CD19 Is Linked to the Integrin-associated Tetraspans CD9, CD81, and CD82*

(Received for publication, May 13, 1998, and in revised form, August 26, 1998)

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The CD19-CD21-CD81 complex regulates signal transduction events critical for B lymphocyte development and humoral immunity. CD81, a molecule with 4 transmembrane domains, member of the tetraspan superfamily, is engaged, together with other tetraspans such as CD9, CD53, CD63, and CD82, in multimolecular complexes containing β1 integrins and major histocompatibility complex antigens. Here we demonstrate that two other tetraspans, CD82 and the early B cell marker CD9, are coimmunoprecipitated with CD19 from Brij97 lysates of B cell lines. Moreover, CD9 was coprecipitated from lysates of purified CD10+ early B cells. These associations were confirmed by the cocapping of CD19 with CD9 or CD82. The CD9/CD81 association was disrupted in the presence of digitonin, contrary to the CD81/CD19 association, indicating that CD9 and CD81 interact with CD19 in different ways. The CD9/CD81 association is also disrupted in the presence of digitonin, suggesting that CD9 associates with CD19 only through CD81. To characterize the regions involved in the CD81/CD19 association, two reciprocal CD9/CD81 chimeric molecules were tested for the association with CD19, but none of them could be coprecipitated with CD19 in digitonin, indicating that the domain of CD81 responsible for its association with CD19 is complex. Finally, engagement of CD9 could induce the tyrosine phosphorylation of different proteins, including CD19 itself, suggesting that the CD9/CD19 association is functionally relevant. Thus, a physical and functional link is formed between the CD19-CD21-CD81 complex and the integrin-tetraspan complexes, which is dynamically modulated in the process of B cell differentiation.

CD19, the earliest cell surface molecule related to B lineage differentiation, has been shown to provide a costimulatory signal for activation through the B cell receptor. Co-ligation of CD19 with the B cell receptor decreases the threshold for antigen-dependent stimulation by at least 2 orders of magnitude (1). The important role of CD19 in B lymphoid lineage is further supported by transgenic experiments. CD19-deficient mice have an important reduction in serum immunoglobulins levels and an impaired response to T cell-dependent antigens (2, 3). Because the ability of CD19-deficient B cells to undergo differentiation, secrete antibodies, and switch isotype in vitro is not overtly impaired, it has been suggested that the hypogammaglobulinemia could result from defects at earlier stages of B cell activation (3, 4). Conversely, mice overexpressing CD19 have a dramatic alteration in B cell development at earlier stages of B cell lineage (18). It has been suggested to be a general regulator of signaling thresholds in B cells (3). One way of CD19 cross-linking to the B cell receptor is through its association with CR2 (complement receptor 2/CD21) after preimmune recognition of an immunogen by the complement system (5). The presence of at least 2 other molecules in the CD19-CD21 complex (6–8), Leu-13/9–27, an interferon-inducible protein and CD81, a member of the tetraspan family, offers alternative or complementary means for the triggering of the CD19 transducing function. Indeed, CD81 mAb stimulates peripheral blood B cell proliferation and induces an increase of intracellular calcium concentrations in the presence of suboptimal concentrations of anti-IgM. CD81 antibodies also inhibit proliferation and trigger homotypic aggregation of B cell lines, as do CD19 mAb (1, 6). The use of chimeric CD19 molecules has provided evidence that CD21, CD81, and Leu-13 are not required for CD19 to trigger a costimulatory signal (9, 10). This is consistent with the fact that CD19 comprises a large, conserved cytoplasmic domain able to bind directly intracellular signaling proteins (3). By contrast, chimeric CD19 molecules unable to associate with CD81 are no longer able to trigger homotypic aggregation, suggesting that CD81 is responsible for this effect (9, 10). On the other hand, a strain of CD81 knock-out mice shows a reduced mobilization of cytoplasmic Ca2+ upon CD19 ligation (11). Altogether, these data suggest that CD81 could partially relay or amplify the signal initiated by CD19.

We and others (12–15) have demonstrated that CD81 is in multiple cell types, a component of large molecular complexes (the tetraspan web) that include such other tetraspans as CD9, CD37, CD53, CD63, and CD82, β1 integrins (in particular αβ1, αβ2, and αβ6), and HLA-DR in B cells. The components of the tetraspan web, tetraspan or non-tetraspan, are recognized by mAbs that induce similar effects such as the inhibition of cell migration in Transwell chambers (12, 16, 17) or homotypic aggregation (6, 17–21).

Lymphoid B cells express the tetraspan molecules CD53, CD81, and CD82 at all stages of ontogeny and CD9 or CD37 at specific stages of differentiation. CD37 is not detected before specific stages of differentiation. CD37 is not detected before the appearance of surface immunoglobulins and is lost upon terminal differentiation into plasma cells. By contrast CD9 is expressed during the early stages of differentiation, lost, and

* This work was supported by the Association Nouvelles Recherches Biomédicales, by the Ligue Nationale Contre le Cancer, by the Associaton pour la Recherche contre le Cancer, and by the Institut de Cancérologie et d’Immuno génétique. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PVDF, polyvinylidene difluoride.
reexpressed after B cell activation. These data raise the question of the roles of CD37 and CD9 in the differentiation of B cells. Because ectopic expression of CD9 stimulates the integrin-dependent migration of the Burkitt cell line Raji on fibroectin and laminin substrates (22), it has been suggested that CD9 could interfere with B cell differentiation by modulating integrin activity. We now demonstrate that together with CD81, 2 other tetraspans, CD9 and CD82, which associate with integrins in B cells (14), also associate with CD19. Consequently, a physical and functional link is formed between the CD19-CD21 complex and the tetraspan web, which is dynamically modulated in the process of B cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Biotin Labeling of mAbs, and Immunofluorescence**—The anti-tetraspan mAbs used for this study were SYB-1 and ALB-6 (CD9) (12), γC11 (CD82) (23), and M38 (CD81) (24). The CD10 mAb ALB-1 (25) and the CD45 mAb ALB-11 were produced in our laboratory. The CD19 mAb B4 was purchased from Coulter (Miami, FL). The anti-phosphotyrosine mAb 4G10 was from UBI (Lake Placid, NY). The FITC-labeled CD40 mAb was from Serotec (Oxford, United Kingdom) and the biotinylated CD38 mAb from L. Chloupek-Dickson (San Jose, CA). An anti-CD9 polyclonal antibody was produced by immunizing rabbits with a synthetic peptide corresponding to a portion of the small extracellular loop of CD9, RFDSTQKSFIEQTN, cross-linked to keyhole limpet hemocyanin. Immunofluorescence and fluorochrome labeling of mAbs were performed as described previously (12). Biotinylation of mAbs with EZ-link-Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) was performed according to the manufacturer's instruction. Flow cytometry analysis was performed with a FacsAlibur (Becton-Dickinson).

**Plasmids**—The CD19 plasmid in pCDM8 was a generous gift from Dr. B. Seed (26). The two chimeric molecules CD81 × 9 and CD9 × 81 have been previously described (17).

**Cells, Cell Culture, and Transfection**—The megakaryocytic cell line HEL and the pre-B cell line NALM-6 cells have previously been described with respect to CD9 association with integrins (12). Daudi and Raji are CD9-negative Burkitt cell lines. COS-7 cells are fibroblast-like kidney monkey cells that strongly express CD9. Monkey CD9 is recognized by most anti-human CD9 mAbs, including the mAb SYB-1 used in this study. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (all from Life Technologies, Cergy-Pontoise, France). Transient transfection of COS-7 cells and stable expression of CD9 and chimeric molecules in Raji and Daudi cells were performed as described previously (14).

Bone marrow samples were obtained from healthy individuals undergoing hip prosthesis surgery. All samples were collected on preservative-free heparin. Mononuclear cells were isolated by density gradi- ent centrifugation on Ficol-Hypaque (density: 1.077 g/ml) (Pharmacia, Uppsala, Sweden). Light-density mononuclear cells were washed several times in phosphate-buffered saline containing 1 mM EDTA to avoid platelet aggregation, resuspended at a concentration of 5 × 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum and incubated overnight in plastic culture flasks at 37 °C with 5% CO_2 in air to remove accessory adherent cells. CD34^+ cells were first recovered for other studies, and the remaining CD10^- cells were purified by the immunomagnetic bead (MACS) technique according to the description of the manufacturer (Miltenyi Biotec, Bergish Gladbach, Germany) by using a combination of biotin-labeled mAb ALB-1 and streptavidin coupled to submicroscopic magnetic beads.

**Cell Labeling, Immunoprecipitation, and Western Blotting**—Surface labeling of cells with EZ-link-Sulfo-NHS-LC-biotin was performed as described previously (14). Labeled or unlabeled cells were lysed in 1.5 ml of lysis buffer containing 1% detergent, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM CaCl_2, 0.5 mM MgCl_2, 0.02% NaN_3, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 10 kallikrein inhibitory units/ml aprotinin. The detergent CHAPS (Sigma) and CHAPS (Boehringer, Meylan, France) were directly dissolved in the lysis buffer. Digitonin (Boehringer Mannheim) was first dissolved in methanol at the concentration of 10% (w/v) and then diluted in lysis buffer without CaCl_2 and MgCl_2. After a 30-min incubation at 4 °C, the insoluble material was removed by centrifugation at 10,000 rpm, and the proteins were immunoprecipitated as described previously (14). They were then separated by 5–15% SDS-polyacrylamide gel electrophoresis under non-reducing conditions to obtain a better separation of CD9 and CD81 on the one hand and of the α and β chains of β1 integrins on the other hand and transferred to a PVDF membrane (Neogen Science Products). Biotinylated mAbs were probed by incubation with a streptavidin-biotinylated horseradish peroxidase complex (Amersham, Rainham, UK). Specific antigens were probed by a combination of biotin-labeled mAb and the streptavidin-biotinylated horseradish peroxidase complex and revealed by chemiluminescence (Neogen Science Products).

**Coating Assay**—The cells were incubated at 4 °C for 30 min with biotinylated CD81 mAb at 20 μg/ml, washed 3 times with RPMI 1640, and then incubated in the presence of Texas Red-labeled streptavidin (Southern Biotechnology Associates Inc., Birmingham, AL) for 2 h at 37 °C in RPMI 1640. They were then washed three times with ice-cold medium and resuspended in cold medium containing 0.1% NaN_3. Other surface antigens were probed with fluorescein-labeled mAbs. Fluorescence staining was visualized using a Leica DMR HC microscope equipped with discriminating sets of excitation and barrier filters. Appropriate controls were run to ensure the specificity of the staining observed with each set of filters.

**Detection of Protein Tyrosine Phosphorylation**—The cells were washed three times in RPMI 1640, resuspended at 10⁶/ml, and incubated for 30 min with 10 μg/ml mAbs at 4 °C. After three washings, they were resuspended at the same concentration and exposed for various times at 37 °C to 20 μg/ml F(ab')_2 goat anti-mouse IgG. The cells were then washed once in ice-cold phosphate-buffered saline containing 1 mM sodium orthovanadate and lysed in a lysis buffer containing 1% Triton X-100, 50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3, 1 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors. The proteins were separated by SDS-PAGE and transferred as described above to a PVDF membrane that was then blocked with 5% bovine serum albumin in TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween 20 (pH 7.6)), incubated with the anti-phosphotyrosine mAb 4G10 (UBI), which was probed with a peroxidase-labeled F(ab')_2 goat anti-mouse IgG and revealed by chemiluminescence.

**RESULTS**

**CD9 Associates with CD19 in Transfected B Cell Lines**—CD81 has been shown to be associated with CD19 in Burkitt B cell lines, such as Raji and Daudi, that do not express CD9. Because CD9 is the tetraspan that has the highest level of homology with CD81, with about 45% identity at the amino acid level, it was interesting to check whether CD9 could also associate with CD19. CD9-transfected Raji (Raji/CD9, Table I) cells that had been surface-labeled with biotin were solubilized in buffer containing different detergents, and the different kinds of complexes were analyzed by immunoprecipitation.

As described previously (14), the pattern of proteins coprecipitated with CD81 or CD9 in the presence of CHAPS or Bri797 is similar and complex (Fig. 1A). In the CD9-transfected Raji cell line, CD9 coprecipitated associated molecules with a higher intensity compared with the CD81 mAb. This is due to the presence of CD9, which hampers the precipitation of CD81 by specific mAbs (14). Some of the proteins coprecipitated have been identified as HLA-DR and integrins (14). Moreover, in CD9-transfected cells, CD9 and CD81 coprecipitated each other in these conditions. The patterns of precipitation were strikingly different after lysis with digitonin, since most of the complexes were disrupted, including the CD9-CD81 complexes.

As described previously, CD19 was not present in CD81

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**Table I**

| Cell line   | CD9 | CD81 | CD9/CD81 |
|-------------|-----|------|----------|
| NALM-6     | ++++| +    | ++       |
| Raji        | --  | ++++ | ++       |
| Raji/CD81   | ++++| +    | ++       |
| Daudi       | --  | ++++ | ++       |
| Daudi/CD9   | ++++| +    | ++       |
immunoprecipitates collected from digitonin extracts (27). By contrast, CD81 (~22 kDa) was present in CD19 immunoprecipitates of digitonin and Brij97 extracts of Raji cells, but not in CHAPS extracts. In accordance with data from the literature (6, 27), CD21 (~140 kDa, unreduced) was clearly immunoprecipitated with CD19 after lysis with digitonin, but was barely detected in the presence of CHAPS or Brij97.

Fig. 1 shows that a band comigrating with CD9 (~24 kDa) is present in CD19 immunoprecipitates from Raji/CD9 cells. This association is specific because it was not observed in the CD45 immunoprecipitate. Neither this 24-kDa protein nor CD81 was coprecipitated by CD19 mAbs in 1% CHAPS. Contrary to CD81, the 24-kDa band was not detected in the CD19 immunoprecipitates collected from digitonin extracts. Similar results were obtained when using CD9-transfected Daudi cells (Daudi/CD9, not shown).

Interestingly, molecules comigrating with \( \beta_2 \) integrins and HLA-DR antigens were also present at low levels in the CD19 immunoprecipitates of Brij97 lysates.

To check that the 24-kDa band coprecipitated with CD19 was actually CD9, immunoblotting were performed. As shown in Fig. 1B, CD19 coprecipitated CD9 after lysing the cells in Brij97 but not in digitonin or CHAPS buffer. This was observed with both Raji/CD9 (Fig. 1B) and Daudi/CD9 cells (not shown).

It was also confirmed by Western blotting that CD19 coprecipitated CD81 in the presence of digitonin or Brij97, but not in the presence of CHAPS (not shown).

**CD9 Associates with CD19 in COS-7 Cells Transfected with CD81** — To check for the specificity of the CD19/CD9 association, we transiently transfected COS-7 cells with CD19. These cells express both CD9 and CD81. The CD19 mAb B4 could precipitate CD9 from Brij97 extracts of CD19-transfected cells but not from untransfected cells (Fig. 2). CD9 was not coprecipitated with CD19 in the presence of digitonin. This also shows that the CD9/CD19 association can occur in a non-lymphoid environment.

**CD9 Associates with CD19 in Pre-B Cells** — CD9 is constitutively expressed at high levels in the pre-B cell line NALM-6 (which does not express CD21). We wanted to determine whether a CD9/CD19 association could be detected in this cell line. Similar to the results obtained with Raji/CD9 and Daudi/CD9 cells, CD19 immunoprecipitated CD81 from both digitonin and Brij97 extracts and CD9 only from Brij97 extracts (Fig. 3). These results were obtained after surface labeling with biotin (Fig. 3A) or after Western blotting with a CD9 mAb (Fig. 3B).

As a control, no CD9 was coprecipitated by the control CD45 mAb.

CD10~+~ early B cells purified from normal human bone marrow express both CD9 and CD19, as shown in Fig. 4A. Fig. 4B shows that, in these cells, CD9 and CD81 are associated. Moreover, the CD19 mAb, but not the CD45 mAb, could also coprecipitate CD9 in Brij97 buffer, as determined by Western blotting (Fig. 4B).

**CD82 Associates with CD19 in Raji Cells** — CD82 is another tetraspan expressed by mature B cell lines. We attempted to determine whether this tetraspan could also associate with CD19. As shown in Fig. 5, CD82 was coprecipitated with CD19 from Brij97 extracts of Raji cells. By contrast, no CD82 could be detected in the CD10 immunoprecipitate.

**CD19 Cocaps with CD9 and CD82** — To demonstrate that the association of CD19 with CD9 and CD82 occurred at the surface of living cells, cocapping experiments were performed. Biotin-labeled CD9 and CD82 mAbs were bound to the cells and cross-linked by Texas Red-labeled streptavidin at 37 °C. The cells were then chilled and treated with FITC-labeled CD19, CD38, or CD40 mAbs. CD19 strongly co-capped with CD9 in Raji/CD9 and NALM-6 cells (Fig. 6). As a control, CD40, in Raji/CD9, or CD38, in NALM-6 cells, did not copac with CD9. Similarly, CD19 was found to co-cap with CD82 in Raji cells. Here again, CD40 did not co-cap with this antigen.

**CD9 Does Not Coprecipitate CD9/CD81 Chimeric Molecules in Digitonin** — The two chimeric CD9/CD81 molecules have previously been described (17). CD9 × 81 is composed of the first half of CD9 joined to the second half of CD81, the junction being located approximately 5 amino acids after the third transmembrane domain of CD9. CD9 × 81 is the reciprocal construction (Fig. 7A). The two chimeric molecules were transfected in Raji cells. CD9 × 81, which is recognized by CD81 mAbs and the polyclonal antibody directed to the short extracellular domain of CD9 associated with CD82, and CD81 × 9, recognized by CD9 mAbs associated with both CD81 and CD82 (data not shown). None of these chimeric molecules were coprecipitated with CD19 in digitonin (Fig. 7B). This suggests that sequences in both halves of the CD81 molecule are involved in the interaction with CD19.

**Ligation of CD9 Induces Tyrosine Phosphorylation in Transfected B Cells** — CD81 has been shown to trigger tyrosine phosphorylation of several proteins in mature B cells (28). Whether CD9 could similarly induce tyrosine phosphorylation of pro-
proteins was investigated by immunoblotting stimulated cell extracts with the 4G10 mAb. Fig. 8 shows that ligation of CD9 on the surface of Daudi/CD9 cells induces the phosphorylation of proteins of apparent molecular masses of about 195, 150, 120, 95, 80, and 72 kDa. The maximum effect was observed after a 1-min incubation. Similar molecules were phosphorylated after engagement of CD81 in Daudi cells. By contrast, in Raji/CD9 cells, only an 95-kDa molecule was phosphorylated after engagement of either CD9 or CD81.

**CD19 Is Phosphorylated following CD9 Engagement**—One of the bands phosphorylated by CD9 and CD81 mAb in Raji/CD9 and Daudi/CD9 has the apparent molecular mass of CD19 (95 kDa, reduced). Moreover, CD19 ligation on the surface of either Raji/CD9 (Fig. 8) or Daudi/CD9 (Fig. 9) cells induced the phosphorylation of this molecule. Because CD19 is phosphorylated after being cross-linked on cell surface (29), we examined whether the 95-kDa molecule phosphorylated after CD9 engagement was indeed CD19. Cell lysates were precleared with a CD19 mAb before Western blot analysis with the anti-phosphotyrosine mAb. As shown in Fig. 9, this led to a marked reduction of this band, but not of other bands. Conversely, analysis of the CD19 immunoprecipitates showed an increase of the level of CD19 phosphorylation following ligation of CD9. By contrast, the CD82 mAb failed to phosphorylate CD19 in Daudi/CD9 cells. That CD9 ligation could induce phosphorylation of CD19 was confirmed in Raji/CD9 cells (data not shown).

**DISCUSSION**

One of the most striking features of the tetraspans is their ability to associate together and with identical non-tetraspan

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**FIG. 3. Immunoprecipitations of proteins from NALM-6 cells.** A, the cells were surface-labeled with biotin before lysis in CHAPS, digitonin, or Brij97 buffers and precipitation as indicated. After under non-reducing conditions and transfer to a PVDF membrane, the precipitated material was revealed by chemiluminescence. The blots reveal in particular the presence of a 24-kDa band comigrating with CD9 in the CD19 immunoprecipitate, but not the CD45 immunoprecipitate of Brij97 extracts. B, immunoprecipitates of unlabeled NALM-6 cells were analyzed by Western blot with a biotin-labeled CD9 mAb (SYB-1). CD19 coimmunoprecipitates CD9 in Brij97 buffer but not in CHAPS buffer.

**FIG. 4. CD9 associates with CD81 and CD19 in purified CD10+ immature B cells.** A, bone marrow CD10+ cells were purified by immunomagnetic separation using a biotin-labeled ALB-1 mAb and magnetic beads coupled to streptavidin and analyzed for the expression of CD9 and CD19 by flow cytometry using the FITC-CD9 mAb SYB-1 and the PE-CD19 mAb B4. B, immunoprecipitates of unlabeled CD10+ cells were analyzed by Western blot with a biotin-labeled CD9 mAb (SYB-1). The two panels are from different experiments and are at different exposures.

**FIG. 5. CD19 associates with CD82 in Raji cells.** Brij97 extracts of Raji cells were immunoprecipitated as indicated and analyzed by Western blot for the presence of CD82 in the different immunoprecipitates.

**FIG. 6. CD19 cocaps with CD9 or CD82.** The capping of CD9 or CD82 was induced by first incubating the cells at 4 °C with biotinylated mAb and then for 2 h at 37 °C in the presence of Texas Red-labeled streptavidin. The cells were then stained at 4 °C with FITC-labeled mAbs directed against CD19 or control mAbs to CD38 or CD40 and visualized by fluorescence microscopy.
molecules, including several β1 integrins (in particular α9β1, α4β1, and α6β1), HLA class II in B cells (13–15, 30), and CD4 or CD8 in T cells (24). These complexes are of high molecular weight as determined by cross-linking and continuous sucrose gradients (15), and they contain several tetraspans, including multiple copies of one given tetraspan (14). The complexes may contain several types of non-tetraspan molecules, for instance β1 integrins and HLA-DR antigens (14). When a tetraspan is expressed after transfection, it is apparently included into pre-existing complexes (14). These features, together with the fact that mAbs directed to tetraspans and associated molecules produce similar effects (17), indicate the existence on cell surface of macromolecular complexes that we call “the tetraspan web” (14).

The CD81 tetraspan belongs in B cells to another molecular complex whose central element, CD19, plays a role in the regulation of B cell activation (3, 5). It is not known whether, in B cells, CD81 is included into two different types of complexes or whether larger types of complexes are formed by the combination of these complexes. In favor of this latter hypothesis, we provide evidence that two other tetraspans, CD9 and CD82, are associated with CD19 in B cell lines. Moreover, CD9 is associated with CD81 and CD19 in purified CD10− early B cells.

CD9 and CD81 interact with CD19 in different manners since the CD19-CD9 complex is disrupted in the presence of digitonin, but not the CD19-CD81 complex (Figs. 1 and 3). It is possible that the association of CD19 with CD9 is weaker than with CD81. Alternately, CD9 could associate indirectly with CD19, whereas CD81 has been proposed to interact directly (3). In favor of this latter hypothesis, it should be noted that the digitonin buffer appears to be more stringent since much fewer molecules are coprecipitated with CD9 or CD81 in these conditions, compared with Brij97 or CHAPS extraction buffers. Interestingly, the CD9/CD81 association is disrupted in digitonin, which further argues for an indirect CD9/CD19 association, possibly mediated through CD81. Surprisingly, none of the two reciprocal CD9/CD81 chimeric molecules transfected in Raji cells could be coprecipitated with CD19, suggesting that the CD81/CD19 association requires sequences present into both halves of the CD81 molecule. It has been previously demonstrated that the interaction of CD81 with CD19 only required the extracellular domain of CD19 (10). It is therefore possible that both the short and the large extracellular domains of CD81 are involved in the association with CD19.
Only a fraction of CD9 or CD82 was coprecipitated with CD19 in Brij97. This may be partly explained by the fact that CD9 is expressed at a higher level than CD19 in the cell lines tested. Moreover, it is likely that partial disruption of the complexes occurs during the lysis with Brij97. With this respect, CD21 was barely detectable in the CD19 immunoprecipitates collected in these conditions (Fig. 1). It should also be noted that CD19 failed to coprecipitate CD81 in the presence of CHAPS (Figs. 1 and 3). That CD19 could associate with CD9 and CD82 was confirmed by capping experiments. Interestingly, after the induction of capping by CD9 or CD82 mAbs, the CD19 almost completely colocalized with these antigens (Fig. 6), suggesting that the majority of CD19 molecules is associated with CD9 and CD82.

We have previously demonstrated that several molecules associated with tetrascans, i.e. HLA-DR and β2 integrins, were present in the same complexes (14). The data reported in this work show that proteins migrating with integrins and HLA-DR are coprecipitated with CD19 from Brij97 lysates (Fig. 1). It is interesting to note that the coprecipitation of these molecules was not observed in conditions that disrupt the association of CD19 with the tetrascans web, in the presence of CHAPS or digitonin. This suggests that CD19 associates with these molecules through the tetrascans web, which probably explains why they are detected at low levels in CD19 immunoprecipitates. An association between HLA-DR and CD19 is suggested by the capping of these two molecules (6) and is strengthened by the fact that anti-HLA-DR mAbs produce the same effects as mAbs to CD19, including homotypic aggregation and inhibition of proliferation of B cell lines (6, 21). CD19 has been reported to associate with VLA-4 and VLA-5 integrins (31), an association that is functionally relevant since CD19 mAbs could induce the adhesion of B cells to the interfollicular stroma of frozen tonsil by a mechanism involving VLA-4 (32). Reciprocally, integrin cross-linking through specific antibody homomucroseptides activates CD19-associated protein-tyrosine kinases and induces the phosphorylation of CD19 itself in mature and pre-B cell lines (31). Our data demonstrate that CD9 or CD82 engagement induces similar patterns of tyrosine phosphorylation, one of the phosphorylated proteins being CD19. This is the first demonstration that CD9 engagement can induce the activation of tyrosine kinases in nucleated cells.

In the absence of known function for tetrascans and of identified soluble extracellular ligands or counterreceptors, their ability to form organized complexes has led to the suggestion that they could be surface organizers (14), or facilitators (33), interconnecting other surface molecules. Indeed, chimeric CD19 molecules that do not associate with CD81 were no longer able to deliver a signal leading to homotypic aggregation (10), suggesting that such a signal is transmitted to the cell through CD81. In addition, if the studies of several CD81-deficient mice have demonstrated that the initial signals following BCR engagement do not necessitate CD81 (11, 34, 35), it was shown in one of these reports that CD81-deficient B cells had an impaired calcium mobilization in response to CD19 mAbs (11). Although the possibility that this defect could be due to a lower expression of CD19 was not ruled out, it is possible that a part of this calcium mobilization requires the presence of CD81. An interesting hypothesis is that in the absence of CD81, CD19 begins to act as a link to the tetrascans web and therefore cannot recruit other molecules necessary for the full signal.

CD19 has been proposed to regulate signaling thresholds for transmembrane signal, in particular those mediated through the B cell receptor (3, 5). The effects of hyperexpression or inactivation of CD19 indicate that it plays a critical role during the selection process of B cells, by tightly regulating the B cell receptor function (2–4). Its role at earlier stages is poorly understood and it may just anticipate the appearance of the B cell receptor. However, CD19 has been shown to be associated in early B cells with β2 integrins (31) whose role in B cell differentiation is suggested by several experiments. First, it was shown that B cell precursor growth on stromal cells can be inhibited by VLA-4 mAbs in vitro (36, 37). Second, chimeric mice originating from RAG-1−/− or RAG-2−/− blastocysts injected with ES cells deleted for β2 or α5 integrins have a dramatic deficiency of B cells in the bone marrow, blood, and lymphoid organs (38, 39). Since the engagement of VLA-4 and VLA-5 induces the phosphorylation of CD19 (and associated tyrosine kinases), it is possible that CD19 mediates signals triggered by these integrins during early B cell differentiation. Our results suggest that CD81 could be responsible for the connection of CD19 to the tetrascans web and its associated molecules such as integrins. The changes in tetrascans expression during B cell differentiation, in particular with the disappearance of CD9, concomitant to the expression of surface immunoglobulins, may modify the way CD19 and integrins are connected. This may modulate the cross-talk between CD19 and integrins.

Acknowledgments—We are grateful to Dr. Bréard, Dr. Conjeaud, Dr. Seed, and Dr. Yoshie for the generous gift of DNA and antibodies. We thank Dr. Bené for critical review of the manuscript.

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