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# Molecular and biological characterization of fusion regulatory proteins (FRPs): anti-FRP mAbs induced HIV-mediated cell fusion via an integrin system

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**Anti-FRP mAbs induced polykaryocyte formation of U2ME-7 cells ($CD4^+$U937 cells transfected with the HIV gp160 gene). Anti-FRP-1 mAb immunoprecipitated gp80-85, gp120 and homodimers of these peptides, and anti-FRP-2 mAb reacted with gp135 identically to the $\alpha 3$ subunit of integrin. Both anti-FRP-1 and anti-FRP-2 mAb-induced cell fusion was blocked by anti-$\beta 1$ integrin antibody, fibronectin or inhibiting anti-FRP-1 antibody. Therefore, anti-FRP mAbs were thought to induce the fusion via an integrin system(s). FRP-mediated fusion was temperature, cytoskeleton, energy and $Ca^{2+}$ dependent. These experiments showed a possible regulatory function of cell fusion by an integrin system(s).**

*Key words:* fusion regulation/HIV/integrin

## Introduction

Membrane fusion is an important event in the functioning of a living organism (Burger and Verkleij, 1990; White, 1990). Life starts as a sperm fuses with the membrane of an egg, leading to its fertilization. Membrane fusion is also required for myogenesis and osteogenesis. Furthermore, exocytosis, endocytosis, organelle formation and intracellular organelle traffic are intimately related to membrane fusion. However, giant multinucleated cells are not found physiologically except for osteoclasts, myotubes, etc. Giant multinucleated cells are associated with granulomatous lesions formed in response to foreign bodies, viruses and bacteria. Cell surface membrane adheres closely to cell surface membranes of adjacent cells and there are exchanges of some molecules between neighboring cells. Nevertheless, these cells physiologically show no cell fusion, although aged culture cells often show polykaryocytes, suggesting the existence of a mechanism(s) regulating cell fusion.

Syncytium formation is induced by many enveloped viruses including paramyxoviruses, indicating that mechanisms preventing cell fusion are readily overcome by virus infection (Burger and Verkleij, 1990; White, 1990). We reported previously that anti-$L_{929}$ cell antisera enhanced syncytium formation in Newcastle disease virus (NDV)-infected $L_{929}$ cells and suggested the presence of factor(s) on the cell surface of host cells capable of regulating virus-induced cell fusion (Ito *et al.*, 1987b). We have recently isolated monoclonal antibodies (mAbs) which enhance cell fusion in NDV-infected human cells (Ito *et al.*, 1992). These mAbs immunoprecipitated gp80 or gp135, both of which were detected on the cell surface of HeLa cells. These molecules had the ability to regulate NDV-induced cell fusion, and thus were designated as fusion regulatory proteins (FRPs)-1 and -2, respectively. However, NDV-infected HeLa cells showed cell fusion at 24 h post-infection without anti-FRP antibody, although the cell fusion appeared in the presence of anti-FRP antibody by 12 h post-infection. The spontaneous cell fusion of NDV-infected HeLa cells raised difficulty in further investigating the roles of FRP in cell fusion.

Human immunodeficiency virus (HIV) is the etiological agent of acquired immunodeficiency syndrome (AIDS) (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984) which infects mainly helper T lymphocytes and cells of monocyte-macrophage lineage expressing the CD4 cell surface glycoprotein (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1988). The CD4 molecule serves as the receptor for HIV. The major virus envelope glycoprotein, gp120, attaches to CD4 molecules expressed on the host cell surface. After binding to CD4 on the target cells, HIV is internalized via direct, pH-independent fusion of the viral and cell membranes (Stein *et al.*, 1987; McClure *et al.*, 1988). However, attachment of HIV to CD4 on the target cells is not sufficient for the fusion (Somasundaran and Robinson, 1987; Hildreth and Orentas, 1989; Chesebro *et al.*, 1990; Valentin *et al.*, 1990). Although mouse cells expressing human CD4 bind HIV, they do not become infected, apparently because of a block in membrane fusion (Maddon *et al.*, 1988). The results suggest that other cellular factor(s), in addition to CD4, may play a role in the internalization of HIV. These factors could be either helper molecules expressed in human cells or inhibitory components present in mouse cells. Syncytium formation is also induced by interaction of the gp160-expressing cells with neighboring cells bearing surface CD4 molecules. Syncytium formation and subsequent generalized cell fusion have been reported as a potentially important mechanism of virus-induced cytotoxic effects (Lifson *et al.*, 1986a,b; Sodroski *et al.*, 1986). Ashorn *et al.* (1990) have recently reported that there may be a specific surface component other than CD4 that must interact with the HIV envelope glycoprotein in order for membrane fusion to occur. $\alpha$ and $\beta$ subunits of LFA-1 were found to be related to HIV-mediated cell fusion (Hildreth and Orentas, 1989; Valentin *et al.*, 1990). This conclusion was derived from experiments that HIV-mediated cell fusion did not occur in the presence of either anti-LFA-$1_\alpha$ or anti-LFA-$1_\beta$ antibody. However, antibody shows the

suppressive effects not only directly, but also indirectly, that is, a steric hindrance. Furthermore, since syncytium formation is a complex process, if any step in the process is disturbed directly or indirectly, syncytium formation is blocked. On the other hand, induction or facilitation of cell fusion is considered to be a more specific event. Consequently, if fusion-inducing or -enhancing antibody is isolated, novel and important information about the regulatory mechanism of cell fusion can be obtained.

U2ME-7 cell is a $CD4^+$U937 cell line transfected with the HIV gp160 gene, and the expression of gp160 is induced by cadmium chloride (Koga *et al.*, 1990). However, at no time after induction of HIV gp160 was formation of synctia found (Koga *et al.*, 1990). In this study, whether anti-FRP antibodies could induce HIV-mediated syncytium formation was investigated using U2ME-7 cells. When U2ME-7 cells treated with cadmium chloride ($Cd^+$U2ME-7 cells) were cultured in the presence of mAb directed against FRP-1 or -2, syncytial cells were induced. In addition, FRP-2 proved to be identical to the integrin $\alpha 3$ subunit of very late activation antigen 3 (VLA-3). On the other hand, the N-terminal amino acid sequence of FRP-1 showed no homology with any integrin subunit previously reported, but an integrin system(s) was found also to be involved in induction of the cell fusion mediated by FRP-1.

## Results

### *Detection of FRP-1 and FRP-2 on the surface of HeLa and U2ME-7 cells*

In our previous study (Ito *et al.*, 1992), FRP-1 and FRP-2 were found on the surface of HeLa cells. First of all, to determine whether FRP-1 and FRP-2 were also detected on the surface of U2ME-7 cells, unfixed cells were immunostained using purified mAbs directed against FRP-1 or FRP-2. These mAbs reacted with the corresponding molecules on the cell surface of U2ME-7 cells, although staining of FRP-2 was carried out using a higher concentration of the antibody (data not shown). These findings were confirmed by FACS flow cytometric analysis using a saturating amount of each antibody (Figure 1), indicating that expression of FRP-2 was lower than that of FRP-1. The expression of Fc receptor could not be detected on U2ME-7 cells by flow cytometric analysis (data not shown).

Subsequently, cell lysates of HeLa, U2ME-7 or $Cd^+$ U2ME-7 cells labeled metabolically with [$^{14}$C]glucosamine were immunoprecipitated by anti-FRP-1 mAbs. Two or three glycoproteins [gp80 (gp75, gp85), gp120] of HeLa cells (Figure 2A and B) and a broad band (gp80–gp100) of U2ME-7 cells (Figure 2C) were detected under reducing conditions, although gp120 was occasionally not found. Furthermore, SDS–PAGE of material immunoprecipitated from $^{125}$I-surface-labeled HeLa cells with anti-FRP-1 mAbs revealed three protein bands at 120, 80 and 37 kDa under reducing conditions (Figure 2D), and four protein bands at 120, 105, 90 and 37 kDa were found under reducing conditions in $^{125}$I-labeled U2ME-7 cell lysates. When these immunoprecipitates were analyzed under non-reducing conditions, bands showing more diffuse, 37 kDa polypeptide and higher molecular weight proteins such as 240 kDa protein could be detected (Figure 2B and D). The 37 kDa polypeptide was precipitated from [$^{35}$S]methionine-labeled HeLa cell lysates by mAb 4-5-1 or 6-1-13, but was not found

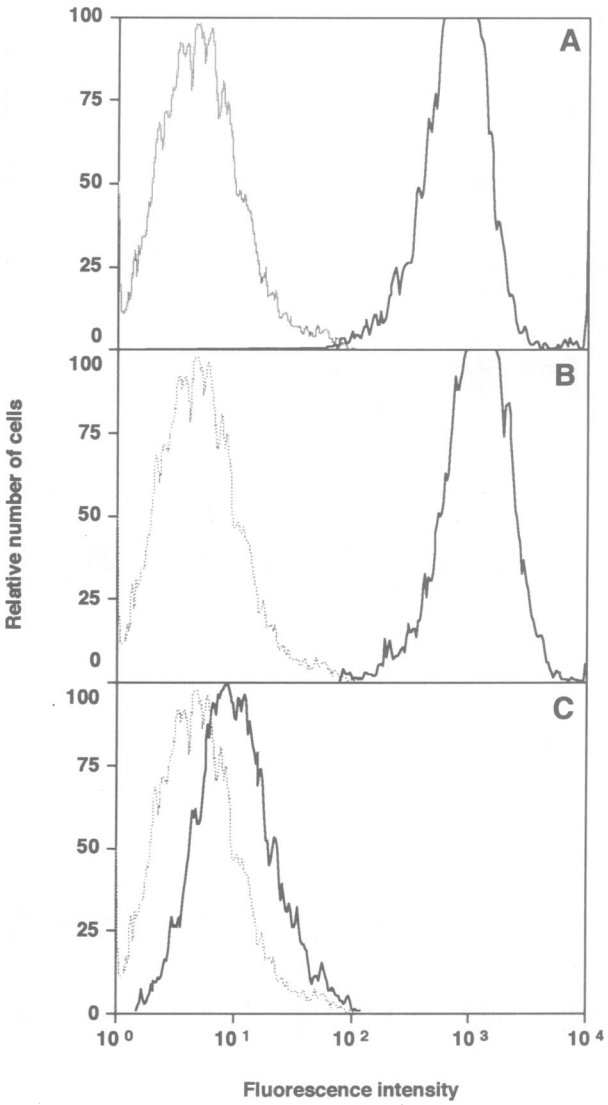


**Fig. 1.** Unfixed $Cd^-$U2ME-7 cells were immunostained with saturating amounts of purified mAb 4-5-1 (**A**), 6-1-13 (**B**), 7-2-1 (**C**) or 25A (control: dotted lines), and analyzed by cytofluorometry. Saturating amounts of anti-FRP mAbs were determined as follows: U2ME-7 cells were stained using 500, 250 or 125 μg/ml of purified mAbs 4-5-1, 6-1-13, 7-2-1 or 25A (isotype-matched control antibody) and FITC-conjugated anti-mouse IgG antibody. Fluorescence intensity scarcely varied with these concentrations of each mAb. Therefore these concentrations were considered to be saturating amounts. Staining patterns using 500 μg/ml of each mAb are shown.

in the immunoprecipitates of [$^{14}$C]glucosamine-labeled lysates (Figure 2B; data not shown), suggesting that the 37 kDa polypeptide is non-glycosylated. To analyze these polypeptides further, two-dimensional SDS–PAGE was carried out using materials immunoprecipitated from [$^{14}$C]glucosamine-labeled HeLa cells with mAb 6-1-13. The electrophoresis in the first dimension (horizontal) was carried out without 2-mercaptoethanol, while the samples were reduced prior to electrophoresis in the second dimension (vertical), indicating that four forms, i.e. gp80 (gp75, gp85), gp120, disulfide-linked oligomers (dimer, trimer and polymer) of gp80 and the disulfide-linked dimer of gp120 were detected (data not shown). Furthermore, two-dimensional Western blotting analysis was used to determine which form(s) reacted directly with the mAb, showing that

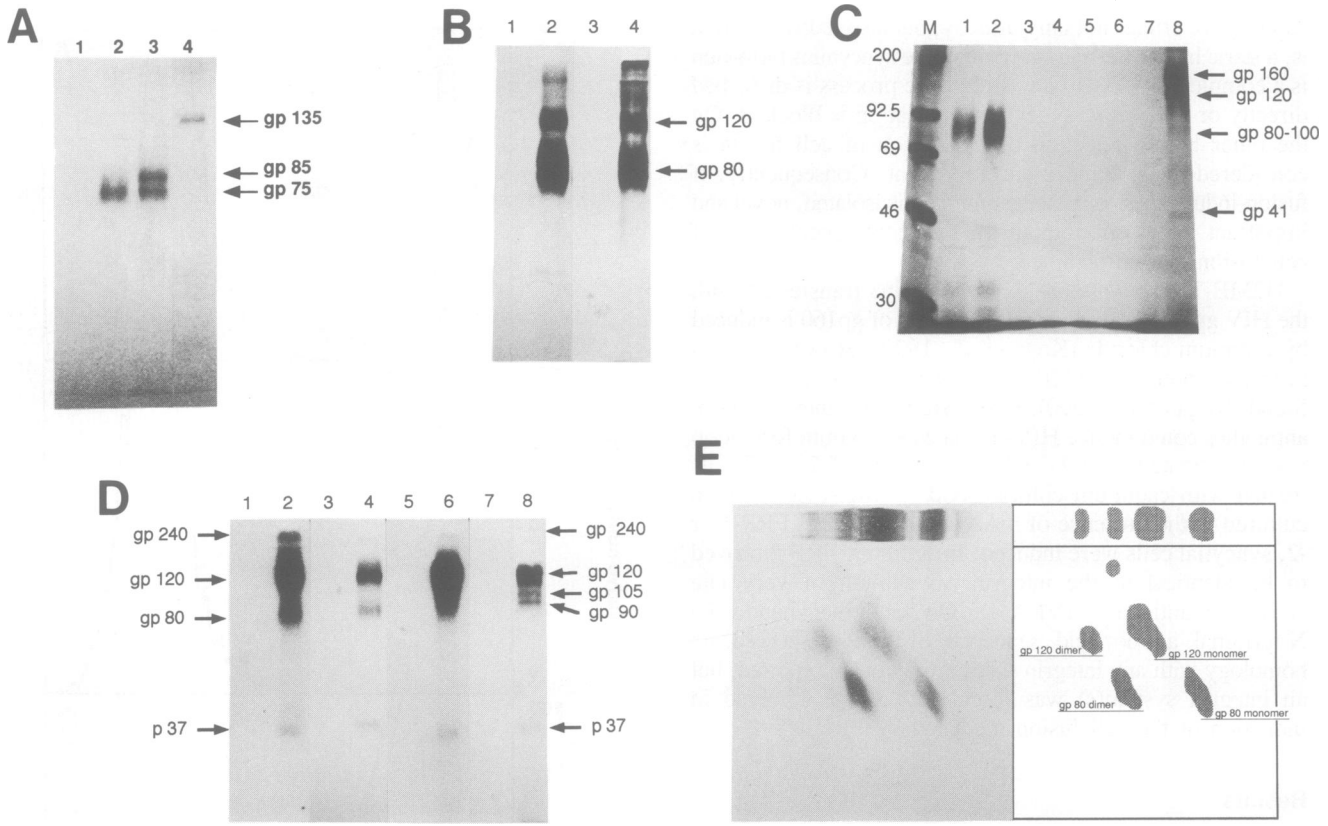


**Fig. 2.** Immunoprecipitation of FRP-1 and FRP-2 from HeLa or U2ME-7 cells. **(A)** HeLa cells were incubated with [$^{14}$C]glucosamine (10 $\mu$Ci) for 24 h and the cell lysates were immunoprecipitated with mAb against mumps virus (control, lane 1), mAb 4-5-1 (lane 2), 6-1-13 (lane 3) or 7-2-1 (lane 4). The immunoprecipitates were analyzed under reducing conditions by SDS−PAGE. **(B)** HeLa cells were incubated with [$^{14}$C]glucosamine (10 $\mu$Ci) for 3 days, and the cell lysates were immunoprecipitated with mAb against mumps virus (lanes 1 and 3) or mAb 6-1-13 (lanes 2 and 4). The precipitates were analyzed under reducing (lanes 1 and 2) or non-reducing conditions (lanes 3 and 4) by SDS−PAGE. **(C)** Either Cd$^-$U2ME-7 (lanes 1, 3, 5 and 7) or Cd$^+$U2ME-7 cells (lanes 2, 4, 6 and 8) were incubated with [$^{14}$C]glucosamine (10 $\mu$Ci) for 8 h, and the cell lysates were immunoprecipitated with mAb against FRP-1 (6-1-13, lanes 1 and 2), FRP-2 (7-2-1, lanes 3 and 4), CD4 (Leu3a, lanes 5 and 6) or gp120 of HIV (KD1053, lanes 7 and 8). The immunoprecipitates were analyzed by SDS−PAGE. M, molecular markers. **(D)** Either HeLa (lanes 1, 2 3 and 4) or U2ME-7 cells (lanes 5, 6, 7 and 8) were $^{125}$I-surface-labeled and extracts were immunoprecipitated with mAb against parainfluenza type 4A virus (control, lanes 1, 3, 5 and 7) or FRP-1 (6-1-13, lanes 2, 4, 6 and 8). The immunoprecipitates were analyzed under non-reducing (lanes 1, 2, 5 and 6) or reducing conditions (lanes 3, 4, 7 and 8) by SDS−PAGE. **(E)** Two-dimensional Western blotting analysis. Purified membrane fraction of HeLa cells was separated by two-dimensional LDS−PAGE (the first dimension, non-reducing; the second dimension, reducing) and blotted to a nitrocellulose membrane. The membrane was immunostained with mAb 6-1-13.

all of these four forms were recognized by the anti-FRP-1 mAb (Figure 2E). Intriguingly, only homodimers were found and the heterodimer (gp80−gp120 dimer) could not be detected (Figure 2E). Remarkably, p37 protein was not recognized by anti-FRP-1 mAb, suggesting that p37 and FRP-1 molecules were antigenically unrelated and both molecules were physically associated.

One band (gp135) was found in the immunoprecipitate of [$^{14}$C]glucosamine-labeled or $^{125}$I-labeled HeLa cell lysate by anti-FRP-2 mAb (7-2-1) (Figure 2A; and data not shown), but this band could not be detected in U2ME-7 cells under the above conditions (Figure 2C). gp160, gp120 and gp41 of HIV were expressed in only Cd$^+$U2ME-7 cells (Figure 2C), but CD4 molecule could not be detected in this experiment (Figure 2C).

### *Polykaryocyte formation of Cd$^+$U2ME-7 cells cultured in the presence of anti-FRP mAbs*

The effect of mAbs directed against FRP-1 and FRP-2 on HIV gp160-mediated polykaryocyte formation was examined using U937-2 and U2ME-7 cells (Table I and Figure 3). Even if U937-2 cells were treated with cadmium chloride and any mAb, syncytia were not induced, although these mAbs could induce cell agglutination (Figure 3C). U2ME-7 cells either with or without cadmium chloride showed neither syncytium formation nor cell agglutination (Figure 3A and B), that is, gp160 expression was induced in Cd$^+$U2ME-7, but polykaryocytes did not appear. Cd$^-$U2ME-7 cells showed cell agglutination, but no cell fusion in the presence of mAb against FRP, 4-5-1, 6-1-13 or 7-2-1. When mAb 4-5-1, anti-FRP-1 antibody, was added to the culture fluid of Cd$^+$U2ME-7 cells, cell agglutination and syncytium formation were induced (Figure 3D and I). Within 5 min, the cells began to form small clusters and cell agglutination reached its maximum level within 1−2 h of incubation. Furthermore, polykaryocytes began to appear at ~10 h and increased until ~15 h of incubation with mAb 4-5-1. However, mAb 6-1-13, another anti-FRP-1 antibody, induced cell agglutination, but not cell fusion in Cd$^+$U2ME-7 cells (Figure 3E), showing that the cell fusion is not a direct result of cell agglutination. mAb 6-1-13-induced aggregates became loose at 2−3 h after incubation (Figure 3F). mAb 7-2-1, anti-FRP-2 antibody, also induced cell agglutination and polykaryocytes in Cd$^+$U2ME-7 cells (Figure 3G). It is

**Table I.** Cell agglutination and syncytium formation of U2ME-7 cells

| Cells | $Cd^{2+}$ | Antibody | Cell agglutination | Syncytium formation |
|---|---|---|---|---|
| U937-2 | − | − | − | − |
| U937-2 | + | − | − | − |
| U937-2 | − | 25A | − | − |
| U937-2 | − | 4-5-1 | ++++ | − |
| U937-2 | − | 6-1-13 | ++++/+ | − |
| U937-2 | − | 7-2-1 | ++ | − |
| U937-2 | + | 25A | − | − |
| U937-2 | + | 4-5-1 | ++++ | − |
| U937-2 | + | 6-1-13 | ++++/+ | − |
| U937-2 | + | 7-2-1 | ++ | − |
| U2ME-7 | − | − | − | − |
| U2ME-7 | + | − | − | − |
| U2ME-7 | − | 25A | − | − |
| U2ME-7 | − | 4-5-1 | ++++ | − |
| U2ME-7 | − | 6-1-13 | ++++/+ | − |
| U2ME-7 | − | 7-2-1 | ++ | − |
| U2ME-7 | + | 25A | − | − |
| U2ME-7 | + | 4-5-1 | ++++ (67 ng)[a] | ++++ (269 ng)[a] |
| U2ME-7 | + | 6-1-13 | ++++/+ (332 ng)[a] | − |
| U2ME-7 | + | 7-2-1 | ++ (494 ng)[a] | +++ (2 μg)[a] |
| U2ME-7 | + | anti-CD4 (Leu3a) | − | − |
| U2ME-7 | + | anti-HIV gp160 (0.5β) | − | − |

For these experiments, the cell number of each cell population was adjusted to $1 \times 10^6$ in 1 ml of DMEM supplemented with 10% FCS. These cell suspensions were put into 96-multiwell dishes either with or without cadmium chloride (10 μM). Antibodies other than anti-FRP mAbs were used at a dilution of 1:25−1:100. Each purified antibody was added into culture fluids of these cells, the cells were observed with microscopy at appropriate periods, and at 20 h after incubation, final judgement was carried out. Cell agglutination scores ranged from − to ++++ where − indicates that essentially no cells were aggregated in clusters, + that <30% of the cells were found in loose clusters, ++ that <80% of the cells formed loose clusters, +++ that >80% of the cells were found in compact aggregates and ++++ that >90% of the cells formed large compact clusters. Cell fusion scores also ranged from − to ++++ where − indicates no detectable syncytia, + that the diameter of syncytium was <30 μm, ++ a diameter of 30−60 μm, +++ a diameter of 60−80 μm and ++++ indicated a syncytium diameter >80 μm. To judge degrees of cell agglutination or cell fusion, total fields in two wells were surveyed by two or three observers. When mAb 6-1-13 was added to culture fluids of either U937-2 or U2ME-7 cells, strong cell agglutination was induced within 1−2 h, but these aggregates became loose at 2−3 h after incubation. Isotypes of mAb 4-5-1, 6-1-13, 7-2-1, 25A, Leu3a and 0.5β are identical, IgG1.

[a]Minimum concentrations showing fusion or cell agglutination-inducing activity. When a lot of other control mAbs including anti-class I MHC, -α3 (P1B5), -α4, -α5, -β1, -β2 and -β3 subunits of integrin, anti-CD11a, -CD11b, -CD44 and -HIV gp120 (A4-33, KD1053) antibodies were added to culture fluids of $Cd^+$U2ME-7 cells, none of them other than anti-integrin α5 antibody induced cell agglutination or cell fusion. About 10-fold higher concentrations than anti-FRP-1 mAb 4-5-1 were required for induction of cell agglutination or cell fusion by anti- FRP-2 mAb 7-2-1. However, it could be concluded on the following bases that induction of cell agglutination and cell fusion by mAb 7-2-1 was specific. (i) 2 μg or 494 ng/ml, minimum concentration showing fusion or cell agglutination-inducing activity of mAb 7-2-1, respectively, is not an extraordinarily high concentration. (ii) A lot of isotype (IgG1)-matched control antibodies showed neither cell agglutination nor cell fusion at any concentration. The maximum concentrations used in this experiment were 20 (25A, anti-CD11a, -β2 integrin, A4-33 and 0.5β antibodies), 4 (anti-CD44, -α3, -β1 and -β3 integrin antibodies) and 2 μg/ml (anti-CD11b and -CD4 antibodies). (iii) mAb 6-1-13 (IgG1) showed no cell fusion activity at a concentration of 200 μg/ml, and (iv) P1B5, a representative mAb against α3 integrin, competed (suppressed) the fusion-inducing activity of mAb 7-2-1 as mentioned later.

clarified from these findings that mAbs 4-5-1 and 7-2-1 can induce syncytium formation of the cells expressing both HIV gp160 and CD4 molecule. When a lot of control mAbs including those against class I MHC, α3 (P1B5), α4, α5, β1, β2 and β3 subunits of integrin, -CD11a, -CD11b, -CD44, -CD4 (Leu3a) and HIV gp120 (A4-33, KD1053, 0.5β) antibodies were added into culture fluids of $Cd^+$U2ME-7 cells, none of them other than anti-integrin α5 antibody induced cell agglutination or cell fusion (Table I and data not shown). Intriguingly, anti-integrin α5 antibody induced large aggregates of U2ME-7 cells within 0.5−1 h, but thereafter the aggregates were loosened and could not be found ~2 h after incubation, and cell fusion was not induced (data not shown).

When mAb 4-5-1 was added into the culture fluids of TALL-1 cells persistently infected with lymphoadenopathy-associated virus (TALL-1/LAV cells) co-cultured with $Cd^-$U2ME-7 cells, small polykaryocytes were induced (Figure 3H). TALL-1/LAV cells showed little fusion in the absence of the antibody when co-cultured with $Cd^-$U2ME-7 cells (data not shown). Furthermore, TALL-1/LAV cells alone did not fuse in the presence of the anti-FRP antibody (data not shown). This result showed that the effect of anti-FRP mAb was not restricted to cells transfected with the *env* gene.

### *Inhibition of anti-FRP mAbs-induced fusion by anti-CD4 or anti-HIV gp120*

To investigate roles of HIV gp160 and CD4 molecules in FRP-mediated cell fusion, anti-CD4 (Leu3a, T4, Hu-TH/1, OKT4), anti-HIV gp120 (0.5β, A4-33, KD1053) or other control antibodies containing anti-CD3, anti-CD8, anti-CD14, anti-CD16 and anti-CD19 were added into culture fluids of $Cd^+$U2ME-7 cells together with mAb 4-5-1 or 7-2-1. Neither anti-gp160 nor anti-CD4 antibody blocked formation of cell agglutination induced by anti-FRP mAbs, while, however, these antibodies other than OKT4 inhibited mAb 4-5-1-induced cell fusion (Figure 4A, H and I; data not shown). OKT4 and control antibodies showed no effect on the agglutination and cell fusion (data not shown). These findings indicate that CD4−gp160 interaction is required for the FRP-mediated cell fusion.

### *Absence of enhancement of HIV gp160 and CD4 molecule expression by stimulation of anti-FRP mAbs*

HIV-induced cell fusion is mediated by interaction between HIV gp160 and CD4 molecule on the surface of $Cd^+$U2ME-7 cells (Lifson *et al.*, 1986a,b; Sodroski *et al.*, 1986). Thus, the effect of anti-FRP mAbs on the expression of HIV gp160 and CD4 in U2ME-7 cells both with and without cadmium chloride was investigated. Either $Cd^-$U2ME-7 or $Cd^+$U2ME-7 cells were incubated at 37°C with mAb 25A (control mAb against mumps virus), 4-5-1, 6-1-13 or 7-2-1, and after 8 h of incubation, the cells were labeled with [$^{35}$S]methionine in the presence of each mAb for 12 h. Subsequently, they were dissolved in RIPA buffer and a part

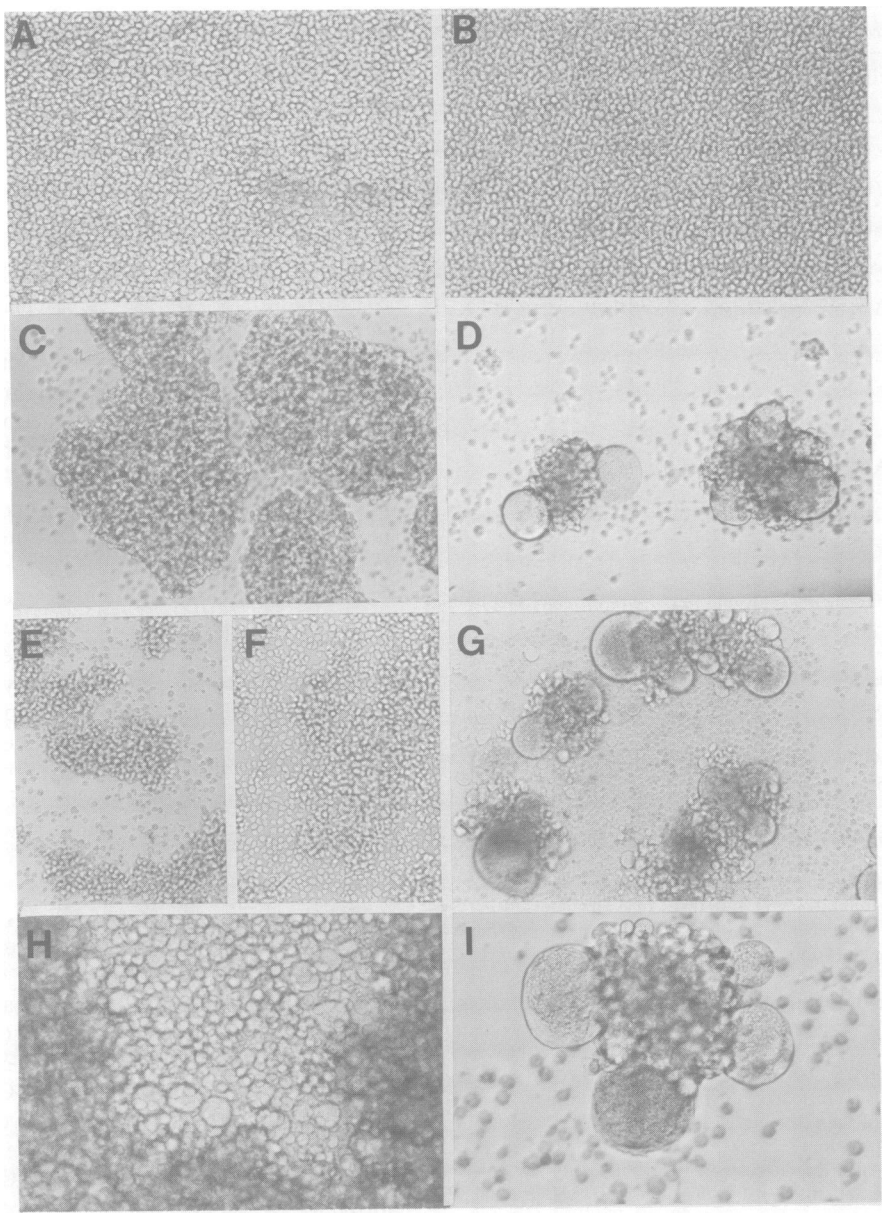


**Fig. 3.** Syncytium formation of $Cd^+$U2ME-7 cells by anti-FRP mAbs. $Cd^-$U2ME-7 (**A** and **C**) or $Cd^+$U2ME-7 cells (**B**, **D**, **E**, **F**, **G** and **I**) were incubated in the presence of mAb 25A (control: A and B), 4-5-1 (C, D and I), 6-1-13 (E) or 7-2-1 (G) for 20 h or mAb 6-1-13 for 3 h (F). TALL-1/LAB cells co-cultured with $Cd^-$U2ME-7 cells were incubated with mAb 4-5-1 for 20 h (**H**). H and I, higher magnification. Concentration of each mAb was 10 μg/ml.

of each cell lysate was analyzed by SDS−PAGE. These mAbs showed no distinct effect on the total protein synthesis of the host cells (Figure 5A). In addition, a distinctly enhanced expression of gp160 was not detected in the cells with any antibody (Figure 5A). Subsequently, each cell lysate was immunoprecipitated with anti-HIV gp120 antibody and the immunoprecipitates were also analyzed by SDS−PAGE. The immunoprecipitation assays were carried out three times under rigorous conditions (see Materials and methods), and in any case the expression of HIV envelope protein was not found to be enhanced by treatment with any mAb (Figure 5B). In the next experiment, in order to detect gp160 on the cell surface of the cells treated with these antibodies, membrane fluorescence was examined with viable cells by a FACS flow cytometry using FITC-conjugated anti-gp160 antibody. As shown in Figure 5C, $Cd^+$U2ME-7 cells incubated with control antibody had a weak fluorescence intensity and little or no fluorescence intensity was detected on the surfaces of the cells treated with any anti-FRP mAb. Little detection of gp160 on the surface of $Cd^+$U2ME-7 cells treated with anti-FRP mAbs may be due to steric hindrance or cell agglutination and consequent changes of the cell membrane induced by anti-FRP mAbs. CD4 expression was also analyzed by FACS flow cytometry with Leu3a mAb in $Cd^+$U2ME-7 cells treated with mAb 25A, 4-5-1, 6-1-13 or 7-2-1, indicating that the expression level of CD4 molecules on the host cells was not influenced by treatment with any mAb (Figure 6). These results showed that induction of the cell fusion by anti-FRP mAbs was not due to the enhancement of either HIV envelope protein or

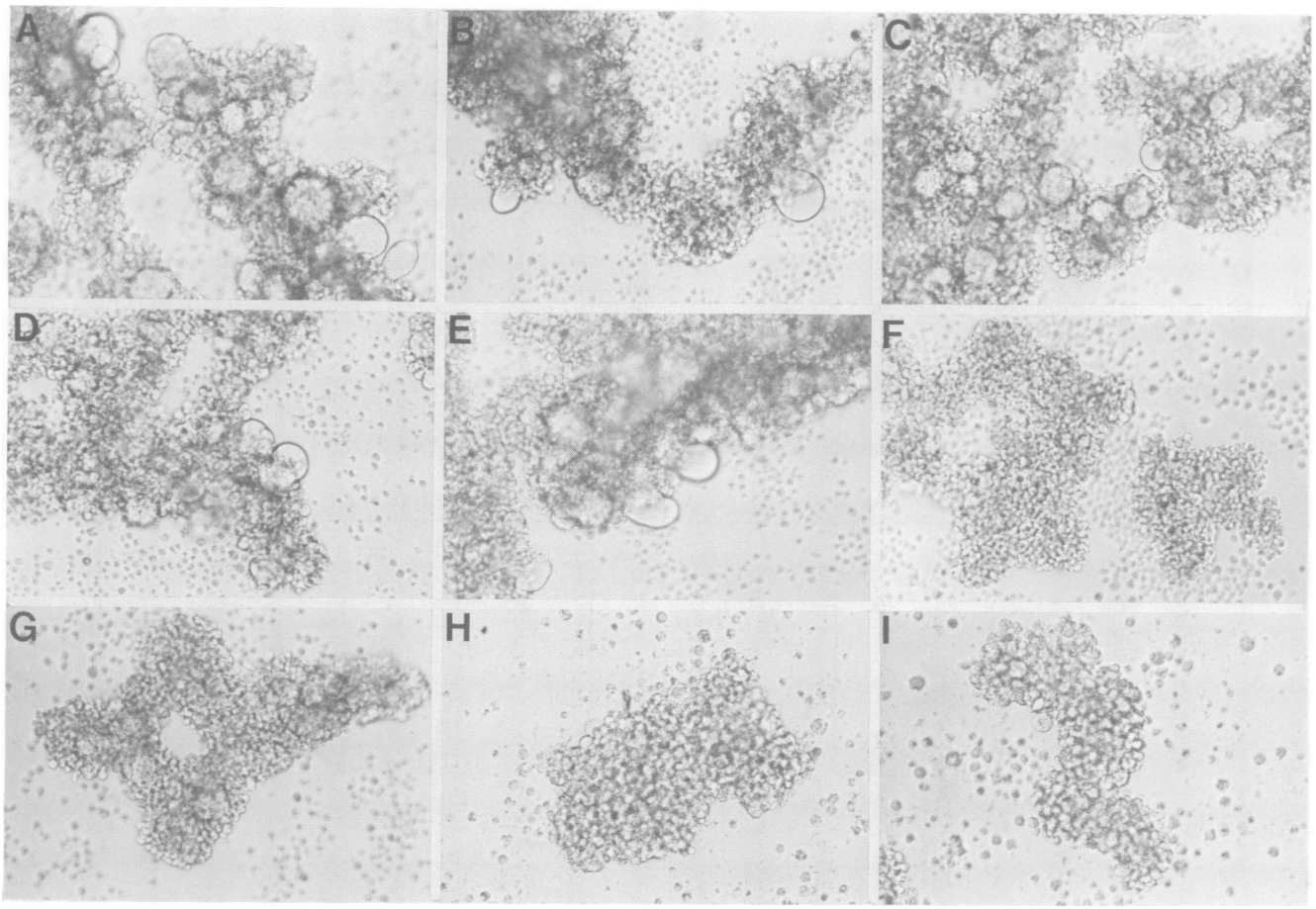


**Fig. 4.** Inhibition of anti-FRP mAb-induced cell fusion by anti-CD4 or anti-gp120 mAb. $Cd^+$U2ME-7 cells were incubated for 20 h in the presence of anti-mumps virus (25A) (control, **A**), -α2 integrin (**B**), -α4 integrin (**C**), -LFA-1β (**D**), -ICAM-1 (**E**), -β1 integrin (**F**), -LFA-1α (**G**), -CD4 (Leu3a, **H**) or -gp120 (KD1053, **I**) together with mAb 4-5-1 (10 μg/ml). All the mAbs other than mAb 4-5-1 were used at a dilution of 1:100. Of anti-CD4 mAbs, Leu3a, T4 and NUTH/1 inhibit, but OKT4 is unable to inhibit HIV binding to CD4 molecule and syncytium formation (Sattentau *et al.*, 1986).

CD4 molecule expression. Furthermore, they did not indicate that anti-FRP antibody treatment could allow an interaction between the CD4–gp120 complex and another member, β1 integrin, of the membrane protein population (data not shown).

### *Purification and N-terminal sequencing of FRP-1 and FRP-2*

To determine the primary amino acid sequences of FRP-1 and FRP-2, these molecules were purified by immunoaffinity chromatography (Figure 7A and B). Although the N-terminal amino acid sequence of FRP-1 could not be determined conclusively, none of the possible sequences showed any homology to already known sequences (data not shown). On the other hand, a sequence of 16 amino acids that matched the sequence of integrin α3 could be determined from the N-terminus of FRP-2 (Takada *et al.*, 1987b) (Figure 7D). Since integrin α3 was associated non-covalently with integrin β1 (VLA-3) on the surfaces of many cells (Takada *et al.*, 1988), there was a possibility that the purified FRP-2 fraction was also associated with integrin β1. Therefore, we tried to examine the reactivities of purified FRP fractions by ELISA using various antibodies containing anti-integrin α3 (P1B5) and anti-integrin β1. Purified FRP-1 fraction reacted with anti-FRP-1 mAbs only, while purified FRP-2 fraction was recognized by mAb against either FRP-2, integrin α3 (P1B5) or β1 (Figure 7C). Anti-human heterotypic adhesion receptor mAb (anti-CD44 mAb, P1G12) did not react with purified FRP-1 and FRP-2 fractions (data not shown). From this result, there was another possibility that FRP-2 might be integrin β1 and integrin α3 might be co-purified. Thus, a membrane fraction from HeLa cells was analyzed by Western blotting with anti-FRP-2, anti-integrin α3 (P1B5) or β1 antibody, clearly showing that FRP-2 is identical to integrin α3 (Figure 7E). Since no specific band had been found in immunoprecipitates of [$^{14}$C]glucosamine-labeled U2ME-7 cell lysate by anti-FRP-2 mAb (7-2-1) (Figure 3C), we tried to detect α3 integrin mRNA in U2ME-7 cells by using reverse polymerase chain reaction (RPCR). As shown in Figure 7F, mRNA of α3 integrin was detected in both HeLa and U2ME-7 cells. The specificity of the amplified products was confirmed by direct sequencing (data not shown).

### *Effects of fibronectin, laminin, collagen and anti-integrin antibodies on the mAb-induced polykaryocyte formation of U2ME-7 cells*

Although identification of FRP-1 has not yet been determined, FRP-2 proved to be integrin α3 (α subunit of VLA-3), which is the receptor for fibronectin, laminin, collagen and epiligrin (Takada *et al.*, 1988; Carter *et al.*, 1991). Therefore,

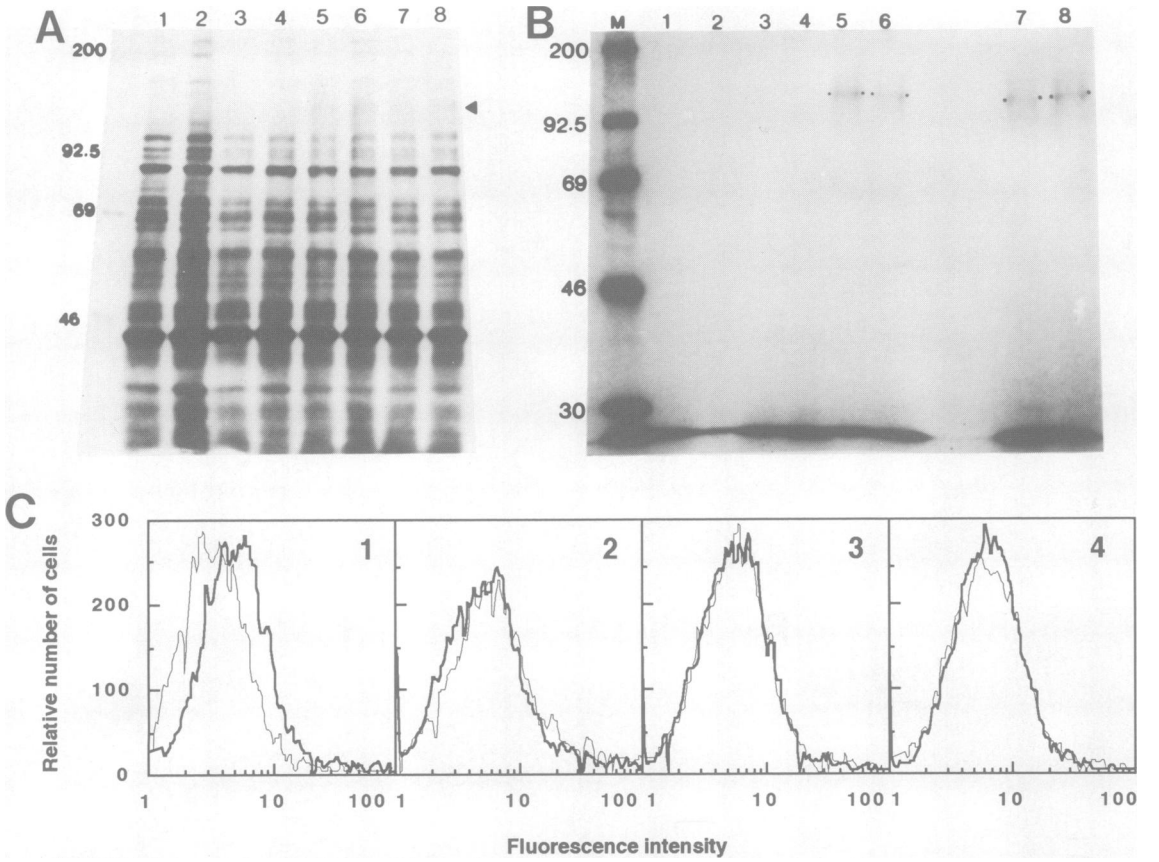


**Fig. 5.** (**A** and **B**) Both $Cd^-$U2ME-7 (lanes 1–4) and $Cd^+$U2ME-7 cells (lanes 5–8) were incubated with mAb 25A (lanes 1 and 5), 4-5-1 (lanes 2 and 6), 6-1-13 (lanes 3 and 7) or 7-2-1 (lanes 4 and 8), and at 8 h of incubation these cells were labeled with [$^{35}$S]methionine (20 $\mu$Ci) in the presence of each mAb for 12 h. The antibody content of each mAb was 10 $\mu$g/ml. Subsequently, the cell lysates were analyzed by SDS–PAGE (**A**). Furthermore, each cell lysate was immunoprecipitated with anti-HIV gp160 (KD1053) followed by SDS–PAGE (**B**). The immunoprecipitation assays were carried out three times under careful conditions [e.g. that input samples to be compared were equivalent, that anti-HIV gp160 mAb (KD1053, IgG2a) was used in excess as compared with amount of antigen, which is an important condition for complete precipitation], and in each case, the expression of HIV envelope protein was not found to be enhanced by treatment with any mAb. ◀ indicates band of HIV envelope protein. (**C**) Both $Cd^+$U2ME-7 cells and $Cd^-$U2ME-7 cells were incubated in the presence of mAb 25A (1), 4-5-1 (2), 6-1-13 (3) or 7-2-1 (4) for 8 h, and then these cells were stained with FITC-conjugated anti-HIV gp160 (KD1053). Expression of HIV envelope protein was analyzed by FACS flow cytometry. (—), $Cd^+$U2ME-7 cells; (–), $Cd^-$U2ME-7 cells.

whether these ligands substituted for anti-FRP antibody was investigated. When $Cd^+$U2ME-7 cells were incubated in the presence of these ligands, neither cell agglutination nor polykaryocyte formation was induced (data not shown). However, fibronectin inhibited syncytium formation induced by either mAb 4-5-1 or 7-2-1 (Figure 8A and B), while neither laminin, nor collagen type I or type IV showed any effect on the anti-FRP antibody-induced cell agglutination and syncytium formation (data not shown). mAb 7-2-1 or mAb 4-5-1-induced cell agglutination was suppressed or delayed, respectively, by a high concentration (100–50 $\mu$g/ml) of fibronectin (Figure 8E and F). Even when fibronectin was added to culture fluids after cell agglutination was established, induction of polykaryocyte formation was inhibited (data not shown). Thus, it is inferred from these findings that the fibronectin–fibronectin receptor system is involved in anti-FRP mAb-induced cell fusion. However, anti-fibronectin antibody did not influence the biological activity of anti-FRP antibodies (data not shown). Furthermore, an oligopeptide containing RGD sequence showed no effect on cell agglutination and cell fusion induced by mAb 4-5-1 or 7-2-1 (data not shown).

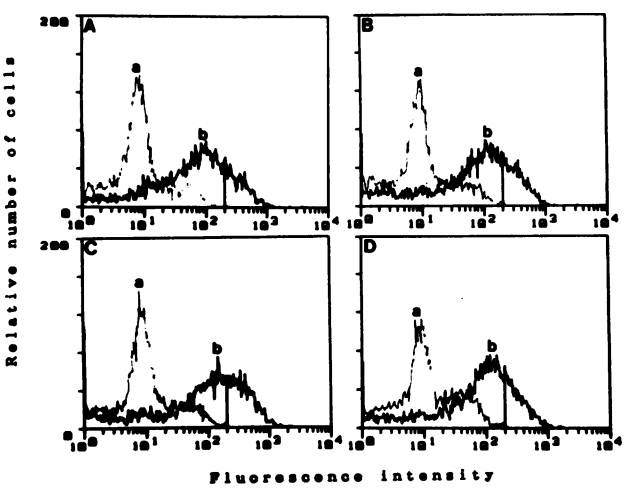


**Fig. 6.** $Cd^+$U2ME-7 cells were cultured for 6 h with mAb 4-5-1 (**A**), 6-1-13 (**B**), 7-2-1 (**C**) or 25A (**D**), incubated with FITC-conjugated control (a) or anti-CD4 mAb (Leu3a, b) and analyzed by a FACS cytofluorometer.

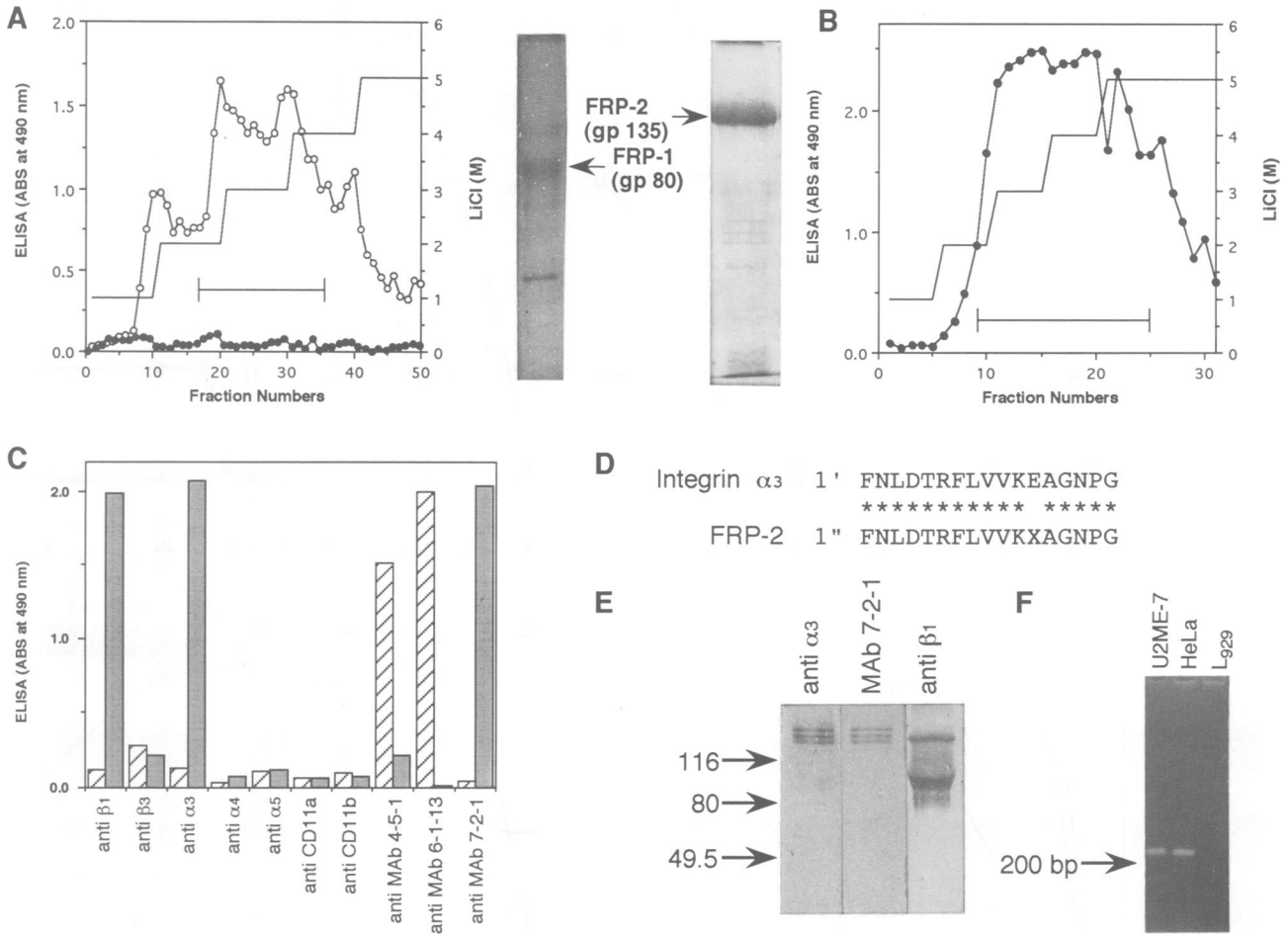


**Fig. 7.** (**A** and **B**) Elution profiles from mAb 6-1-13 immunoaffinity column (A) and mAb 7-2-1 immunoaffinity column (B). An extract from purified cell membrane was applied to mAb 6-1-13 immunoaffinity column, and the flow-through fraction from the column was successively applied to mAb 7-2-1 immunoaffinity column. The columns were washed free of unbound proteins and bound protein was sequentially eluted stepwise with LiCl in 50 mM Tris−HCl (pH 7.5). The eluates were monitored with ELISA using mAb 6-1-13 (○-○) or 7-2-1 (●-●). Fractions indicated by ⊢⊣ were concentrated and were analyzed by SDS−PAGE. (**C**) Antigenicity of the eluates from mAb 6-1-13 (▨) and mAb 7-2-1 (▩) affinity column was examined by ELISA using various antibodies. (**D**) The N-terminal amino acid sequence of FRP-2 and alignment with that of integrin α3. (**E**) Identification of FRP-2 fraction with Western blotting. Purified FRP-2 fraction was analyzed by Western blotting using mAbs against α3 and β1 integrins and FRP-2. (**F**) Detection of mRNA of α3 integrin in U2ME-7, HeLa and $L_{929}$ cells by reverse PCR. Total cellular RNA was isolated from murine $L_{929}$, HeLa or U2ME-7 cells according to the guanidinium isothiocyanate method and the poly(A)-containing RNA was purified. Complementary DNA was synthesized from 1 μg of mRNA by reverse transcriptase and an antisense primer of human integrin α3 chain, and used as primer for amplification of the cDNA fragment that encoded human α3 integrin. PCR was carried out in 30 cycles and 5 μl aliquots of the PCR were separated on a 2% agarose gel.

Subsequently, to clarify further the interaction between the fibronectin−fibronectin receptor system and cell fusion, effects of anti-integrin α and β subunit antibodies on anti-FRP mAb-induced fusion were investigated. Anti-integrin β1 antibody blocked not only mAb 7-2-1-induced, but also mAb 4-5-1-induced cell fusion (Figures 4F, 8C and 8D). In addition, formation of cell agglutination induced by anti-FRP mAbs was delayed by anti-β1 integrin antibody (Figures 4F, 8G and 8H). When anti-β1 integrin antibody was added to the culture fluids of U2ME-7 cells with mAb 4-5-1 or 7-2-1 after cell agglutination had occurred, formation of syncytium cells was blocked (data not shown). It is the first time that anti-integrin β1 antibody has been shown to have an ability to block HIV-mediated cell fusion, suggesting that very late antigen (VLA) is involved in anti-FRP-induced cell fusion. A representative mAb directed against α3 integrin (P1B5) was incapable of inducing cell agglutination and cell fusion (data not shown), suggesting that the epitope recognized by mAb 7-2-1 is different from that recognized by P1B5. However, P1B5 enhanced cell agglutination induced by either mAb 7-2-1 or 4-5-1, while, on the other hand, it suppressed the cell fusion induced by mAb 7-2-1 (Figure 8H and data not shown). Hildreth and Orentas (1989) and Valentin *et al.* (1990) reported that anti-LFA-α antibody inhibited HIV-mediated cell fusion. Anti-LFA-α antibody also inhibited anti-FRP mAb-induced cell fusion, although the antibody did not influence the cell agglutination (Figure 4G; data not shown). The other antibodies against β2, β3, α2, α4 and α5 integrins and CD11b, and ICAM-1 and CD44 antibodies showed no effect on mAb 4-5-1-induced cell agglutination and cell fusion (Figure 4A, B, C, D and E; data not shown). Interestingly, mAb 6-1-13 suppressed induction of cell fusion by either mAb 4-5-1 or 7-2-1 (data not shown).

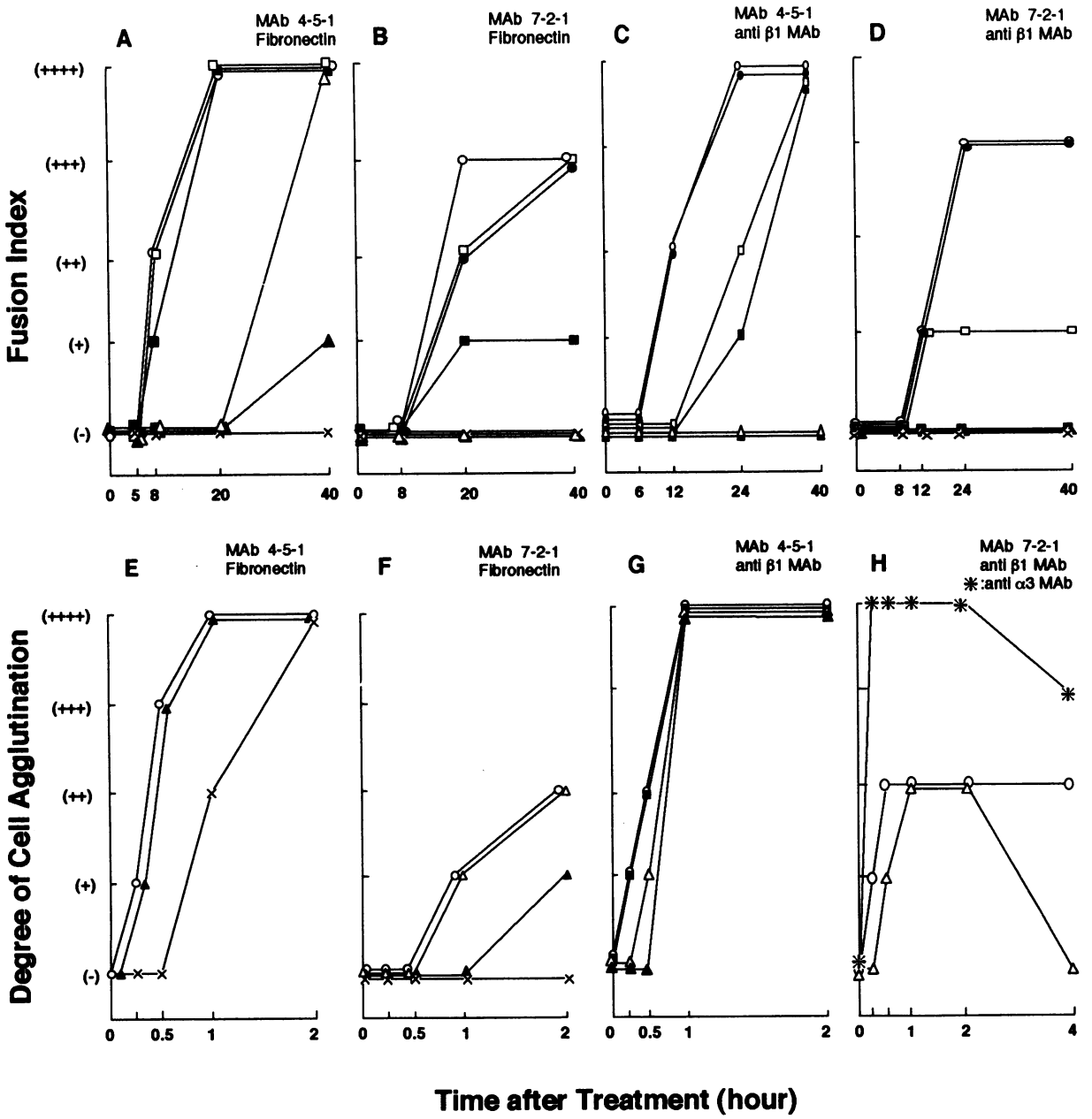


**Fig. 8.** Effects of fibronectin and anti-β1 integrin antibody on the mAb-induced cell agglutination and cell fusion of $Cd^{+}$U2ME-7 cells. Various concentrations of fibronectin (**A**, **B**, **E** and **F**) or anti-β1 integrin mAb (**C**, **D**, **G** and **H**) were added to culture fluids of $Cd^{+}$U2ME-7 cells together with mAb 4-5-1 (A, C, E and G) or 7-2-1 (B, D, F and H). Concentration of anti-FRP mAb was 10 μg/ml. ○, in the absence of fibronectin; in the presence of: ●, 3 μg/ml fibronectin; □, 6 μg/ml; ■, 12.5 μg/ml; △, 25 μg/ml; ▲, 50 μg/ml; **X**, 100 μg/ml (A, B, E and F); ○, in the absence of anti-β1 and α3 integrin mAb, in the presence of: ●, 0.125 μg/ml anti-β1 integrin mAb; □, 0.25 μg/ml; ■, 0.5 μg/ml; △, 1.0 μg/ml; ▲, 2.0 μg/ml; *, 1 μg/ml anti-α3 integrin mAb (C, D, G and H).

### *Effects of various treatments on activities of anti-FRP mAb*

In order to clarify further the processes leading to cell fusion, the effects of various treatments, that is, addition of drugs, low temperature incubation, $Ca^{2+}$ deprivation, etc., on the activities of mAb 4-5-1 or mAb 7-2-1 were investigated (Table II). When $Cd^{+}$U2ME-7 cells were incubated at 37°C with either mAb 4-5-1 or mAb 7-2-1 ($mAb^{+}$cells), uneven distribution of cells occurred within 1−2 min; thereafter, cell agglutination appeared and reached its maximum level within 1−2 h. When $Cd^{+}$U2ME-7 cells were incubated at 4°C, the cells were evenly distributed for a long period and no cell agglutination appeared in the presence of mAb 4-5-1 or mAb 7-2-1. Cytochalasin B (anti-microfilament agent) treatment completely blocked the cell agglutination and even distribution of the cells persisted throughout. Intriguingly, cytochalasin B inhibited the polykaryocyte formation whenever it was added during incubation (even after cell agglutination had already taken place), although existing cell agglutination was not broken up by cytochalasin B (data not shown). When the cells were cultured in the serum-free medium, uneven distribution of

**Table II.** Effects of various treatments on anti-FRP mAb-induced syncytium formation and cell agglutination

| | | Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Serum deficient | $Ca^{2+}$ deficient | Cytochalasin B (50 μM) | DMSO (0.5%) | Sodium azide | Vinblastine (0.1 μM) | Cycloheximide (50 μg/ml) | H-7 (50 μM) | Herbimycin A (0.5 μg/ml) |
| Cell agglutination | 4-5-1 | – | – | – | ++++ | ++++ | ++++ | ++++ | + | ++++ |
| | 7-2-1 | ND | ND | – | ++++ | ++++ | ++++ | ++++ | + | ++++ |
| Syncytium formation | 4-5-1 | – | – | – | ++++ | – | – | – | – | – |
| | 7-2-1 | ND | ND | – | ++++ | – | – | – | – | – |

Cell agglutination and syncytium formation were judged at 2 and 20 h of incubation, respectively. Various doses of drugs were used in this experiment, and representative results are described in the table.

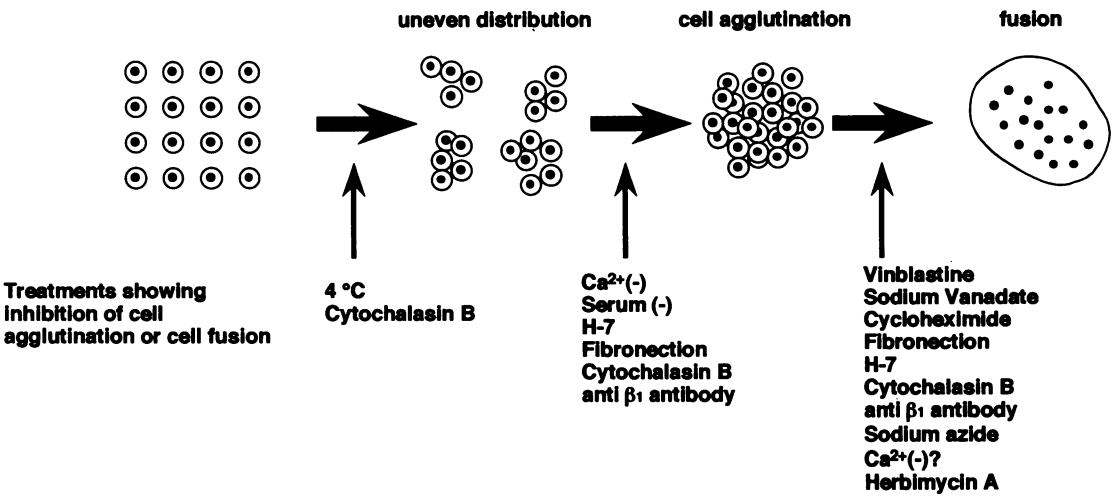


**Fig. 9.** Summary of the effects of various treatments on processes leading to cell fusion.

the cells occurred, but neither cell agglutination nor cell fusion was induced in the presence of mAb 4-5-1. When the cells treated with mAb 4-5-1 were incubated in $Ca^{2+}$-deficient medium with EGTA (1 mM), cell agglutination was delayed until 8 h of incubation, but cell fusion did not appear. A metabolic poison, sodium azide (0.1%), showed no effect on the cell agglutination, while addition of sodium azide at 0.006% or 0.003% resulted in prevention of syncytium cell formation induced by mAb 4-5-1 or 7-2-1, respectively. Treatment of $mAb^{+}$Cell by vinblastine (anti-microtubule agent), sodium vanadate (inhibitor of phosphatase and dynein ATPase) or cycloheximide (inhibitor of protein synthesis) showed no effect on the cell agglutination, but no cell fusion occurred in the presence of these drugs. H-7, protein kinase C inhibitor, suppressed cell agglutination and cell fusion, while control compound, HA1004, showed no effect (data not shown). When H-7 was added to the culture fluid of $mAb^{+}$Cell after cell agglutination had been induced, induction of cell fusion was blocked, although existing cell agglutination was not reversed (data not shown). Herbimycin A, inhibitor of tyrosine kinase, blocked mAbs 4-5-1 and 7-2-1 induced cell fusion, but showed no effect on cell agglutination.

These findings are summarized in Figure 9, showing that anti-FRP mAb-induced cell agglutination is temperature, microfilament and $Ca^{2+}$ dependent, and that the agglutination does not require newly synthesized protein and integrity of microtubule function. The FRP-mediated cell fusion depends on microtubule function, energy and newly synthesized protein(s). Protein phosphorylation is also involved in cell fusion and/or cell agglutination.

## Discussion

This study clearly shows that some host cell molecules other than CD4 are involved in HIV-mediated cell fusion. Anti-FRP mAbs 4-5-1, 6-1-13 and 7-2-1, were first isolated by a screening test for an activity enhancing NDV-mediated syncytium formation. HIV envelope glycoprotein gp41 serves as the fusion protein for HIV in a manner similar to fusion proteins of other viruses including paramyxoviruses (Gallaher, 1987). These findings show that common host cell factors are involved in HIV- and paramyxovirus-mediated cell fusion.

FRP-2 proved to be integrin α3 subunit and anti-FRP-2 mAb reacted with VLA-3 which is the receptor for fibronectin, laminin and collagen IV (Takada *et al.*, 1988). Induction of HIV-mediated cell fusion by anti-FRP-2 mAb was inhibited by addition of fibronectin, anti-integrin β1 or α3 subunit antibody, suggesting that the cell fusion was regulated by the fibronectin–VLA-3 system. Furthermore, the mechanism(s) by which anti-FRP-1 mAb-induced HIV-mediated cell fusion was intimately regulated to the fibronectin–integrin system for the following reasons: (i) anti-α3 integrin antibody (P1B5) enhanced and accelerated mAb 4-5-1-induced cell agglutination, (ii) anti-β1 integrin antibody blocked the cell fusion induced by mAb 4-5-1, (iii) exogenous addition of fibronectin suppressed mAb

4-5-1-induced cell fusion, and (iv) even when either anti-β1 integrin antibody or fibronectin was added to culture fluid after cell agglutination had been completed, formation of syncytia was blocked. On the other hand, the fusion-inducing mechanism of the VLA-3 system is also regulated by the FRP-1 system, because anti-FRP-1 antibody that does not induce fusion blocked mAb 7-2-1-induced cell fusion.

The integrins are a family of transmembrane glycoproteins comprising non-covalent heterodimers (Hynes, 1987, 1992; Takada *et al.*, 1987a). They interact with a variety of ligands including extracellular matrix glycoproteins, complement and other cells (Hynes, 1987, 1992). They participate in cell−matrix and cell−cell adhesion in many physiologically important processes including embryogenesis, hemostasis, thrombosis, wound healing, immune and non-immune defense mechanisms, and oncogenic transformation (Hynes, 1992). Mature skeletal muscle cells are distinguished by being multinucleated, a state resulting from the fusion of myoblasts. Mammalian myogenesis is a multistep process, that is, primary myoblasts fuse to form primary myotubes, then secondary myoblasts align along the primary myotubes and form secondary myotubes (Rosen *et al.*, 1992). Antibodies to N-CAM and N-cadherin have been shown to delay myoblast fusion in culture and N-CAM and N-cadherin might predominate in myoblast−myoblast fusions (Knudsen *et al.*, 1990a,b). Recently, Rosen *et al.* (1992) have reported that antibodies to VLA-4 and VCAM-1 inhibit myotube formation and that VLA-4−VCAM-1 interactions influence alignment of secondary myoblasts along primary myotubes and/or the fusion of secondary myoblasts. In addition, members of the β1 subgroup of integrins, including α5β1, α3β1 and α7β1, have been identified in muscle (Rosen *et al.*, 1992). Thus, various adhesion molecules, particularly β1 subgroup integrins, are involved in myoblast fusion, that is, fusion between primary myoblasts and between secondary myoblasts and primary myotubes. The union of sperm and egg is a special membrane fusion event that gives a signal to begin development. α and β subunits of PH-30, a sperm surface protein involved in sperm−egg fusion, have recently been found to be type I integral membrane glycoproteins (Blobel *et al.*, 1992). The α subunit contains a putative fusion peptide typical of viral fusion protein and the β subunit contains a domain related to a family of soluble integrin ligands found in snake venom. Our study clearly indicates that an integrin(s) system is involved in the mechanism regulating cell fusion in non-muscle cells.

The addition of anti-FRP mAb resulted in an extremely rapid and vigorous homotypic agglutination among cells, including lymphocyte, macrophage and epithelial cell lines. No cell agglutination occurred if the cells were incubated with mAb 4-5-1 at 4°C or in the $Ca^{2+}$- or serum-deprived medium. Cytochalasin B blocked the cell agglutination, indicating a requirement for integrity of microfilament and the possibility of an association between the adhesion receptors and cytoskeletal components. On the other hand, vinblastine, an anti-microtubule agent, showed no effect on cell agglutination although cell fusion was suppressed by this drug. In addition, this study clarified that anti-α5 antibody also had an ability to aggregate cells, although the aggregates were loosened after 1 h and could not be found ~2 h after incubation. Anti-α4 integrin antibody was reported to induce homotypic lymphocyte aggregation (Bednarczyk and McIntyre, 1990). Furthermore, anti-β2 type integrin antibodies have been shown to induce homotypic lymphocyte aggregation (Keizer *et al.*, 1988). These findings suggest a role for integrin in homotypic cell agglutination. The cell agglutination was not a sufficient step for cell fusion, because (i) mAb 6-1-13, anti-FRP-1 mAb, induced vigorous cell agglutination, but not cell fusion, (ii) sodium azide, vinblastine, sodium vanadate, herbimycin A and cycloheximide did not interfere with cell agglutination, but inhibited formation of polykaryocytes, (iii) even when anti-β1 integrin antibody, fibronectin or H-7, an inhibitor of protein kinase, was added after cell agglutination had already taken place, induction of cell fusion was blocked, and (iv) TPA, phorbol ester, induced small clusters of $Cd^{+}$U2ME-7 cells, whereas it did not induce cell fusion (unpublished data).

From these results, we speculate possible mechanisms for antibody-induced fusion as follows. There are at least two fusion regulatory systems, that is, FRP-1 and FRP-2 (integrin) systems. These systems exist separately from each other and have their own signal transduction pathways. Conformational change triggered by direct interaction between the system and antibody/ligand can induce cell fusion. However, since anti-integrin β1 subunit antibody blocks FRP-1-mediated cell fusion and anti-FRP-1 antibody that does not induce fusion inhibits FRP-2-mediated fusion, these systems do not exist independently of each other and there is cross-talk between them. Therefore, it is a very strong possibility that FRP-1 is a new type of adhesion molecule distinct from known integrin molecules as well as CD antigens, and the most fascinating idea is that FRP-1 is a counter receptor for FRP-2 (VLA-3). The molecular weight of FRP-1 was heterogeneous, that is, 80 kDa, 120 kDa and homooligomers. Furthermore the 80 kDa polypeptide was subdivided into 75 kDa and 85 kDa polypeptides. A unique 37 kDa protein was coprecipitated with FRP-1 from the surfaces of both HeLa and U2ME-7 cells. p37 was not recognized directly by anti-FRP-1 mAb and was little, if at all, glycosylated. Thus identification of FRP-1 and p37 molecules is a pressing subject.

## Materials and methods

### *Cells*

U937-2 ($CD4^{+}$U937 cells), U2ME-7 (U937-2 cells transfected with the HIV gp160 gene) and HeLa cells were used in this study. The cells were cultured in Dulbecco's minimum essential medium (DMEM) or Eagle's minimum essential medium (EMEM) fortified with 10% fetal calf serum. The properties of U2ME-7 cells were described previously (Koga *et al.*, 1990).

### *Antibodies*

Anti-FRP mAbs (mAbs 4-5-1, 7-2-1 and 6-1-13) were previously described (Ito *et al.*, 1992). Anti-human fibronectin rabbit serum was kindly donated by Dr S.Saga, Nagoya University, Nagoya, Japan. Anti-integrins β1 and β3 and -CD44 (human heterotypic adhesion receptor, P1G12) antibodies were purchased from Locus, anti-integrins α2, α4 and α5 from Telios, anti-α3 (P1B5) antibody from Oncogene Science. anti-LFA-α (CD11a) and -CD4 (HuTH/1) antibodies were bought from Nichirei. Anti-LFA-1b (CD18), -LFA-1α (CD11a) and -ICAM-1 (CD54) antibodies were kindly provided by Dr H.Mizutani, Mie University, Mie, Japan. Anti-CD3, -CD8, -CD14, -CD16 and -CD19 antibodies were purchased from Becton-Dickinson. Anti-HIV gp120 antibodies, 0.5β, A4-33 and KD1053 were offered by Drs S.Matsushita, Kumamoto University, Kumamoto, Japan; Y.Ohmoto, Cell Technology Research Institute, Tokushima, Japan and C.Örvell, Karolinska Institute, Sweden, respectively.

### *Isotopic labeling, RIPA, SDS–PAGE and two-dimensional SDS–PAGE*

Isotopic labeling of HeLa and U2ME-7 cells, radio-immunoprecipitation assay (RIPA), and SDS–PAGE were done as described previously (Ito *et al.*, 1987a; Yokochi *et al.*, 1987).

### *Immunofluorescent staining*

Unfixed $Cd^{-}$U2ME-7 cells were immunostained by using hybridoma supernatant or purified antibody and FITC-conjugated anti-mouse Ig antiserum according to the method described previously (Ito *et al.*, 1989).

### *Western blotting and two-dimensional Western blotting*

Western blotting was performed using lithium dodecyl sulfate (LDS) instead of SDS as described previously (Tsurudome *et al.*, 1989). Anti-FRP mAbs reacted weakly to the Western blotting antigens under the denatured condition by SDS. Therefore, antigens were treated with LDS and electrophoresis was performed at 4°C using LDS-containing buffer. For two-dimensional Western blotting, lysates of HeLa cell membrane fraction were electrophoresed in the first dimension without 2-mercaptoethanol on an 0.75 mm thick 10% acrylamide gel. Individual lanes of the first dimension slab gel were excised, incubated in reducing solution (10% 2-mercaptoethanol in Tris–glycine buffer) for 2 h and affixed to another 10% slab gel (1 mm thick) with 1.5% agarose in reducing solution for the second dimension.

### *Purification of FRPs*

Plasma membranes were prepared from HeLa cells ($2 \times 10^9$) by the method of Maeda *et al.* (1983). The cell membranes were solubilized with 30 ml of 50 mM Tris–HCl, 1% Triton X-100 using an ultrasonic cell disruptor (Heat Systems) and centrifuged at 25 000 *g* for 20 min. The supernatant was passed through Sepharose 4B and control IgG (anti-parainfluenza virus type 4A mAb) column to remove non-specifically adhering materials. Flow-through fraction was applied to mAb 6-1-13 immunoaffinity columns, and the flow-through fraction from the columns was successively applied to mAb 7-2-1 immunoaffinity columns. The columns were washed free of unbound proteins with 10 vol of solubilizing buffer and bound protein was sequentially eluted stepwise with LiCl in 50 mM Tris–HCl (pH 7.5). The LiCl eluate was dialyzed against NANOpure water and concentrated by using a Speedvac concentrator.

### *Determination of the N-terminal amino acid sequences of FRPs*

Fractions containing FRPs were separated by SDS–PAGE and electroblotted to polyvinylidine difluoride (PVDF) membrane. Blotted proteins were identified by Coomassie blue staining and sequenced using an Applied Biosystems model 477A gas-phase sequencer equipped with a phenylhydantoin amino acid analyzer.

### *Detection of α3 integrin mRNA in U2ME-7, $L_{929}$ and HeLa cells by reverse PCR*

PCR analysis was used to detect mRNA of integrin α3 in U2ME-7 cells. Total cellular RNA was isolated from $L_{929}$, HeLa or U2ME-7 cells according to the guanidinium isothiocyanate method. The poly(A)-containing RNA was purified by the batch method using Oligotex$(dT)^{30}$. Complementary DNA was synthesized from 1 μg of mRNA by reverse transcription using Moloney leukemia virus reverse transcriptase and the antisense primer of human integrin α3 chain, and used as primer for amplification of the cDNA fragment that encoded human α3 integrin. The oligonucleotide primers used were 20mers 5′-TGAGTCCGCTGTCTTCCACG-3′ and 5′-TTGTTGTCAGGCCCGCACTC-3′ which were prepared on the basis of the nucleotide sequence (Takada *et al.*, 1989). PCR was carried out in 30 cycles and 5 μl aliquots of the PCR were separated on a 2% agarose gel.

### *Drugs and chemical reagents*

Fibronectin prepared from pooled plasma was purchased from Cappel and a synthetic peptide, Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro, was bought from Sigma. H-7 and the control compound, HA1004, were kindly donated by Dr H.Hidaka, Nagoya University School of Medicine, Nagoya, Japan.

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