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CDw149 antibodies recognize a clustered subset of CD47 molecules associated with cytoplasmic signaling molecules

Key words:
CD47; CDw149; G-protein; integrin-associated protein; antibody affinity; Src-family kinases

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Abstract: One of the recently described antigens broadly expressed on human leukocytes is CDw149, which was defined at the 6th Human Leukocyte Differentiation Antigen (HLDA) Workshop by means of 2 monoclonal antibodies (mAbs). Molecular characterization of this antigen has been lacking. In the present study we demonstrate that these anti-CDw149 mAbs actually recognize a clustered subset of a well-defined membrane protein, CD47, also known as integrin-associated protein (IAP). This clustered subset is present on leukocytes but not erythrocytes. The anti-CDw149 mAbs bind with only low affinity to a monomeric (unclustered) subset of CD47 but with high avidity to the CD47 clusters. A fraction of CD47 is associated with large complexes containing cytoplasmic signaling molecules (Src family kinases and heterotrimeric G-proteins) similar to glycosphingolipid-enriched microdomains (GEMs), which may explain the previously described signaling capacity of CD47. The low-affinity anti-CD47 mAbs may be useful tools targeting specific receptor complexes involved in cell activation. Specific reactivity of low-affinity mAbs with clustered subsets of cell surface antigens may more generally explain the nature of poorly defined "activation forms" or activation neoepitopes described previously for several cell surface molecules.

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Numerous proteins have been identified on the surface of various types of leukocytes. Most of the well-defined human leukocyte surface proteins have been characterized as to their functions and most of them were given CD names (1, 2). A basic characteristic of a CD molecule is its pattern of expression on various cell types as determined by immunostaining by specific monoclonal antibodies (mAbs). However, such results can be greatly affected by binding characteristics of the mAbs used, i.e. by their affinity toward the respective antigen. Low-affinity antibodies preferentially bind to the cells expressing higher levels of the antigen. In addition, such low-affinity mAbs are expected to preferentially bind to oligomers or clusters of the antigen. In both these situations (high level of expression and clustering) the low-affinity mAb has the opportunity to bind simultaneously with two (IgG) or multiple (IgM) binding

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sites and thus achieve a stable, high-avidity attachment to the cell surface. If the low-affinity mAb is used for immunostaining of cells expressing relatively low levels of monomeric antigen molecules, only monovalent interactions with the antigen are possible which are kinetically unstable; the mAb rapidly dissociates from the antigen during the washing step and such cells then appear as negative. Low-affinity mAbs may thus falsely appear to define novel cell surface molecules with unique expression patterns. Recently, such cases were described for anti-CDw78 mAbs (actually low-affinity mAbs to major histocompatibility complex (MHC) class II molecules) (3, 4) and certain mAbs recognizing an "activation epitope" of CD147 (actually low-affinity anti-CD147 mAbs reactive with activated cells expressing higher levels and aggregated forms of CD147 molecules) (5). In the present paper we describe a similar case – mAbs which apparently defined a "novel" molecule named CDw149 (6); these are now actually identified as low-affinity mAbs to a structurally and functionally well established molecule, CD47, also known as integrin-associated protein (IAP) (7). These mAbs recognize an aggregated or oligomerized subset of CD47 molecules present on leukocytes but not on erythrocytes. Interestingly, this aggregated subset of CD47 is associated with cytoplasmic signaling molecules within specific membrane microdomains.

Material and methods

Cells

T-cell line HPB-ALL was obtained from Dr. J. L. Strominger (Harvard University, Cambridge, MA, USA), T-cell line Jurkat and B-cell line Raji from American Type Culture Collection (ATCC; Rockville, MD, USA). Human peripheral blood cells of healthy volunteers were drawn into K_3EDTA Vacuettes (Greiner; Kremsmünster, Austria). Leukocytes and erythrocyte membranes were isolated by brief osmotic lysis (30 s in distilled water) followed by differential centrifugation. CD47 transfectants of ovarian carcinoma clone OV10 (8) were kindly provided by Dr. F. P. Lindberg (Washington University School of Medicine, St. Louis, MO, USA). Formaldehyde-fixed Jurkat cells were prepared by incubation of a suspension of 10^7 cells washed with phosphate-buffered saline (PBS) in 1 ml of 3.7% formaldehyde for 30 min at 20°C followed by washing and blocking in 1% glycine and 0.2% gelatin for 30 min at 4°C.

Reagents and antibodies

CNBr-activated Sepharose 4B and molecular mass standards were purchased from Pharmacia (Uppsala, Sweden), pre-stained standards and anti-Ig-peroxidase conjugates were from Bio-Rad (Hercules, CA, USA), chemicals for buffers and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as well as detergents were from Sigma (St. Louis, MO, USA), enhanced chemiluminiscence Western blotting kit from Amersham Pharmacia Biotech (Little Chalfont, UK), Immobilon-P membrane from Serva (Heidelberg, Germany), nitrocellulose from Schleicher & Schuell (Dassel, Germany). Anti-CDw149 mAbs MEM-120 (IgM) and MEM-133 (IgG2a) were obtained by standard techniques from mice immunized with HPB-ALL cell line and characterized and clustered within the 6th Human Leukocyte Differentiation Antigen (HLDA) Workshop (6). MAbs MEM-28 (IgG1; anti-CD45), MEM-122 (IgM; anti-CD47), MEM-59 (IgG1; anti-CD43), MEM-53 (IgG1; anti-CD53), MEM-97 (IgG1; anti-CD20) and MEM-78 (IgG1; anti-CD10) were also produced and characterized in our laboratory; their specificities were confirmed in previous HLDA Workshops. MAb BRIC126 (9) (IgG2b; anti-CD47) was kindly provided by Dr. D. Anstee (International Blood Group Reference Laboratory, Bristol, UK) and TS2/9 (IgG1; anti-CD58) was obtained from ATCC. MAb HI172 (IgG1) was obtained as a part of the Non-Lineage Panel of the 6th HLDA Workshop (provided to the Workshop by Dr. De-Cheng Shen, Institute of Hematology, Tianjin, China) and was subsequently identified as an anti-CD47 mAb in our laboratory (K. Drbal, unpublished results). Rabbit antiserum to protein tyrosine kinase (PTK) Fyn was kindly provided by Dr. A. Veillette (McGill University, Montreal, Canada), rabbit antisera to PTK Lyn and to $G_{\alpha\ i\text{-}3}$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mAbs were used either as diluted ascitic fluids or purified by Protein A-Sepharose (Pharmacia) affinity chromatography (IgG) or by precipitation (IgM). Fluorescein isothiocyanate (FITC)-labeled mAbs were prepared in our laboratory by coupling FITC (Sigma) to purified mAbs. FITC-labeled goat $F(ab')_2$ anti-mouse Ig (fluorescein-GAM) used as the secondary antibody in indirect immunostaining was from Jackson Immunoresearch (West Grove, PA, USA). Fab fragments were prepared by papain treatment essentially as described elsewhere (10), undigested antibody and Fc fragments were removed by Protein A–Sepharose and the Fab fragment monomers were finally purified by high-performance gel chromatography (Superdex 200 HR 10/30 column; Pharmacia). FITC-labeled mAb monomers were also purified using gel filtration immediately before use.

Cytofluorometric methods

After 10 min blocking of Fc receptors by 20% human AB serum, cells were incubated on ice with solutions of mAbs (10 µg/ml) in Hanks' balanced salt solution (HBSS) containing 0.2% gelatin and

0.1% NaN_3 without Ca^{2+} and Mg^{2+} (HBSS-GA) for 30 min. After two brief washes (3 min), fluorescein-GAM (10 μg/ml) was added for additional 30 min in indirect immunostaining experiments. In whole blood staining experiments, cells were blocked using 1 mg/ml irrelevant mouse Ig and phycoerythrin-labeled anti-CD45 mAb MEM-28 (10 μg/ml) was added for 20 min. Prior to measurement on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA, USA) propidium iodide (PI) (0.1 μg/ml; Sigma) and LDS-751 (0.2 μg/ml; Exciton, Dayton, OH, USA) were added and 10,000 viable cells (PI-negative) were collected for each sample. 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, USA). In competition (cross-blocking) experiments, cells were first incubated 10 min on ice in a solution of an unlabeled mAb (approx. 0.5 mg/ml); then the tested fluorescein-mAb was added (final concentration 5 μg/ml), the mixture was incubated on ice for further 60 min and, after washing, the cells were analyzed by flow cytometry.

All kinetic studies were done using real-time flow cytometry (11, 12). Cells were suspended in HBSS-GA, equilibrated for 30 min at 4°C or 37°C, and the concentration was adjusted to give an event rate of 200–300 cells per minute at low differential pressure. During the time-course of all measurements, the assay temperatures 4°C or 37°C were carefully maintained by submerging sample tubes in ice contained in an insulated beaker or connecting samples to the water bath at 37°C, respectively. An integrated fluorescence signal (FL1-Area) was acquired at linear setting versus time. Attention was drawn to set the amplifier gain not too high to allow most cells (>95%) to appear on the scale. Only living cells (gated on forward versus side scatter and PI negative) were collected over 512 s. Formaldehyde-fixed cells were gated on forward versus side scatter only without the addition of PI. Non-specific fluorescein-mAb binding and free fluorescein-mAb background was determined in the presence of blocking concentration of MEM-122 mAb (1 mg/ml) and subtracted from total binding to give specific binding. After the initial background detection (30 s) fluorescein-mAb (1.5 μg/ml final concentration) was added with brief mixing and placed immediately back onto the cytometer. The kinetics of mAb binding was monitored with occasional mixing. After 512 s the sample was diluted 10 times and briefly centrifuged, diluted again 10 times (<1 min) and the measurement of dissociation kinetics started for additional 512 s. Finally, all list mode data files were analyzed using WinMDI 2.8 software (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA) and mean fluorescence value over time was saved to tab delimited text file, which was further read into a Microsoft Excel spreadsheet. Background values (autofluorescence and nonspecific fluorescein-mAb solution fluorescence) were subtracted and the graphs were smoothed using moving average trend.

Immunoprecipitation of CD47-associated molecules and *in vitro* kinase assay

The CD47-containing complexes were immunoisolated from mild detergent (Brij-58) lysates of Raji cells using the solid phase immunoisolation technique as previously described (13). The immunoprecipitates obtained in this way in plastic antibody-coated wells were then eluted with non-reducing sample buffer and analyzed by SDS-PAGE and Western blotting. Alternatively, the immunoprecipitates were subjected to a self-phosphorylating *in vitro* kinase assay (without the addition of any exogenous substrate) as described in detail before (13) and the *in vitro* phosphorylated immunoprecipitates were analyzed by SDS-PAGE under reducing conditions followed by autoradiography. *In vitro* kinase assays were performed also on fractions obtained by gel chromatography on minicolumns of Sepharose 4B (see below).

Immunoaffinity chromatography and gel chromatography

Preparation of cell lysates, preparation and detergent solubilization of cell membranes, SDS-PAGE and Western blotting (employing luminographic detection) were all performed as described in detail elsewhere (13, 14). 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 (0.15 M NaCl, 0.1 M Tris/HCl buffer pH 8.2, 1% NP40 and protease inhibitors 5 mM iodoacetamide and 1 mM Pefabloc). Twenty-five μl of detergent lysate of HPB-ALL cells (5×10^7 per ml of the lysis buffer) or erythrocyte membranes were applied at the top of the column, which was then eluted with the lysis buffer (at the rate of 15 μl/min), and eleven 50-μl fractions were collected. All fractions were mixed 1:1 with 2× concentrated non-reducing SDS-PAGE sample buffer and non-boiled samples were analyzed by SDS-PAGE followed by Western blotting.

Gel chromatography on Sepharose 4B was performed on 3-ml columns of gel equilibrated in the appropriate lysis solution, fractions were collected and analyzed by SDS-PAGE followed by Western blotting or *in vitro* kinase assay as described before (13).

Native electrophoresis

Native, non-SDS-PAGE (blue native electrophoresis (BNE)) was performed essentially as described elsewhere (15). Briefly, 5×10^6 cells

were solubilized in 200 μl of the native lysis buffer (1% n-dodecyl β-D-maltoside and 5 mM iodoacetamide and 1 mM Pefabloc in 750 mM aminocaproic acid, 50 mM Bis-tris, pH 7.0), centrifuged for 3 min at 11,000 ×*g* and Coomassie Brilliant Blue G was added to the supernatant to the final concentration of 0.25%. Five-μl volumes of the 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 Immobilon-P membrane and visualized by immunoperoxidase staining. The size standards used were monomers and oligomers (obtained by chemical cross-linking) of bovine serum albumin and mouse IgG1 mAb.

Results

CDw149 mAbs recognize CD47 molecules

Anti-CDw149 mAbs (MEM-120 and MEM-133) recognize a broadly expressed antigen; the expression pattern on blood cells is similar to that of CD47, except that erythrocytes are negative (Fig. 1A). Our repeated attempts at biochemical characterization of the CDw149 antigen have failed – immunoprecipitation from surface biotinylated and iodinated leukocytes yielded repeatedly negative results (data not shown); Western blotting after standard SDS-PAGE detected in some experiments a faint spurious doublet of 120–130 kDa (6). How-

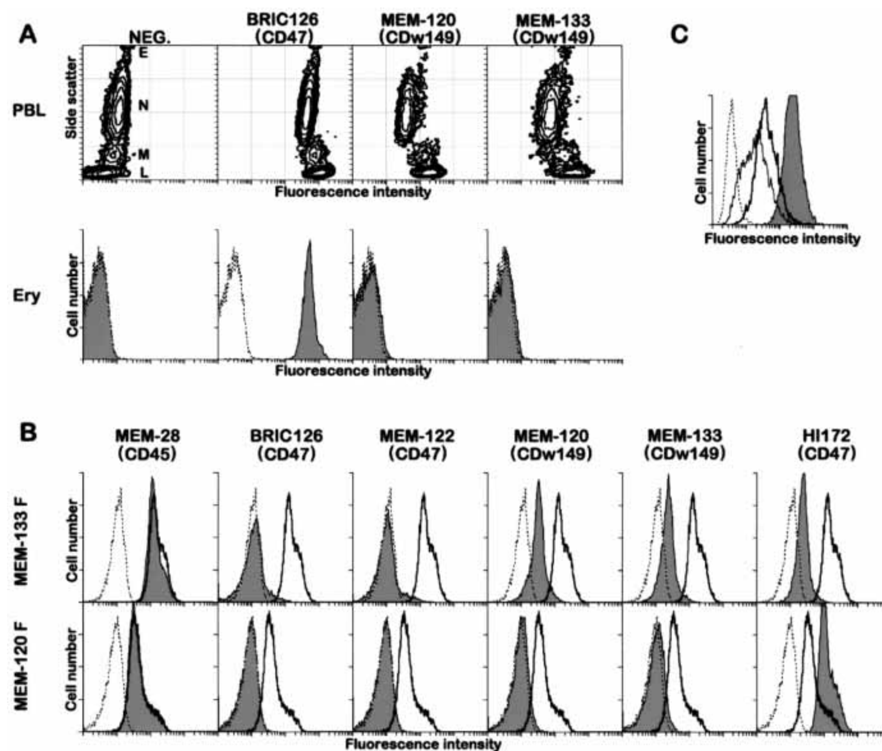

Fig. 1. **Flow cytometric demonstration of the relationship between CD47 and CDw149. A) Reactivity of a standard anti-CD47 mAb (BRIC126) and two anti-CDw149 mAbs (MEM-120 and MEM-133) mAbs with blood cells.** Contour plots in the upper panel show reactivities of the indicated mAbs with peripheral blood leukocytes (L – lymphocytes, M – monocytes, N – neutrophils, E – eosinophils); NEG. represents staining with an irrelevant negative control. Histograms in the lower panel show reactivities on erythrocytes (irrelevant mAb binding shown as dotted line). The leukocytes were mixed with an appropriate number of erythrocytes before adding mAbs to ensure the same staining conditions. As described in Material and methods, the immunostaining was done on ice and all peripheral blood cell populations were resolved during analysis on the basis of CD45 expression, LDS-751 staining and forward vs. side scatter properties. **B) Competition (cross-blocking) of FITC-labeled MEM-133 (MEM-133 F; upper panel) and FITC-labeled MEM-120 (MEM-120 F; lower panel) with other unlabeled mAbs (indicated at the top).** The histograms of peripheral blood leukocytes stained with MEM-133 F or MEM-120 F alone are shown as the solid lines, negative controls (staining with irrelevant isotype matched mAbs) as dotted lines. The shaded peaks represent the staining obtained under the conditions of competition with the respective unlabeled mAb. **C) Reactivity with CD47 transfectants (CD47-OV10).** Negative control (irrelevant mAb) is shown as dotted line, MEM-120 staining corresponds to the solid thin line, MEM-133 staining to the bold line, BRIC126 to the shaded peak.

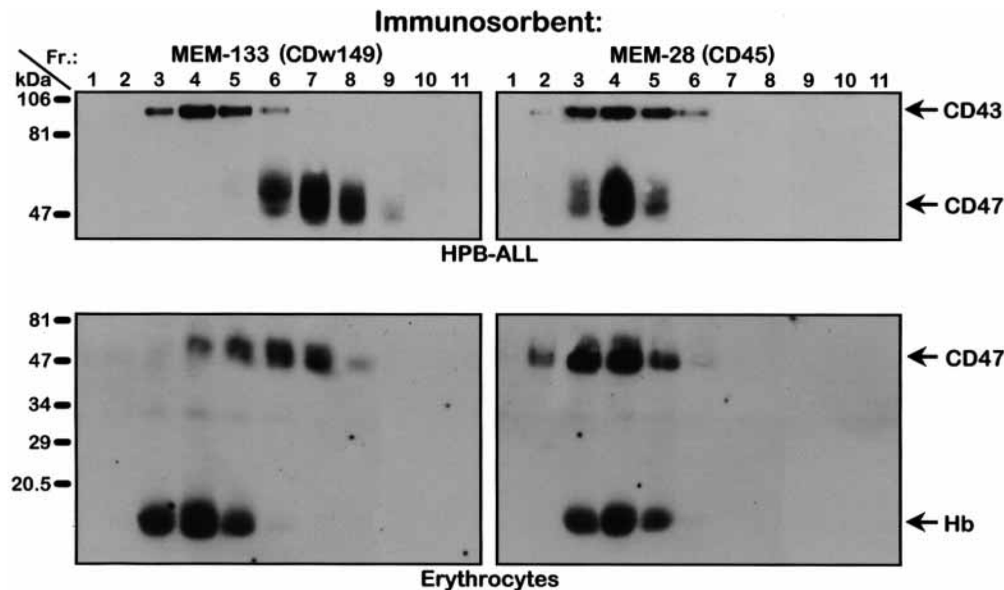

Fig. 2. **Immunoaffinity chromatography on anti-CDw149 immunosorbent.** HPB-ALL cells (upper panel) or erythrocyte membranes (lower panel) were solubilized by a rather stringent detergent (1% NP40) and subjected to immunoaffinity chromatography on a minicolumn made of immobilized mAb MEM-133 (left column) or a control mAb MEM-28 (right column). The fractions collected (numbered at the top) were subjected to SDS-PAGE, electroblotting and immunoperoxidase staining with mAbs to CD47 (mAb BRIC126) and to a control antigen CD43 (mAb MEM-59). Hemoglobin was used as a control in the case of erythrocytes and visualized based on its intrinsic peroxidase activity. The relevant proteins are indicated by arrows.

ever, two standard anti-CD47 mAbs, BRIC126 and MEM-122, strongly blocked the binding of anti-CDw149 mAbs to leukocytes (Fig. 1B) indicating that the CDw149 antigen may actually be a leukocyte-specific form of CD47. Interestingly, one CD47 mAb strikingly enhanced the binding of CDw149 mAbs (Fig. 1B); this phenomenon is discussed below. Anti-CDw149 mAbs bound to CD47 transfectants (Fig. 1C). Reactivity of anti-CDw149 mAbs with the CD47 glycoprotein was further directly confirmed by the results of immunoaffinity chromatography of cell detergent lysates on anti-CDw149 immunosorbent: the CD47 antigen was specifically retarded on the immunosorbent column (Fig. 2). Both leukocyte (HPB-ALL) and erythrocyte CD47 was retarded on the immunosorbent (Fig. 2). It should be noted that CD47 molecules were only retarded by the column and not firmly bound; this indicates a low-affinity, easily reversible, interaction of the immobilized mAb with the solubilized CD47 molecules. The conditions used for solubilization (a rather stringent detergent NP40) dissociated most of the weakly associated protein-protein complexes, and thus the lysates probably contained mainly monomeric CD47 molecules. This behavior was reminiscent of anti-CDw78 mAbs, which were recently demonstrated to be low-affinity mAbs recognizing preferentially a clustered subset of MHC class II molecules (4).

Kinetics of the mAbs binding to the cells

Binding kinetics of both of the anti-CDw149 mAbs MEM-133 and MEM-120, to the Jurkat cells was typical for low-affinity mAbs, and in a clear contrast to a reference high-affinity anti-CD47 mAb MEM-122 (Fig. 3A). At 4°C the anti-CDw149 mAbs bound rapidly only to a fraction of the binding sites and then slowly approached the level of saturation; in contrast, the high-affinity anti-CD47 mAb rapidly saturated all available binding sites. In addition, temperature dependence of the association rate was markedly different for the low-affinity mAbs as compared to the high-affinity one: whereas at 37°C the binding curves were similar for both the low- and high-affinity mAbs, they markedly differed at 4°C (Fig. 3A). These results obviously reflect the fact that at 37°C the membrane is more fluid, diffusional movement of monomeric CD47 molecules is relatively rapid and even low-affinity mAbs have opportunities to bind with high frequency to transiently formed pairs of antigen molecules. At a low temperature, rapid binding of the low-affinity mAb is possible only to a presumed stable clustered (or at least dimerized) subset of the CD47 molecules and transient (accidental) formation of spatially close enough pairs of monomeric molecules, subsequently stabilized by bivalent binding is a slow process. Clearly, stable monovalent

binding of the high-affinity mAb is rapid even at low temperature. The difference in association kinetics of high-affinity vs. low-affinity mAbs is even more striking when their binding is tested to Jurkat T-cells fixed by formaldehyde: whereas no marked difference is observed in the kinetics of high-affinity mAb binding to the fixed vs. unfixed cells at 4°C (stable monovalent binding with slow rate of dissociation), low-affinity mAbs bind only to a minor fraction of presumably clustered antigen on the surface of fixed cells, and the amount of the bound mAb essentially does not increase with time (due to the drastically limited mobility of the CD47 molecules on the surface of fixed cells preventing random formation of transient dimers). In agreement with this idea, a clearly detectable binding of anti-CDw149 mAbs even to erythrocytes could be achieved when the incubation temperature was raised to 37°C (Fig. 3A), which pre-

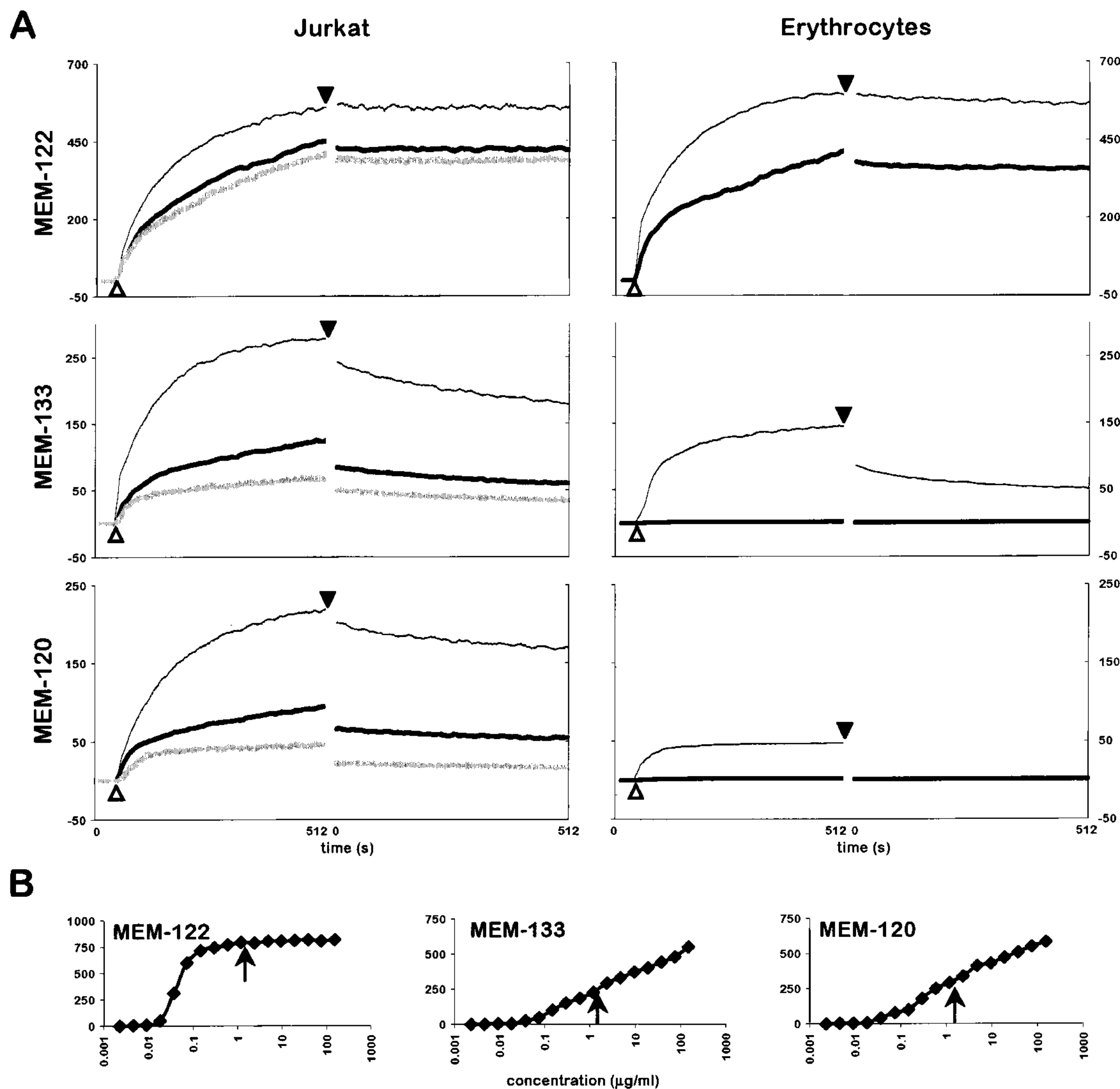

Fig. 3. **Kinetics of the mAbs association and dissociation to/from leukocyte (Jurkat T-cells) and erythrocyte surface. A) Real time flow cytometry (see Material and methods) was used to follow the kinetics of FITC-labeled mAbs (MEM-122 F, MEM-133 F, MEM-120 F) to unfixed Jurkat cells (left column) or erythrocytes (right column) at 4°C (thick black line) or 37°C (thin black line) or to formaldehyde-fixed Jurkat cells at 4°C (thick grey line).** Open arrowheads indicate the moment when the FITC-labeled mAb was added. Closed arrowheads indicate the point when the mAb was removed from the suspension and spontaneous dissociation from the cell surface started. Corrected fluorescence intensities (arbitrary units; y-axis) are plotted versus time (in seconds; x-axis). Note different scales on y-axis for different cells and different antibodies. The absence of the thin line in the dissociation part of the last panel (MEM-120 F on erythrocytes) is due to very rapid and complete dissociation of the mAb under the conditions used. The data shown are from one representative experiment out of three performed. **B) Concentration dependence of the respective mAbs binding to unfixed Jurkat cells at 4°C.** The arrows point to the concentration used for the kinetic experiments.

sumably allowed to a limited extent for random, transient dimerization of the otherwise monomeric erythrocyte CD47 and thus bivalent binding of low-affinity mAbs to these pairs of CD47 molecules. Nevertheless, the rate of dissociation of anti-CDw149 mAbs from erythrocyte surface was high (Fig. 3A). As expected, there was no binding of anti-CDw149 mAbs to formaldehyde-fixed erythrocytes, even at 37°C (not shown).

Finally, as expected for low-affinity mAbs, saturation of anti-CDw149 mAbs binding to the T-cell line surface was difficult to achieve even at high mAb concentration at 4°C (Fig. 3B). Monovalent Fab fragments of CDw149 mAbs did not measurably bind even to leukocytes either at 0°C or 37°C when intact bivalent mAbs did bind (data not shown).

A clustered subset of CD47 molecules is present on leukocytes

Because of these indications that a clustered subset of CD47 molecules may exist on leukocyte but not erythrocyte surface, we tested this hypothesis directly by biochemical methods. A T-cell line and erythrocytes were solubilized in a mild detergent n-dodecyl β-D-maltoside (which is supposed to preserve most protein-protein interactions) and subjected to gel chromatography on Sepharose 4B or to "native" (non-SDS) polyacrylamide gel electrophoresis. Two fractions containing CD47 molecules and differing in size were detected in the T-cell line lysate but not in the erythrocyte membrane lysate (Fig. 4A, B). These results demonstrate that a fraction of leukocyte, but not erythrocyte, CD47 molecules exists which is aggregated or associated with other proteins within large complexes (as indicated by the gel filtration results) or possibly noncovalently dimerized (as indicated by the results of the native electrophoresis).

Association of membrane complexes containing clustered CD47 with cytoplasmic signaling molecules

Cells of the lymphoid cell line Raji were solubilized by a mild detergent Brij-58 (which apparently preserves most complexes based on protein-protein and some protein-lipid and lipid-lipid interactions) and the lysate subjected to immunoprecipitation followed by *in vitro* kinase assay. The immunoprecipitates obtained on immobilized anti-CD47 and anti-CDw149 mAb MEM-133 exhibited a strong protein kinase activity. A similar pattern of *in vitro* phosphorylated proteins was observed in the case of the immunoprecipitate obtained by means of immobilized mAb to CD58, a GPI-anchored protein, as well as to the transmembrane protein CD10 which was previously shown to be a component of large detergent resistant complexes corresponding to glycosphingolipid-enriched membrane domains (GEMs) containing also GPI-anchored proteins and several cytoplasmic signaling molecules (16). In contrast, immunoprecipitates obtained by means of mAbs to a number of other strongly expressed Raji cell membrane proteins were negative (Fig. 5A and data not shown). The complexes containing CD47-associated protein kinase activity were very large as judged by gel chromatography on Sepharose 4B (Fig. 5B). The CD47 immunoprecipitates obtained by means of both standard and low-affinity (anti-CDw149) mAbs contained protein tyrosine kinases Lyn and Fyn and trimeric G-proteins (Fig. 6); the immobilized low-affinity (anti-CDw149) mAb was reproducibly more efficient in immunoprecipitation of these large complexes containing the signaling molecules (see discussion on this phenomenon below).

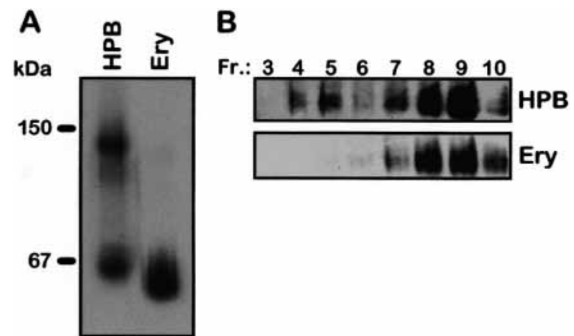

Fig. 4. **Differential arrangement of CD47 molecules in leukocyte vs. erythrocyte membranes. A) T-cells (HPB-ALL) and erythrocyte membranes solubilized in a mild detergent (1% n-dodecyl β-D-maltoside) were subjected to blue native electrophoresis and electroblotting followed by immunoperoxidase staining with the anti-CD47 mAb BRIC126. B) T-cells (HPB-ALL) and erythrocyte membranes solubilized in a mild detergent (1% n-dodecyl β-D-maltoside) were subjected to gel chromatography on Sepharose 4B.** Fractions were analyzed by SDS-PAGE (non-reducing conditions), electroblotting and immunoperoxidase staining with the anti-CD47 mAb BRIC126. Only the relevant parts of the blots are shown (around 50 kDa). Elution volumes of fractions 4, 6 and 8 correspond to the void volume, IgM and IgG, respectively.

Discussion

Our data demonstrate the following: 1) anti-CDw149 mAbs are actually low-affinity mAbs to CD47; 2) the organization of CD47 molecules is different on leukocytes vs. on erythrocytes; and 3) leukocyte surface CD47 molecules are components of large membrane complexes containing cytoplasmic signaling molecules. CD47 appears to be oligomerized on leukocytes but not on erythrocytes, and this "conformational" difference is responsible for the apparent

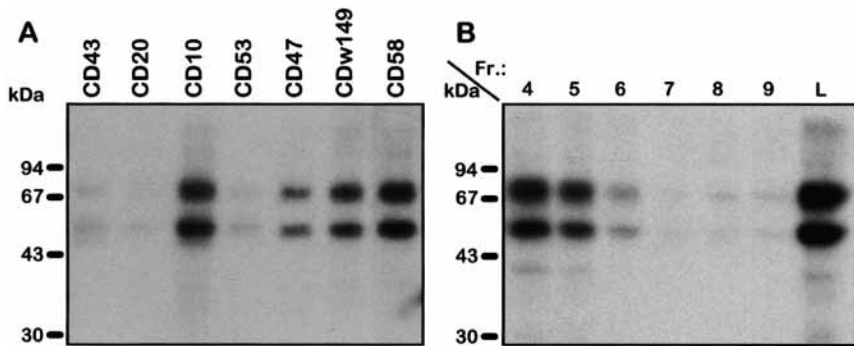

***Fig. 5. In vitro* kinase assays. A) Raji cell lysate (3% Brij-58) was subjected to immunoprecipitation on immobilized mAbs directed to antigens indicated at the top (anti-CD43 – MEM-59; anti-CD20 – MEM-97; anti-CD10 – MEM-78; anti-CD53 – MEM-53; anti-CD47 – BRIC126; anti-CDw149 – MEM-133; anti-CD58 – TS2/9) followed by *in vitro* kinase assay.** The *in vitro* phosphorylated immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Qualitatively similar but weaker profile of *in vitro* phosphorylated proteins was observed also when using other cell lines (not shown). **B) The same cell lysate as in (A) was subjected to gel chromatography on a column of Sepharose 4B.** The fractions (4–9) and lysate (L) were subjected to immunoprecipitation on immobilized MEM-133 followed by *in vitro* kinase assay, SDS-PAGE and autoradiography. Elution volumes of fractions 4, 6 and 8 correspond to the void volume, IgM and IgG, respectively.

difference in CD47 vs. CDw149 expression. It should be noted that the strikingly different reactivity of the low-affinity anti-CD47 (anti-CDw149) mAbs with leukocytes vs. erythrocytes is not due to higher expression of the CD47 antigen on leukocytes – the expression levels (as detected by standard high-affinity mAbs) are essentially identical on both cell types.

The existence of oligomeric or clustered forms of CD47 molecules was directly demonstrated by the results of gel filtration and native electrophoresis of mild detergent lysates (Fig. 4A, B). Although we have not measured rigorously the affinities of the mAb-CD47 interactions, several pieces of evidence strongly suggest that the anti-CDw149 mAbs are low affinity ones: 1) monovalent Fab fragments apparently did not bind to leukocyte surface; 2) kinetics of binding of intact mAbs to cells was characteristic for low-affinity mAbs (and distinct from standard, high-affinity anti-CD47 mAbs); 3) binding to erythrocytes was enhanced by increased temperature (which increases membrane fluidity and mobility of CD47 monomers); and 4) monovalent Fab fragments did not bind even at 37°C (K. Drbal, unpublished data), excluding the possibility that the increased binding at elevated temperature was due to a temperature-dependent intramolecular conformational change. The observed enhancement of anti-CDw149 mAbs binding by a unique anti-CD47 mAb probably indicates that the anti-CD47 mAb (recognizing a different epitope) crosslinked a fraction of CD47 molecules, and this newly formed aggregated fraction was then suitable for stable, bivalent binding by the low-affinity anti-CDw149 mAbs. A similar, apparently paradoxical, enhancement of mAb binding by another mAb was recently observed for CDw78 (4) and for Thy-1 (17). Recently, another anti-CD47 mAb was described which also did not react with erythrocytes under standard conditions (18); it is not entirely clear whether this pattern of reactivity is due to a low affinity or rather due to recognition of a cell-type specific epitope. Affinity of binding, number of binding sites on myeloid cells and erythrocytes and temperature dependence of the binding parameters of standard, high- and medium-affinity anti-CD47 mAbs was thoroughly studied by Rosales et al. (19). Interestingly, these

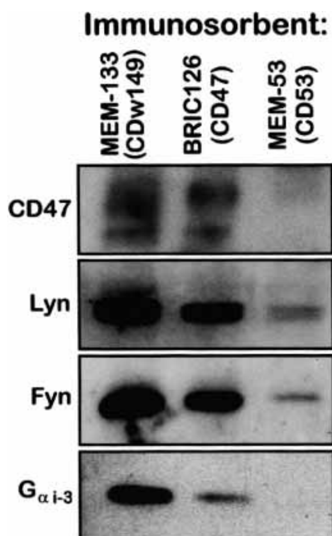

Fig. 6. **Association of cytoplasmic signaling molecules with CD47/CDw149.** Immunoprecipitates obtained from Raji cells solubilized in 3% Brij-58 on the indicated immunosorbents were subjected to SDS-PAGE (non-reduced samples), electroblotting and immunoperoxidase detection of the indicated molecules. Only the relevant parts of blots are shown (corresponding to the molecular mass of the respective protein).

authors found that whole IgG mAbs and $F(ab')_2$ fragments bound mostly bivalently to the cell surface and suggested that CD47 may undergo a temperature-dependent conformational change affecting the affinity of mAb binding. In contrast to these results, our data indicate that a plausible alternative to a conformational change responsible for the increased binding of low-affinity mAbs at 37°C is increased mobility of the antigen and thus increased chance for bivalent binding of the mAbs. Of course, these explanations are not mutually exclusive and critically depend on the nature of the epitopes recognized by the particular mAbs.

The CDw149 mAbs were originally reported to bind under the conditions of Western blotting weakly to a 120–130 kDa antigen (6); in the light of our present data on the nature of the CDw149 epitopes it seems likely that the mAbs either cross-react with an unidentified SDS-denatured protein or recognize an SDS-resistant complex (dimer?) of CD47.

At present it is not clear what is the nature of the CD47 complexes (clusters) detected by us in leukocytes but not erythrocytes; the results of the native electrophoresis (Fig. 4A) are compatible with the existence of CD47 homodimers. It is well established that CD47 is associated with β3 integrins in platelets and placenta (7, 8, 20), and in erythrocytes it is a component of a complex involving Rh proteins (9). This complex was obviously dissociated by the solubilization conditions used for native electrophoresis and therefore only the zone corresponding to monomeric CD47 molecules was detectable.

We demonstrate that the clustered form of CD47 is present in a large complex resistant to mild detergent Brij-58, which contains signaling molecules. CD47 was reported to be associated with G-proteins (21, 22) and this association was suggested to explain the well-established signaling and costimulatory capacity of CD47 (18, 23–28). Moreover, CD47 (IAP) was reported to be associated with Lck in PMA activated but not resting T cells (24) and with Src in platelets (20). Our data indicate that a fraction of CD47 is associated with a larger assembly of signaling molecules involving both heterotrimeric G-proteins and Src-family protein tyrosine kinases. The properties of such a complex (size, *in vitro* phosphorylation pattern, presence of Src-family kinases and G-proteins) are reminiscent of membrane microdomains denoted as GEMs or membrane rafts, which appear to be sites of accumulation of signaling molecules and probably play important roles in immunoreceptor signaling (for review see ref. 29). The major *in vitro* phosphorylated proteins observed in the GEMs immunoprecipitates have been previously identified as Src-family kinases (55–60 kDa) (13) and the the recently cloned transmembrane adaptor protein PAG (70–80 kDa) (30); it is very likely that these are also identical to the major zones observed in the present study (Fig. 5). Very recently, complexes of CD47 with β3 integrins and G-proteins were found in similar detergent-insoluble membrane microdomains of human ovarian carcinoma cells (22). The low-affinity anti-CD47 mAbs may be very useful tools targeting such potentially functionally important multicomponent membrane structures containing CD47 molecules. It should be noted that the low-affinity mAb MEM-133 immunoprecipitated the large, kinase-containing complexes more efficiently than the high-affinity CD47 mAb (Fig. 6). This result is only seemingly paradoxical – in fact, this is due to the fact that under the conditions used, the low-affinity mAb bound sufficiently strongly essentially only the large multivalent complexes while in the case of the high-affinity mAb monomeric CD47 effectively competed with the kinase-rich complexes for binding to the low-capacity immunosorbent. However, it should be noted that the clustering or oligomerization of CD47 molecules as detected by the low-affinity mAbs described here is probably a phenomenon independent from the presence of CD47 in GEMs: large complexes containing CD47 were observed by us even after solubilization of cell membranes by dodecyl-maltoside, a detergent known to disrupt efficiently these lipidic microdomains (Fig. 4B). At present it is not clear whether the oligomerized/clustered fraction of CD47 demonstrated here in Fig. 4 resides within or rather outside of GEMs.

In conclusion, our results demonstrate again that low-affinity mAbs to cell surface antigens may exhibit surprisingly different and specific patterns of reactivity as compared to the mAbs of higher affinity and that they may potentially be useful tools for detection and specific isolation of oligomeric forms of the membrane antigens or of large complexes thereof.

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