contain positively charged residues mostly at the cytoplasmic side (*15*). This finding is in agreement with the structural arrangement of the membrane-spanning region of the Pf3 coat protein.

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19. We thank R. Konings and L. Day for Pf3 bacteriophage; H. Jütte for technical assistance; and E. Amstutz and E. Stoop for preparation of the manuscript. Supported by the Swiss National Science Foundation (Grant 3.533-0.86).

31 May 1990; accepted 7 August 1990

Efficient Incorporation of Human CD4 Protein into Avian Leukosis Virus Particles

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Virus envelope (Env) proteins are thought to contain specific signals for selective uptake by virus particles. In the course of attempting to define these signals by testing virus incorporation of CD4-Env chimeric proteins, normal human CD4 was found to be efficiently and selectively assembled into avian leukosis virus particles in quail cells. Viruses bearing CD4 at their surface may be useful reagents in the design of retrovirus-mediated gene therapy for the acquired immune deficiency syndrome.

RETROVIRUS PARTICLES EFFICIENTLY incorporate viral envelope (Env) proteins from the cell surface and exclude the majority of host cell surface proteins, but the basis of this specificity is poorly understood. Retroviral Env proteins are made up of two polypeptide chains: surface (SU) and transmembrane (TM). Specific interactions between the transmembrane domain of TM Env proteins and matrix (MA) Gag proteins have been proposed to account for the selective uptake of Env proteins by avian leukosis virus (ALV) particles (*1*). On the basis of this hypothesis, we predicted that ALV might incorporate chimeric proteins comprising the extracellular region of human CD4 fused to sequences from the transmembrane and cytoplasmic tail domains of retroviral TM Env proteins.

Two chimeric *CD4-env* genes were constructed, designated *CR* and *CM*, which encode the extracellular domains of human CD4 fused in-frame with the transmembrane and cytoplasmic tail sequences of either the ALV TM protein or the TM protein of murine leukemia virus (MLV) (CR and CM, respectively, Fig. 1A). The *CM* chimeric gene was constructed because previous experiments have shown that MLV can provide Env proteins for ALV pseudotypes (*2*).

Incorporation of CR, CM, or wild-type CD4 proteins into ALV was tested by transiently expressing these proteins, by means of SV40-based expression vectors, in quail QT6 cells (*3*) previously infected with a replication-competent ALV vector. Immunofluorescence studies with CD4-specific antisera demonstrated transient cell surface expression of CD4, CR, or CM chimeric proteins in 5 to 10% of transfected cells (*4*). Transfected cells were metabolically labeled, and cell extracts and virions were assayed by immunoprecipitation. The production of the 95-kD ALV precursor Env protein (*5*) and CD4, CR, or CM proteins in transfected cells was confirmed by means of antisera specific for either viral Env or human CD4 proteins (Fig. 1B, lanes 1 to 6). Equivalent virus production from transfected cells was demonstrated by immunoprecipitation of Gag proteins from virus samples (Fig. 1B, lanes 10, 14, 18). The low concentrations of Env proteins detected in these experiments was due to inefficient

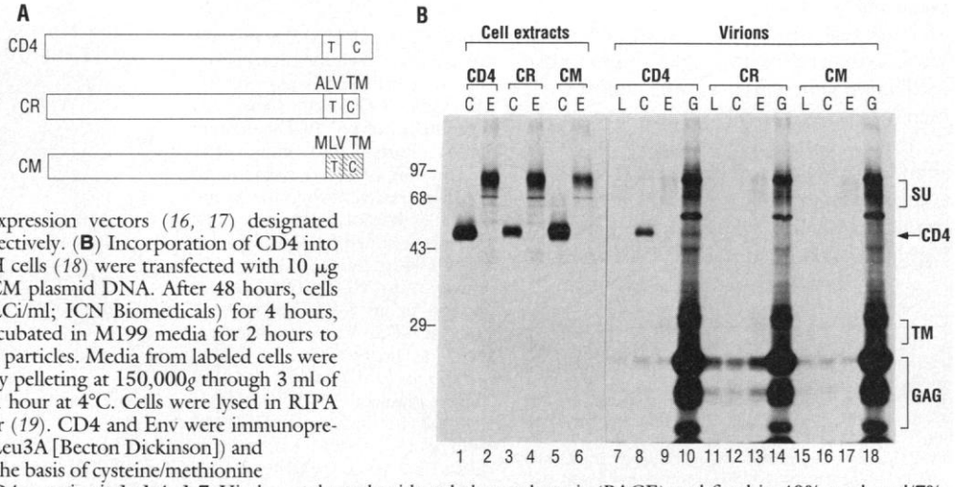

Fig. 1. (**A**) Structure of *CR* and *CM* chimeric CD4-Env proteins. *CD4-env* genes were constructed to encode the extracellular domains of human CD4 (*14*) fused in-frame with the transmembrane and cytoplasmic tail domains of either ALV (CR) or MLV (CM) transmembrane (TM) Env proteins (*15*). A wild-type *CD4* gene (*14*) and the *CM* and *CR* chimeric genes were expressed from SV40-based expression vectors (*16, 17*) designated pSVβCD4, pSV7dCM, and pSV7dCR, respectively. (**B**) Incorporation of CD4 into ALV particles. Approximately 3×10^6 QAH cells (*18*) were transfected with 10 μg of either pSVβCD4, pSV7dCR, or pSV7dCM plasmid DNA. After 48 hours, cells were incubated with [^{35}S]trans-label (350 μCi/ml; ICN Biomedicals) for 4 hours, washed in phosphate-buffered saline, and incubated in M199 media for 2 hours to allow the export of radioactively labeled virus particles. Media from labeled cells were cleared at 2000 *g*, and virions were purified by pelleting at 150,000*g* through 3 ml of 20% sucrose in a Beckman SW41 rotor for 1 hour at 4°C. Cells were lysed in RIPA buffer and viruses were lysed in NP-40 buffer (*19*). CD4 and Env were immunoprecipitated with C (the CD4-specific antibody Leu3A [Becton Dickinson]) and E [an ALV-Env specific antiserum (*20*)]. On the basis of cysteine/methionine compositions, the ratio of labeled TM:SU:CD4 proteins is 1: 1.4: 1.7. Viral Gag was immunoprecipitated with G [an ALV-Gag–specific antiserum (*4*)], and L [the CD8-specific monoclonal antibody Leu2A (Becton Dickinson)] was used as a negative control. Samples were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE) and fixed in 40% methanol/7% acetic acid. The gel was incubated in Amplify (Amersham), dried, and exposed to Kodak XAR-5 film at −70°C for 1 day (cell extracts) or 7 days (virions).

immunoprecipitation of the Env-specific antisera (Fig. 2, lanes 5 and 6).

Unexpectedly, wild-type human CD4 (Fig. 1B, lane 8) was incorporated into ALV particles more efficiently than either of the CD4-Env chimeric proteins (Fig. 1B, lanes 12 and 16). From these and similar experiments, we estimate that CD4 is incorporated into ALV particles at least five- to tenfold more efficiently than either CR or CM chimeric proteins.

To exclude the possibility that ALV nonselectively incorporates cell surface proteins, we asked whether ALV takes up CD4 preferentially over quail cell glycoproteins. The glycoprotein content of cell extracts and virus particles was determined by concanavalin A (con A)–agarose precipitation. In addition, three types of proteins [CD4, Env, and CSAT proteins (three integral membrane glycoproteins of 120 to 160 kD) (*6*)] were tested by specific immunoprecipitation. Examination of cell extracts confirmed that CSAT, Env, and CD4 proteins represent minor subsets of the cellular glycoproteins in transfected quail cells (Fig. 2, lanes 1 to 4). A major virus-associated glycoprotein in the con A–agarose precipitate (Fig. 2, lane 11) was identified as CD4 by first depleting virus samples of CD4 with the CD4-specific antibody Leu3A and then precipitating other glycoproteins with con A–agarose (Fig. 2, lanes 7 and 8). As expected, the Env-specific and CSAT-specific antisera did not deplete CD4 from virus-associated glycoproteins precipitated by con A–agarose (Fig. 2, lanes 5, 6, 9, and 10), and CD4 was not detected in virus samples prepared from untransfected ALV-infected quail cells (Fig. 2, lane 14). The inefficient incorporation of other quail cell glycoproteins into virions (Fig. 2, lanes 1, 2, and 9 to 11), demonstrates that CD4 is selectively taken up by ALV.

Sequential incubations of labeled virus-associated proteins indicated that a single incubation with con A–agarose almost completely precipitates the glycoproteins (Fig. 2, lanes 11 to 13). However, the only abundant species are Env proteins, CD4, and coprecipitated Gag proteins. After correcting for labeling efficiencies (see legend to Fig. 1), the relative amount of CD4 and Env proteins in virus particles was 1:18 (Fig. 2). Given that only a small percentage of ALV-infected quail cells express CD4 after transfection, this value no doubt underestimates the potential for incorporating CD4 into virus particles. We conclude from these experiments that CD4 proteins are efficiently incorporated into ALV, but the inefficient precipitation of cell-associated Env proteins precludes an estimate of the relative efficiencies of incorporation.

Further evidence of CD4 uptake by ALV was obtained by equilibrium density sedimentation experiments, which demonstrated that human CD4 proteins copurify with retrovirus particles. Medium from metabolically labeled ALV-infected quail cells that express CD4 was sedimented to equilibrium through a 20 to 45% sucrose gradient. Immunoprecipitations from gradient fractions demonstrated cofractionation of CD4 and viral Env proteins (Fig. 3, fractions 2, 3, and 4). Furthermore, most of the CD4 and Env proteins were contained on particles that banded at a density of 1.16 g of sucrose per milliliter (Fig. 3, fraction 3), as expected for retroviruses at equilibrium under these conditions (*7*). We conclude from these experiments that CD4 is contained in ALV particles. To exclude the possibility that

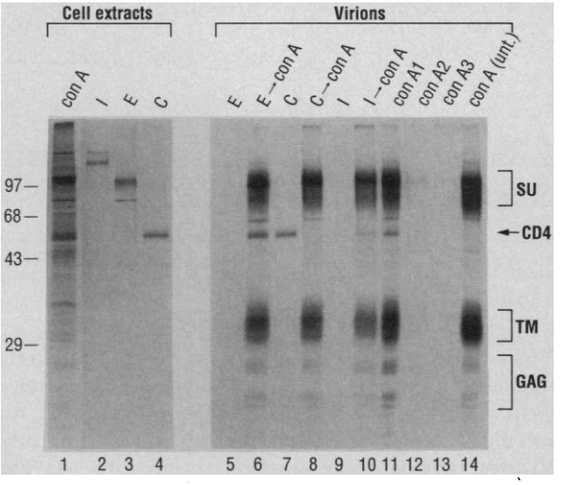

Fig. 2. Specific incorporation of CD4 into ALV particles. Approximately 10^7 QAH cells transfected with 30 μg of pSVβCD4 plasmid DNA, and 3×10^6 untransfected QAH cells, were metabolically labeled as described in the legend to Figure 1. Virions were pelleted from cleared media at 11,000*g* through 3 ml of 20% sucrose. Cell extracts and purified virus samples were resuspended in NP-40 buffer (*19*) containing 10 μM $MnCl_2$. Equal aliquots of cell lysate were either precipitated by con A–agarose (lane 1), or immunoprecipitated with I [lane 2, a CSAT-specific antibody (6)], E [lane 3, an Env-specific antibody (*20*)], or C [lane 4, the CD4-specific antibody Leu3A (Becton-Dickinson)], in the presence of 0.1% SDS. Equivalent amounts of virus samples from pSVβCD4-transfected cells were incubated with antibodies specific for Env proteins (lane 5), CD4 (lane 7), or CSAT (lane 9) before precipitation with con A–agarose (lanes 6, 8, and 10, respectively). An equivalent virus sample was subjected to three sequential precipitations with con A–agarose to test the efficiency of precipitation by this reagent (lanes 11 to 13). Virus samples prepared from untransfected QAH cells were precipitated with con A–agarose (lane 14). Samples were subjected to electrophoresis as described in Fig. 1 and the dried gel was exposed to Kodak XAR-5 film for 1 hour (lane 1) or 30 hours (lanes 2 to 14) at −70°C. The relative amount of CD4 to Env proteins (1:18) in virus particles was determined by liquid scintillation counting of the corresponding bands after con A–agarose precipitation (lanes 6 and 11).

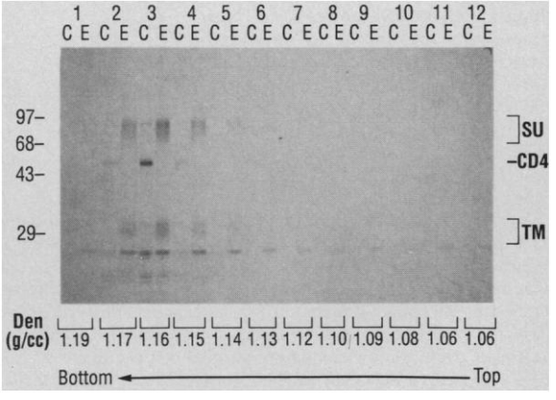

Fig. 3. CD4 and ALV Env proteins cofractionate in equilibrium density sucrose gradients. Approximately 10^7 QAH cells were transfected with 30 μg of pSVβCD4 plasmid DNA. Forty-eight hours after transfection, cells were metabolically labeled as described in the legend to Fig. 1. Radiolabeled virus particles were purified by centrifugation at 150,000*g* through 3 ml 20% sucrose onto a 1 ml 60% sucrose cushion in an SW41 rotor for 1 hour at 4°C. Virus particles removed at the interface were layered on top of a 10 ml 20 to 45% sucrose gradient and centrifuged at 150,000*g* for 16 hours in an SW41 rotor at 4°C. Twelve 1-ml fractions (1 to 12) collected from the bottom of this gradient were each diluted 1:3 before pelleting through 2 ml of 20% sucrose at 190,000*g* in an SW50.1 rotor for 1 hour at 4°C. Pelleted samples were lysed in NP-40 buffer (*19*) and immunoprecipitated with either C [the CD4-specific antisera Leu3A (Becton Dickinson)], or E [an env-specific antisera (*20*)]. Samples were separated by electrophoresis as in Fig. 1, and the dried gel was exposed to Kodak XAR-5 film for 6 days at −70°C. Den, density.

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CD4 was present in cosedimenting membrane vesicles derived from quail cells, we performed control experiments in which CD4 was expressed in uninfected quail QT6 cells. We observed concentrations of CD4 in the media approximately 5% of those found in the media from ALV-infected cells; since CD4 from the media of uninfected cells migrated at a density of 1.12 to 1.14 g/ml in linear sucrose gradients (*4*), we conclude that CD4 in these control experiments is contained in less dense membrane-derived vesicles.

The incorporation of CD4 into ALV particles was an unexpected result, because cell surface proteins are generally not efficiently assembled into virus particles. Indeed, most quail cell glycoproteins were excluded from ALV particles in our experiments (Fig. 2). Since human CD4 is normally expressed in lymphocytes, it seems likely that quail (QT6) fibroblast cells lack the proteins that normally interact with CD4 (for example, $p56^{lck}$) (*8*). Consequently, CD4 might differ from other glycoproteins at the surface of quail cells because it lacks interactions with other proteins. This raises the possibility that virus incorporation of host cell surface proteins might be prevented, at least in part, by the interactions between these proteins and cytoplasmic proteins.

At present we cannot explain why CR and CM proteins were assembled into ALV particles much less efficiently than was wild-type human CD4. However, we have obtained an intriguing result by transiently expressing CD4 and CR proteins in quail [R(-)Q No. 3] cells that contain an avian sarcoma virus genome lacking *env* (*9*). Virus particles produced from these cells incorporate CD4 or CR proteins with equal efficiencies (*4*). This suggests that Env proteins might compete with chimeric proteins, but not with CD4 proteins, for assembly into ALV particles. Alternatively, interactions between chimeric proteins and viral Env proteins might prevent their incorporation into virus particles.

CD4 is the first example of a nonviral cell surface protein with transmembrane and cytoplasmic tail domains shown to be efficiently incorporated into retroviral particles. Other studies have shown that Thy-1, which is attached to the cell surface by a glycosylphosphatidylinositol linkage (*10*), is incorporated into MLV particles (*11*). The uptake of cell surface proteins by virus particles could influence the host cell tropism of a virus if interactions between these proteins and their cognate receptors can direct virus infection of normally resistant cell types. This suggests a strategy to target viral vectors containing therapeutic genes to specific cell types. For example, since CD4 and the gp120 Env proteins of human immunodeficiency viruses type–1 and type–2 (HIV-1 and HIV-2) interact with high affinity (*12*), ALV (CD4) pseudotypes can be used to test if virus-associated CD4 proteins can target ALV vectors to cells expressing HIV Env glycoproteins. If so, it might be possible to use this sytem to specifically deliver retroviral vectors containing dominant negative mutations in *gag*, *tat*, or *rev* genes (*13*) to cells infected with HIV.

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21. The authors thank N. Landau, D. Littman, S. Hughes, M. Stoltzfus, C. Damsky, and R. Katz for generously providing reagents. We also thank members of the Varmus lab, especially P. Pryciak, D. Kaplan, and K. Kaplan for stimulating discussions and for critically reading this manuscript. This work was supported by grants from the NIH. J.A.T.Y. was a European Molecular Biology Organization postdoctoral fellow and is now supported by a postdoctoral fellowship from the Arthritis Foundation. H.E.V. is an American Cancer Society Research Professor.

26 June 1990; accepted 28 September 1990

The Role of β_2-Microglobulin in Peptide Binding by Class I Molecules

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Efficient transport of class I major histocompatibility complex molecules to the cell surface requires association of the class I heavy chain with endogenous peptide and the class I light chain, β_2-microglobulin (β_2M). A mutant cell line deficient in β_2M transports low amounts of nonpeptide-associated heavy chains to the cell surface that can associate with exogenously provided β_2M and synthetic peptide antigens. Normal β_2M-sufficient cells grown in serum-free media devoid of β_2M also require an exogenous source of β_2M to efficiently bind synthetic peptide. Thus, class I molecules on normal cells do not spontaneously bind or exchange peptides.

CYTOLYTIC T LYMPHOCYTES (CTLs) recognize peptide antigens bound within the antigen-binding groove of class I molecules (*1–4*). Peptides derived from processing of endogenously synthesized proteins become associated with class I molecules during their assembly in the endoplasmic reticulum (*5, 6*). Studies of mutant cell lines indicate that efficient transport of class I to the cell surface requires association of the class I heavy chain with both peptide and β_2M (*6–8*). Once expressed on the cell surface, class I molecules can bind or exchange peptide, as demonstrated by the ability of glutaraldehyde-fixed cells (*9*) or purified class I molecules (*10, 11*) to bind and present exogenously provided synthetic peptides for recognition by CTLs. However, this exchange is inefficient, as measured by the small percentage of purified class I molecules that can bind peptide (0.3%)

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