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Immunology

Phenotypic knockout of the high-affinity human interleukin 2 receptor by intracellular single-chain antibodies against the α subunit of the receptor

(anti-Tac/adult T-cell leukemia/gene therapy)

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ABSTRACT **The experimental manipulation of peptide growth hormones and their cellular receptors is central to understanding the pathways governing cellular signaling and growth control. Previous work has shown that intracellular antibodies targeted to the endoplasmic reticulum (ER) can be used to capture specific proteins as they enter the ER, preventing their transport to the cell surface. Here we have used this technology to inhibit the cell surface expression of the α subunit of the high-affinity interleukin 2 receptor (IL-2Rα). A single-chain variable-region fragment of the anti-Tac monoclonal antibody was constructed with a signal peptide and a C-terminal ER retention signal. Intracellular expression of the single-chain antibody was found to completely abrogate cell surface expression of IL-2Rα in stimulated Jurkat T cells. IL-2Rα was detectable within the Jurkat cells as an immature 40-kDa form that was sensitive to endoglycosidase H, consistent with its retention in a pre- or early Golgi compartment. A single-chain antibody lacking the ER retention signal was also able to inhibit cell surface expression of IL-2Rα although the mechanism appeared to involve rapid degradation of the receptor chain within the ER. These intracellular antibodies will provide a valuable tool for examining the role of IL-2Rα in T-cell activation, IL-2 signal transduction, and the deregulated growth of leukemic cells which overexpress IL-2Rα.**

Interleukin 2 (IL-2) is a cytokine of central importance to the immune system, stimulating growth and effector functions in a variety of cell types including T lymphocytes, B lymphocytes, monocytes, lymphokine-activated killer cells, and natural killer cells (1–4). The response of peripheral blood T cells to physiological concentrations of IL-2 is a two-stage, antigen-driven process. Antigenic stimulation of resting T cells first induces the appearance of high-affinity IL-2 receptors (IL-2Rs) and the production of IL-2 (5, 6). Interaction of IL-2 with the high-affinity receptor then initiates a cascade of signaling events (4, 7) that culminate in cell proliferation (5–9). The high-affinity IL-2R is a heterotrimer composed of α, β, and γ chains (4, 10). The β and γ chains (IL-2Rβ and γ_c) form a receptor of intermediate affinity (10), which is present on resting T cells (11). Expression of the 55-kDa α chain (IL-2Rα, Tac), and hence the high-affinity receptor, occurs only transiently following engagement of the T-cell antigen receptor and is a key point of regulation in the T-cell response to antigen (9).

The constitutive upregulation of IL-2Rα in certain T- and B-cell malignancies, and its association with T-cell activation, has made this receptor chain a natural target for suppressive immunotherapy. Strategies based on blockade of the high-affinity receptor by monoclonal antibodies (mAbs) directed against IL-2Rα have been used to dampen a variety of undesirable T-cell-mediated reactions, including allograft rejection, graft-versus-host disease, and some forms of autoimmunity (12). Humanized mAbs against IL-2Rα and immunotoxins such as IL-2 fused to the catalytic domain of pseudomonas exotoxin (IL-2 PE40) have also been used to selectively eradicate leukemic or otherwise harmful T cells bearing the high-affinity IL-2R (13).

The capacity to disrupt the expression of specific IL-2R chains in primary lymphocytes and established cell lines would provide a new dimension to the analysis of IL-2R signaling and be of potential therapeutic value. Intracellular antibodies (intrabodies) (14) targeted to the endoplasmic reticulum (ER) provide an effective means of inhibiting the expression of cell surface or secreted molecules (15, 35). In practice, a single-chain variable-region fragment (sFv) is the most convenient form of an antibody to express inside the cell. A sFv consists of immunoglobulin heavy- and light-chain variable (V_H and V_L) domains joined by a flexible peptide linker and is synthesized as a single polypeptide. The stable introduction of genes encoding single-chain intrabodies provides a powerful alternative to antisense RNA and other methods of gene inactivation. In this paper we demonstrate the use of two single-chain intrabodies to downregulate IL-2Rα and show that the incorporation of an ER retention signal can influence not only the distribution of an intrabody but also its stability, efficacy, and mode of action.

MATERIALS AND METHODS

Cells. C8166 is an immortalized T-cell line which was derived by *in vitro* infection of human cord blood with human T-lymphotropic virus type I (HTLV-I) (16) and expresses high levels of IL-2Rα. The human T-cell line Jurkat does not express IL-2Rα under normal circumstances but can be induced to do so by treatment with phorbol diester (17). The hybridomas HD245-332 and 7G7B6 (ATCC HB8784) were used as a source of anti-Tac and 7G7B6 mAbs. Anti-Tac and 7G7/B6 recognize different epitopes of human IL-2Rα and do not compete for binding (18, 19).

Construction of the sFvTac Gene. Total cellular RNA and cDNA were prepared from the HD245-332 hybridoma cells as described (20). V_H and V_L regions were amplified from the cDNA by PCR using the primers listed in Table 1 (A and B for V_H, C and D for V_L). A DNA segment encoding the interchain linker (ICL) $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ was amplified from an

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Abbreviations: ER, endoplasmic reticulum; HTLV-I, human T-lymphotropic virus type I; IL-2, interleukin 2; IL-2R, IL-2 receptor; IL-2Rα, IL-2R α subunit; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; sFv, single-chain variable-region fragment.

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Table 1. Primers used for construction of the sFvTac and sFvTacKDEL genes

Primer	Description*	Sequence (5′ to 3′)	Restriction site
A	Forward V_H leader	TTTAAGCTTACCATGGAAAGGCACTGGATC	*Hin*dIII
B	Reverse J_H	TGMGGAGACGGTGACCRWGGTCCCT	*Bst*EII
C	Forward V_κ	GAGCTCGTGCTCACMCARWCTCCA	*Sac* I
D	Reverse C_κ	ATTTGCGGCCGCTACAGTTGGTGCAGCATC	*Not* I
E	Forward J_H	GGGACCTCGGTCACCGTCTCCTCA	*Bst*EII
F	Reverse V_κ	TGGAGACTGGGTGAGCACGAGCTCAGATCC	*Sac* I
G	Forward V_H framework 1	TTTACCATGGCCGAGGTTCAGCTGCAGCAGTCTGGG	*Nco* I
H	Reverse C_κ-stop	GGGTCTAGACTCGAGGATCCTTATTATACAGTTGGTGCAGCATC	*Xba* I
I	Reverse C_κ-KDEL-stop	TTTTCTAGATTATTACAGCTCGTCCTTTTCGCTTACAGTTGGTGCAGCATC	*Xba* I

Translation start and stop codons in primers A, H, and I are underscored. In degenerate primer mixes (B and C), M = A or C, R = A or G, W = A or T.
*V_H and J_H, heavy-chain variable and joining regions; V_κ and C_κ, κ light-chain variable and constant regions.

existing sFv gene (anti-Tat 1) (21) by PCR using the primers E and F. The single-chain antibody gene, called sFvTac, was assembled from the V_H, V_L, and ICL fragments by overlap extension (22) followed by amplification with primers A and D. The complete sequence of the assembled sFvTac gene was determined by the dideoxy chain-termination method (23).

Expression of the sFvTac Gene in *Escherichia coli*. For expression in *E. coli*, the assembled sFvTac gene was reamplified with primers G and D (Table 1), digested with *Nco* I and *Not* I, and ligated into the vector pHEN1 (24). In pHEN1, the native anti-Tac heavy-chain leader sequence is replaced by a signal sequence from the bacterial *pelB* gene, which directs the protein into the bacterial periplasm, and a c-Myc peptide is added to the C terminus for detection purposes. Periplasmic extracts containing the sFvTac protein were prepared from isopropyl β-D-thiogalactopyranoside-induced cultures by hypotonic shocking of the cells (21).

Immunostaining of Cells for Flow Cytofluorometry. Jurkat or C8166 cells were incubated sequentially for 45 min on ice with (*i*) bacterial periplasmic extract; (*ii*) mAb 9E10, which recognizes the C-terminal Myc peptide (25); and (*iii*) fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Boehringer Mannheim). Alternatively, steps *i* and *ii* were replaced by a single incubation with one of the following mAbs: anti-Tac for IL-2Rα; W6/32 for class I major histocompatibility complex proteins (ATCC HB95); Leu-3a for CD4, Leu-5b for CD2, and Leu-4 for CD3 (Beckton Dickinson); and pan αβ for the T-cell antigen receptor (AMAC, Westbrook, ME).

Construction and Stable Transfection of Mammalian Cell Expression Vectors. For expression in mammalian cells, the assembled sFvTac gene was reamplified by PCR using primers A and H or A and I. The H primer introduces a stop codon at the end of V_L. The I primer introduces six additional amino acids (Ser-Glu-Lys-Asp-Glu-Leu, SEKDEL) at the C terminus, followed by a stop codon. The amplified fragments were digested with *Hin*dIII and *Xba* I and ligated into the vector pRc/CMV (Invitrogen). The resulting plasmids, designated pCMVTac and pCMVTacKDEL, were linearized at an *Xmn* I site within the β-lactamase gene and introduced into Jurkat cells by electroporation. G418 selection was applied at 0.8 mg/ml, and 2 weeks later, G418-resistant cells were seeded at 0.3 cell per well in 96-well plates.

Metabolic Labeling of Cells and Immunoprecipitation. Jurkat cells (1×10^7) stimulated for 6–8 hr with phytohemagglutinin (PHA, 1 μg/ml) and phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) were washed once in phosphate-buffered saline and incubated for 6–12 hr in cysteine-free RPMI 1640 containing 10% fetal bovine serum, antibiotics, and 50 μCi of [^{35}S]cysteine (DuPont/NEN) per ml (1 μCi = 37 kBq). Cells to be labeled in the presence of 20 mM methionine methyl ester, 50 mM NH_4Cl, or 5 μg of brefeldin A per ml were stimulated for 5 hr with PHA and PMA, preincubated with the compound for 1 hr, washed, and labeled with [^{35}S]cysteine for 4 hr in the presence of the relevant compound. The labeled cells were washed with phosphate-buffered saline, and solubilized in 1 ml of RIPA buffer [0.15 M NaCl/0.05 M Tris·HCl, pH 7.2/1% (vol/vol) Triton X-100/0.1% (wt/vol) sodium dodecyl sulfate (SDS)/1% (wt/vol) sodium deoxycholate). IL-2Rα was immunoprecipitated by incubation of clarified lysates with mAb 7G7/B6 coupled to protein A-Sepharose beads (Pharmacia). The single-chain antibodies were immunoprecipitated with polyclonal rabbit anti-mouse IgG (Sigma). Samples were electrophoresed in SDS/10% polyacrylamide gels and the gels were treated with EN^{3}HANCE (DuPont/NEN) prior to autoradiography.

Pulse–Chase Analysis. Jurkat cells were stimulated for 8 hr with PHA and PMA, washed in phosphate-buffered saline, and incubated at 5×10^6 cells per ml for 2.5 hr in cysteine-free medium. [^{35}S]Cysteine was then added at 100 μCi/ml (0.2 mM) for 30 min and the cells were then diluted 5-fold with complete medium supplemented with 2 mM nonradioactive L-cystine. Cells (4×10^6) were harvested at timed intervals and processed for immunoprecipitation as described above.

Endoglycosidase H Treatment. IL-2Rα immunoprecipitated from Jurkat cells with mAb 7G7/B6 was eluted from the protein A-Sepharose beads by boiling for 3 min in 2× endoglycosidase H digestion buffer [100 mM sodium citrate, pH 5.5/1% (wt/vol) SDS/5% (vol/vol) 2-mercaptoethanol]. An equal volume of distilled water was then added and the sample was incubated for 3 hr at 37°C in the presence or absence of endoglycosidase H (Boehringer Mannheim) at 0.05 unit/ml.

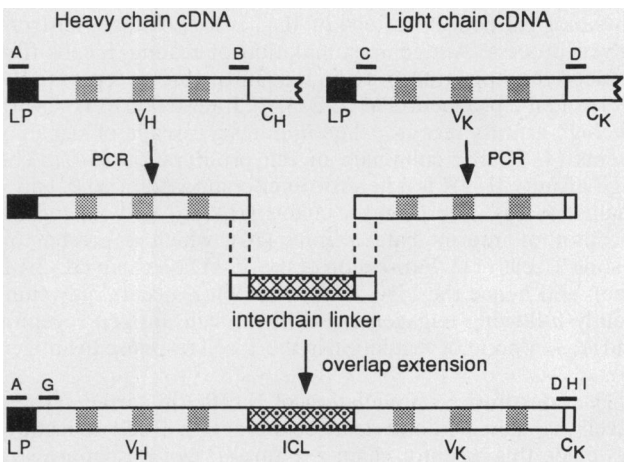

FIG. 1. Construction of sFvTac genes. Stippled areas represent hypervariable regions of the immunoglobulin which form the antigen binding site. A–I are PCR primers listed in Table 1. LP, leader peptide; V_H, V_κ, C_H, and C_κ represent heavy-chain and κ light-chain variable and constant regions; ICL, interchain linker.

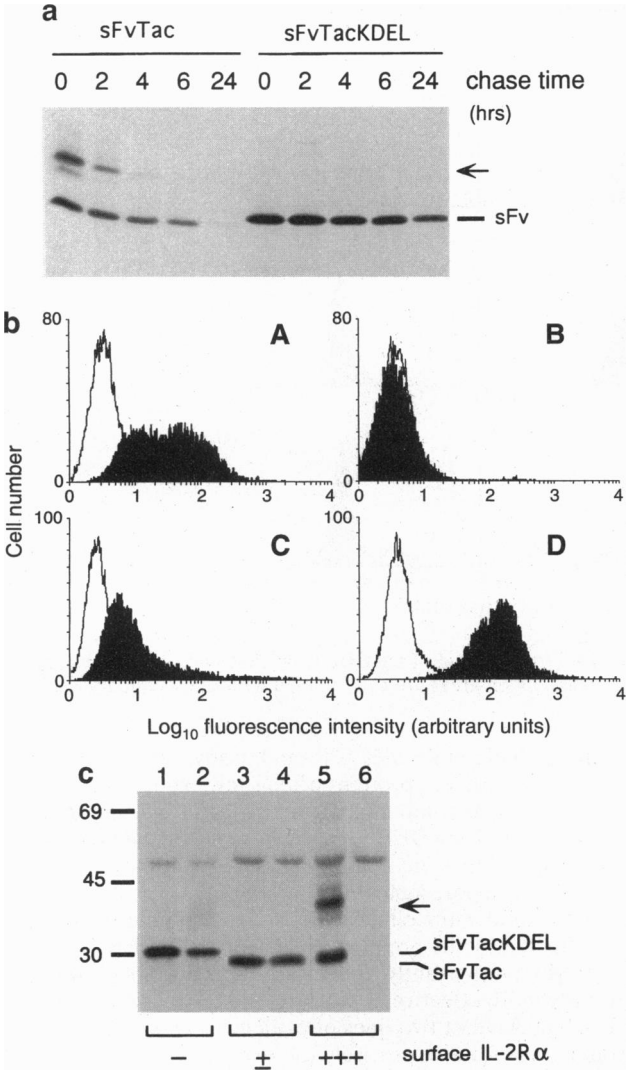

FIG. 2. (*a*) Pulse–chase analysis of sFvTac and sFvTacKDEL in stably transfected Jurkat cells. (*b*) Flow cytometric analysis of IL-2Rα expression in Jurkat clones. Parental Jurkat cells (*A*), sFvTacKDEL clone 8 (*B*), sFvTac clone 17 (*C*), and sFvTac clone 20 (*D*) were stained with anti-Tac mAb before (open area) and after (solid area) 18 hr of stimulation with PHA and PMA. (*c*) SDS/PAGE analysis of sFvTacKDEL and sFvTac immunoprecipitated from PHA/PMA-stimulated Jurkat cells. Lanes 1 and 2, sFvTacKDEL clones 3 and 8; lanes 3–5, sFvTac clones 17, 18, and 20; lane 6, nontransfected Jurkat cells. Molecular size markers (kDa) are at left. Arrows in *a* and *c* indicate a 38-kDa protein that coprecipitates with sFvTac in lysates from some cells.

RESULTS

Construction of sFvTac, a Single-Chain Antibody Against IL-2Rα. A sFv of the anti-Tac mAb was constructed as outlined in Fig. 1. The sFvTac protein was first synthesized in *E. coli* and shown by flow cytometry to bind specifically to a cell line (C8166) which expresses high levels of IL-2Rα (data not shown). A control sFv directed against the human immunodeficiency virus type 1 Tat protein did not bind to the C8166 cells, and neither sFv bound to unstimulated Jurkat cells, which do not express IL-2Rα.

Stable Expression and Intracellular Retention of ER and Non-ER Forms of sFvTac. Two versions of the sFvTac gene, differing only in the presence or absence of a C-terminal ER retention signal (amino acids KDEL), were stably transfected into Jurkat cells and single cell clones were generated. Both versions contain the native V_H leader peptide, to direct the protein into the ER. Pulse–chase analysis revealed the sFv molecules to be extremely stable, exhibiting intracellular half-lives of 4–6 hr for sFvTac and >30 hr for sFvTacKDEL (Fig. 2*a*). Only trace amounts of antibody were detectable in the culture supernatants, indicating that neither sFv protein was secreted to a significant extent. The basis for intracellular retention of the sFvTac antibody is not clear, but the same phenomenon has been noted with other sFv molecules that lack a known retention signal (15, 35, 36).

Intracellular Expression of sFvTac Inhibits Cell Surface Expression of IL-2Rα. The ability of sFvTac intrabodies to downregulate IL-2Rα was assessed in Jurkat cells, which do not express IL-2Rα under normal circumstances but can be induced to do so by treatment with PMA and PHA (ref. 17 and Fig. 2*b*). Sixteen of 16 independent clones expressing the ER antibody sFvTacKDEL showed complete inhibition of IL-2Rα induction, consistent with a block to the cell surface transport of IL-2Rα in these cells (Fig. 2*bB*). Three of 15 clones expressing sFvTac also showed downregulation of IL-2Rα, but this was incomplete, as evidenced by some breakthrough expression of IL-2Rα at the cell surface (Fig. 2*bC*); the remaining 12 clones showed normal or even high levels of cell surface IL-2Rα after PHA/PMA stimulation (Fig. 2*bD*). In each of these 12 clones, an unidentified 38-kDa cellular protein was found to coprecipitate with sFvTac and may have interfered with the formation of sFvTac/IL-2Rα complexes (Fig. 2*c*, lane 5). The 38-kDa protein was present in unstimulated Jurkat cells, had a 90-min half life, and was not recognized by mAb 7G7/B6 (Fig. 2*a* and data not shown). In spite of its similar size, the interfering protein is unlikely, therefore, to be related to the 40-kDa precursor of IL-2Rα.

The IL-2Rα downregulation observed in all sFvTacKDEL and some sFvTac clones was a specific effect as the cell surface transport of other plasma membrane proteins, including class I major histocompatibility complex molecules, the T-cell antigen receptor, CD2, CD3, and CD4, was unaffected (Fig. 3 and data not shown).

IL-2Rα Is Retained as an Endoglycosidase H-Sensitive Precursor in Cells Expressing sFvTacKDEL. In common with most secreted and cell surface proteins, newly synthesized IL-2Rα undergoes extensive posttranslational processing in the ER and Golgi compartments. Electrophoretic analysis of IL-2Rα immunoprecipitated from C8166 cells or PHA/PMA-treated Jurkat cells revealed the presence of two forms: a predominant 55-kDa form (p55), representing the mature receptor, and a less abundant 40-kDa form (p40), which has previously been identified as an immature form of IL-2Rα (Fig. 4*a*, lane 1) (26). Pulse–chase experiments confirmed the 40-kDa protein to be a natural precursor of p55 which is chased into the higher molecular weight form within 2 hr in control Jurkat cells (Fig. 4*a*). PHA/PMA treatment of Jurkat cells expressing sFvTacKDEL was associated with intracellular accumulation of the p40 precursor and the complete absence of p55 (Fig. 4 *a* and *b*, lane 3). Moreover, a 30-kDa protein identical in size to the single-chain antibody coprecipitated with p40 IL-2Rα in these cells (Fig. 4 *a* and *b*). The p40 form of IL-2Rα was sensitive to endoglycosidase H digestion and is therefore a high-mannose glycoprotein (Fig. 4*b*, lanes 1–4). The absence of complex oligosaccharides, which are added in the medial Golgi compartment and render the glycoprotein resistant to endoglycosidase H, suggests that p40 is located in a pre- or early Golgi compartment. As expected, the mature p55 was resistant to endoglycosidase H digestion (Fig. 4*b*, lanes 1 and 2).

Rapid Degradation of IL-2Rα in Cells Expressing sFvTac. In sFvTac clones that showed downregulation of IL-2Rα (e.g., sFvTac.17), very little IL-2Rα (either p55 or p40) could be detected by immunoprecipitation in the cell lysates, suggesting rapid degradation of these antibody/IL-2Rα complexes (Fig. 4*b*, lanes 5 and 6). The degradation appeared to occur by a

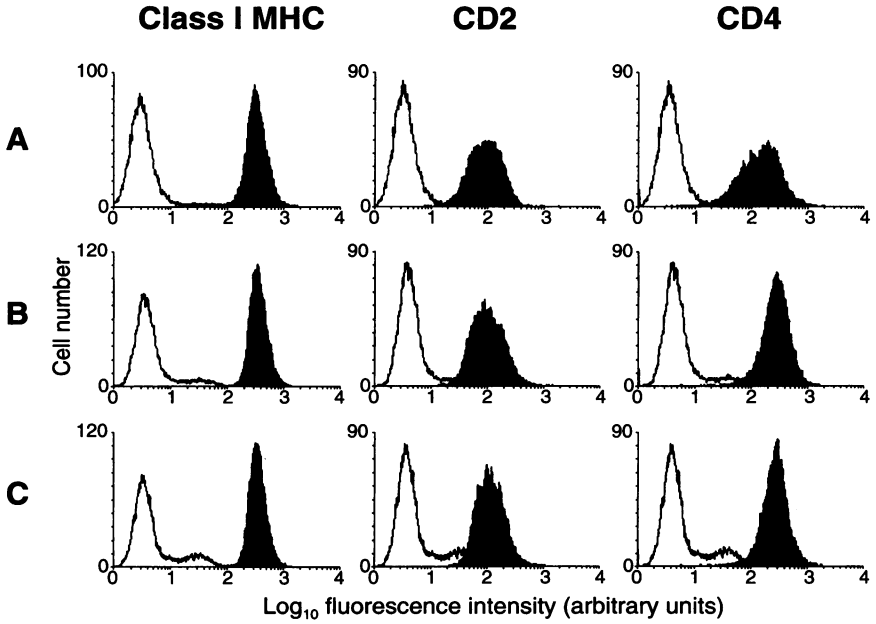

FIG. 3. Flow cytometric analysis of Jurkat cells expressing no sFv (row A), sFvTac (row B), or sFvTacKDEL (row C) stained with mAbs against class I major histocompatibility complex (MHC) proteins, CD2, or CD4 (filled areas). Open areas are cells stained with the secondary antibody alone.

nonlysosomal mechanism, as the presence of methionine methyl ester, a lysosomal inhibitor (27), did not result in the accumulation of IL-2Rα in sFvTac.17 cells (Fig. 4*c*). In addition, no accumulation of IL-2Rα was observed in sFvTac.17 cells treated with brefeldin A (Fig. 4*c*), a fatty acid derivative which causes disassembly of the Golgi apparatus and prevents proteins from exiting the ER (28). This strengthens the suggestion that IL-2Rα degradation occurs within the ER. Some p40 processing was observed in the brefeldin A-treated cells, consistent with the known recycling of Golgi processing enzymes to the ER in the presence of brefeldin A (28).

DISCUSSION

Molecular techniques for inhibiting the expression of specific genes allow a highly refined approach to the analysis and manipulation of cellular pathways. Antisense RNA, ribozymes, dominant negative mutants, and targeted gene disruption have all been successfully used to inhibit the expression or function of specific genes (29–32) but may not be applicable in every situation. Single-chain intrabodies synthesized by the cell and targeted to specific cellular compartments represent the most recent innovation in this field and have been used to inactivate proteins in the ER, cytoplasm, and nucleus (15, 21, 35). For reasons discussed below, this approach may be especially well suited to proteins trafficking through the secretory system, and here we describe the use of two intrabodies to inhibit the cell surface expression of IL-2Rα, an essential component of the high-affinity IL-2R (1, 4).

In theory, there are multiple points within the secretory pathway at which an intrabody could be placed to bind and divert a trafficking protein from its ultimate destination. The ER may be the most strategic location, as the tubular architecture of this organelle, combined with the precise channeling of proteins through the secretory pathway, should maximize the chances of interaction between a resident antibody and the target protein. Unlike more distal parts of the secretory system, peptide signals required for ER retention of soluble proteins are well characterized and consist of the C terminal tetrapeptide KDEL (33). The ER is also the natural site of antibody assembly, being residence to molecular chaperones such as BiP and GRP94, which assist in the correct folding of immunoglobulin molecules (34), and, lastly, offers the advantage that ER-resident proteins often show extended half-lives.

Here, we have compared the activities of two intrabodies, sFvTac and sFvTacKDEL, which differ only by the presence of an ER retention signal on the latter. Multiple aspects of intrabody behavior, including the stability, efficacy, binding specificity, and intracellular fate of the immune complexes, were affected by the presence or absence of an ER retention signal. Most importantly, the ER-resident intrabody was significantly more effective at blocking the cell surface transport of IL-2Rα. At least two lines of evidence suggest that the ER intrabody operates in precisely the expected manner—i.e., by binding and holding the receptor chain in the ER. First, the sFvTacKDEL intrabody was coprecipitated with IL-2Rα, suggesting a physical interaction between the two proteins inside the cell. The reciprocal coprecipitation was difficult to demonstrate due to the vast excess of intrabody over IL-2Rα. Second, IL-2Rα was detectable within these cells as an immature 40-kDa form that was sensitive to endoglycosidase H, consistent with its retention in a pre- or early-Golgi compartment. The sFvTac intrabody was also able to downregulate IL-2Rα but, unlike sFvTacKDEL, gave a "leaky" phenotype characterized by low-level expression of IL-2Rα at the cell surface. In contrast to the stable complexes formed between the IL-2Rα and sFvTacKDEL, IL-2Rα complexed to sFvTac appeared to undergo rapid degradation within the ER.

IL-2Rα is a growth factor receptor of clinical importance, being overexpressed in a variety of T- and B-cell leukemias, most notably in HTLV-I-associated adult T-cell leukemia (13), where it is strongly implicated in the deregulated growth of these cells. The availability of an effective tool for inhibiting the expression of IL-2Rα will enable studies to clarify the role of this receptor chain in HTLV-I leukemogenesis and, in combination with targeted gene delivery, may provide a mechanism for downregulating IL-2Rα in selected cells *in vivo*.

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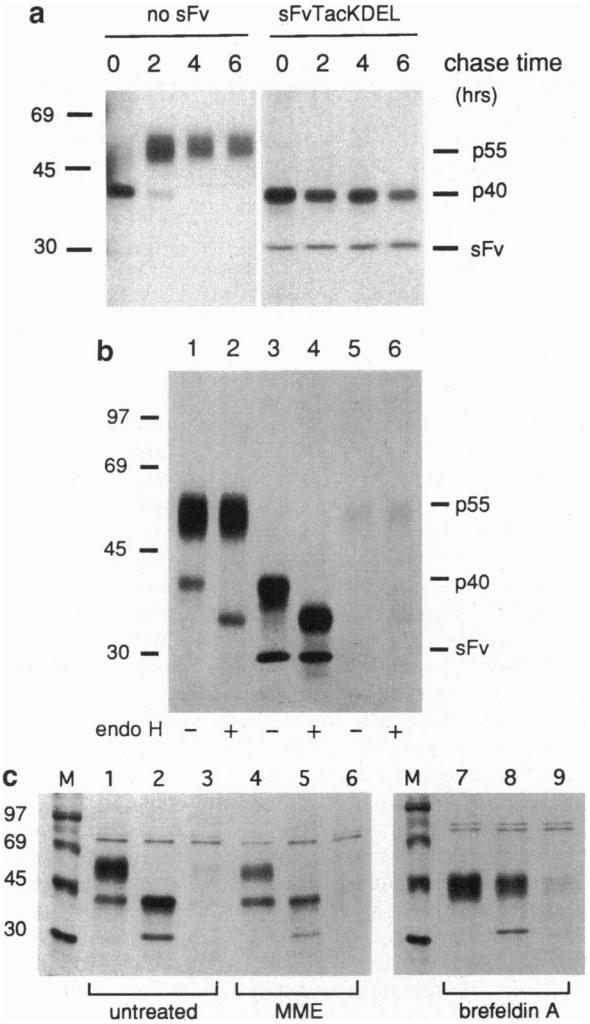

FIG. 4. (*a*) Pulse–chase analysis of IL-2Rα in PHA/PMA-stimulated Jurkat cells and sFvTacKDEL clone 8. (*b*) Endoglycosidase (endo) H sensitivity of IL-2Rα immunoprecipitated with mAb 7G7/B6 from control Jurkat cells (lanes 1 and 2), sFvTacKDEL clone 8 (lanes 3 and 4), and sFvTac clone 17 (lanes 5 and 6). The p55 and p40 forms of IL-2Rα are indicated. (*c*) Immunoprecipitation of IL-2Rα from control Jurkat cells (lanes 1, 4, and 7), sFvTacKDEL clone 8 (lanes 2, 5, and 8), and sFvTac clone 17 (lanes 3, 6, and 9) treated with 20 mM methionine methyl ester (MME) or 5 μg of brefeldin A per ml or not treated. Lane M, molecular size standards (sizes in kilodaltons are at left).

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