 immunosorbent (Fig. 3B). Only the 96 kDa form was surface biotinylated and could be immunoprecipitated as CD11/CD18 heterodimers by mAbs to CD18 and CD11a but not by MEM-148 (Fig. 3C). Similar results were obtained with CD11a,b,c-positive THP-1 myeloid cell line (not shown). Thus, the 96 kDa form corresponds to the surface (CD11-associated) CD18, while the 87 kDa and 78 kDa forms are apparently intracellular and probably unassociated with CD11 chains. To determine whether these intracellular forms are free or perhaps associated with other (non-CD11) molecules, we performed a native non-SDS–PAGE under conditions which preserve the integrity of CD11/CD18 heterodimers. As shown in Fig. 4A, two major CD18-positive species could be distinguished by this method, corresponding to mol. wt. of 90 kDa and approx. 300

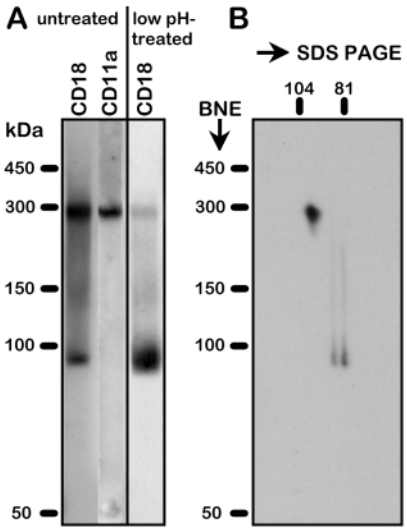

FIG. 4. Blue native electrophoresis (BNE) of Jurkat cell detergent lysates. (A) The separated proteins were electroblotted onto PVDF membrane and immunostained by mAbs to CD18 (MEM-48) or CD11a (MEM-144). The right lane represents the sample from cells treated by pH 3.5 prior to detergent solubilization; immunostained for CD18. (B) Two-dimensional electrophoretic analysis of CD18 forms of Jurkat cells (first dimension, BNE; second dimension, standard nonreducing SDS–PAGE) followed by electroblotting and immunostaining of CD18. Note that the 96 kDa form is present exclusively in the 300 kDa native complex while the 87 and 78 forms are unassociated even under native conditions.

kDa. Only the latter one contained also detectable CD11a molecules and obviously corresponded to intact CD11a/CD18 heterodimers. The 90 kDa species obviously corresponded to free, unassociated CD18 chains. Analysis by second-dimension SDS–PAGE has shown that the 300 kDa zone observed on the native gel contained exclusively the 96 kDa CD18 form whereas the zone corresponding to free CD18 contained the 87 kDa and 78 kDa forms (Fig. 4B). In contrast to the untreated Jurkat cells, the low pH-treated cells contained only trace amounts of the $\alpha\beta$ heterodimers and the majority of CD18 migrated under the mild native conditions as free monomers confirming that the heterodimers were dissociated by the acidic buffer (Fig. 4A).

To make sure that the 87/78 kDa proteins represent variant forms of CD18 and are not some unrelated proteins cross-reactive with the mAbs used, all three forms (96, 87, 78 kDa) obtained by immunoaffinity chromatography on the MEM-148 immunosorbent from low pH-treated Jurkat cells were analyzed by mass spectrometric (MALDI-TOF) peptide mapping. Lys-C peptide fragments produced from all three zones yielded molecular peaks corresponding to those expected for CD18 (Table 1) confirming that all of them represent variant forms of CD18. Moreover, the presence in all three forms of peptides originating from sequences close to N- and C-termini indicates that the forms probably do not differ in the length of the

TABLE 1

Comparison of Peptide Maps Obtained after Lys-C Digestion of the Three Electrophoretically Separated Forms of CD18

CD18 species	Peptides	8–26	102–125	127–152	153–164	153–174	175–187	253–272	273–288	289–306	318–335	341–357	358–392	603–614	615–636	703–718	703–722	723–733	Sequence coverage
96 kDa	Calculated $[M + H]^+$	2068.9	2897.4	2764.5	1367.7	2547.3	1571.8	2242.1	1847.9	2031.1	1884.0	1926.0	3894.9	1433.7	2202.1	2046.1	2544.4	1362.6	
	Measured $[M + H]^+$	2068.8	2897.3	2764.2	1367.7	n.o.	1571.9	2242.1	1847.9	2031.2	1884.0	1926.0	3894.6	n.o.	n.o.	2046.0	n.o.	1362.7	33%
87 kDa	Measured $[M + H]^+$	2068.9	2897.2	2764.5	1367.8	n.o.	1571.9	2242.2	1848.0	2031.2	1884.0	1926.1	n.o.	n.o.	n.o.	2046.2	n.o.	1362.7	30%
78 kDa	Measured $[M + H]^+$	2068.9	2897.5	2764.5	n.o.	2547.3	n.o.	n.o.	1847.9	2031.2	1884.0	1926.1	n.o.	1433.7	2202.2	n.o.	2544.3	1362.7	30%

Note. The peptides observed in the mass spectra are indicated by numbers of their first and last amino acid residues. Some peptides were not observed (n.o.) in the particular forms of the protein. The peptides containing glycosyl moieties or other posttranslational modifications could not be identified, as well as those containing cysteines involved in disulphide bridges.

polypeptide backbone but rather in a posttranslational modification(s) (see Discussion).

Thus, we conclude that leukocytes (especially cell lines) contain a significant pool of intracellular CD18 chains which are of lower apparent mol. wt. (higher mobility on SDS–PAGE) and unassociated with CD11 chains or with other molecules.

DISCUSSION

We demonstrate that a large intracellular pool of free CD18 chains, unassociated with CD11 molecules, is present in lymphoid and myeloid cell lines. These intracellular free CD18 molecules most probably structurally differ from the cell surface CD18 chains present in β_2 integrin heterodimers as their apparent mol. wt. determined by SDS–PAGE is lower (78–87 kDa) compared to the cell surface species (96 kDa). One possibility is that these intracellular molecules are biosynthetic precursors of the mature, cell surface expressed ones; the difference in apparent mol. wt. could be due to differences in the structure of their carbohydrate chains (13).

Another possibility is that the differences in electrophoretic mobilities of nonreduced samples (as analyzed here) of the intracellular vs cell surface CD18 molecules are due to different arrangement of intramolecular cystine bridges. Extracellular domain of CD18 contains in total 54 cysteine residues which probably form up to 27 intramolecular disulfide bridges. Variants with different disulfide arrangements would most likely differ in their shape which would be reflected in different molecular radii of their complexes with SDS and thus in different electrophoretic mobility under the conditions of nonreducing SDS–PAGE. To elucidate this point, antibody reagents reactive with fully denatured, reduced CD18 chains would be needed: such antibodies used for Western blotting would be expected to detect little or no size differences between the cell surface and intracellular CD18 species if they differ in arrangement of intramolecular cystine bridges but not in glycosylation. Obviously, if the CD18 forms differ in glycosylation, size differences should be detectable also in the reduced samples.

It remains to be determined what is possible biological role of the intracellular free CD18. At least a part of them probably represent biosynthetic precursors of the cell surface mature forms still unassociated with the α (CD11) chains (13). On the other hand, it may be speculated that these intracellular CD18 chains may be under certain circumstances externalized and expressed in a monomeric form on the cell surface to serve so far unknown purposes. As mentioned above, we have recently observed large amounts of free, proteolytically truncated CD18 chains on the surface at activated monocytes and neutrophils. It is possible that at least a fraction of these molecules may originate from the intracellular pool of free CD18 chains. The hypothetical possibility that free CD18 species can be expressed on cell surface under some natural circumstances is supported by the fact that free CD18 (in contrast to CD11 molecules) can be easily expressed on the cell surface of cells transfected with CD18 cDNA expression vectors. Further research is needed to clarify possible physiological relevance of the intracellular free CD18 molecules described in this communication.

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