Internalization of the Lymphocytic Surface Protein CD22 Is Controlled by a Novel Membrane Proximal Cytoplasmic Motif*

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CD22 is a key receptor on B-lymphocytes that modulates signaling during antigenic stimulation. We have defined a novel cytoplasmic motif in human CD22 that controls its unusually rapid turnover at the plasma membrane. Chimeric and mutated CD22α cDNA vectors were constructed and stably transfected in CD22-negative Jurkat T-lymphocytic cells. Two assays were employed to measure CD22α internalization: first, cytoplasmic uptake of radiiodinated anti-CD22 monoclonal antibody; and second, lethal targeting of a toxin, saporin, into cells via CD22 using bispecific F(ab')2 (anti-CD22 × anti-saporin) antibody. Results showed that CD22α lacking a cytoplasmic tail was not internalized and that replacement of the cytoplasmic tail of CD19 with that of CD22α resulted in a chimeric molecule that behaved like CD22α and internalized rapidly. Step-wise deletion of the cytoplasmic tail of CD22α located the internalization motif to a polar region of 11 residues (QRRWKRTQSQQ) proximal to the plasma membrane, a part of the molecule predicted to form a coil or turn structure. Interestingly, additional CD22 mutants showed that the two glutamine residues sandwiching the serine are critical to internalization but that the serine itself is not.

CD22 is a 135-kDa B-lymphocyte-specific glycoprotein and a member of the recently described sialoadhesin family of molecules (1–3). It is a key accessory molecule that first appears at the late pro-B-cell stage of differentiation as a cytoplasmic protein and is then expressed simultaneously with IgD as a surface membrane receptor on most mature B-cells (2). Although the nature of the CD22 ligand(s) is not fully defined, it is known to bind sialoglycoconjugate NeuAcα2–6Galβ1–4GlcNAc, which is widely distributed on N-linked carbohydrates (3, 4). The principal function of CD22 is to regulate B-cell responses, which is probably achieved by recruiting key molecules to the antigen receptor complex (5, 6). The cytoplasmic domain is rapidly tyrosine-phosphorylated upon ligation of the B-cell antigen receptor and associates with a range of intracellular signaling molecules that includes tyrosine phosphatase SH2-domain containing tyrosine phosphatases (1, 2, 7). The importance of CD22 in modulating B-cell responses has recently been confirmed in knockout mice, which show augmented antibody responses, expanded peritoneal B-1 cell populations, and increased levels of circulating autoantibodies (8–10).

In humans, two isoforms of CD22 have been identified (11, 12). A larger species, CD22β, which has seven extracellular Ig-like domains and a cytoplasmic domain containing 141 amino acids, and a smaller isoform, CD22a, which has five extracellular Ig-like domains (domains 3 and 4 are deleted) and a cytoplasmic region that is 23 amino acids shorter than that of CD22β. Although CD22β is by far the predominant species, and the only form identified in mouse, a smaller immunoprecipitate has been found that may correspond to the CD22α in human (13). Both isoforms contain tyrosine residues in their cytoplasmic tails, 3 residues in the α form and 6 in the β form. Most of these tyrosine residues are arranged to form immunoreceptor tyrosine-based inhibition motifs (ITIM), consisting of a 6-amino acid stretch with the consensus sequence (I/L/V)XYXX(L/V), and/or potential immunoreceptor tyrosine-base activation motifs (ITAM) (12, 14–16). In addition, CD22 contains a XXYM sequence recognized by the NH2-terminal SH2 domain of the p85 subunit of phosphoinositide 3-kinase. Thus, CD22 has the capacity to modulate B-cell behavior via a wide range of intracellular, tyrosine-dependant signaling molecules.

In addition to any signaling role, CD22 shows an unusual and unexplained capability to internalize rapidly from the cell surface to the cytoplasm. Recent studies have shown that CD22 internalizes constitutively on unstimulated B-cell lines, and this is followed by degradation without detectable recycling (17). This internalization probably explains, at least in part, the success achieved when CD22 has been employed as a target molecule for delivering toxins into neoplastic B-cells using antibody immunotoxins (18, 19) and bispecific antibodies (BsAbs) (20–22). We find BsAbs with specificity for CD22 and the ribosome-inactivating protein, saporin (anti-CD22 × anti-saporin), is unusually effective at delivering saporin into neoplastic B-cells and thereby inhibiting protein synthesis and eradicating tumors in animals (21) and patients (22). Most other membrane molecules do not work as efficiently (20). In the present study, we have investigated the molecular structure of the cytoplasmic domain of CD22 and identified a new motif that controls its internalization from the cell surface and may account for its unusual immunotherapeutic properties.

MATERIALS AND METHODS

Human Cell Lines—The Burkitt’s lymphoma cell line Daudi and the acute T-cell leukemia cell line Jurkat were used in these studies. Both lines were maintained in RPMI 1640 medium containing 10% fetal calf serum (Life Technologies, Inc.) and supplemented with 1 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml benzyl penicillin, and 100 µg/ml streptomycin sulfate. Cells were maintained continuously in the

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§ The abbreviations used are: BsAb, bispecific antibody; mAb, monoclonal antibody; PCR, polymerase chain reaction.
logarithmic phase of growth by passage at regular intervals.

**Antibodies and Preparation of Bispecific Antibodies**—The mAbs used in this study include anti-CD19 (RFB9) (20), anti-CD22 mAb (4KB128) (23), anti-CD38 mAb (AT8/35) (24), and anti-saporin mAb (anti-sap1 and anti-sap9) (25). Hybridoma cells were expanded in stationary culture (95% suspension) in RPMI medium (Gibco, Life Technologies, Inc.) and IgG purified using protein A affinity chromatography as described previously (26). Bispecific Fab ab\(^1\) heterodimers were constructed using well established procedures in which Fab' fragments from two different mAbs are cross-linked via their hinge region SH groups using the bis-maleimide linker o-phenylenediamineimide (26).

cDNA and Construction of CD22 Mutants—cDNA clones of human CD19 and CD22a were kind gifts from Dr. Brian Seed. The CD19 clone provided, which will be called CD19\(^{HS}\) throughout this paper, is not the full-length, wild type transcript, but a truncated version that has its 5' exon and amplified sequence (27). Fortunately, for the present study we were only interested in a full-length, wild type transcript, but a truncated version that has its 3' end. The 5' half of the primer bound to the 3' end of the primer bound to the 3' end of the primer bound to the 3' end was deleted, which was accomplished by PCR using primer pairs 22\(^F\) (TTGAGATCCAGACCGGGAACACGGCTTCGACACG) and 22\(^A\)ctyR (TCGCTAGATCTAGACCCACAGATTGGCAGAAG). To delete the whole cytoplasmic tail, a stop codon (TAG) was introduced after Leu510 in the transmembrane region. PCR was performed in a Protocol Thermal Cycler (AMS Biotechnology) using primer pairs containing 19/22\(^R\) (GCGGAAACAGGCTTGCAC) and 22\(^A\)ctyR. The PCR products were then added, and a further 10 cycles were completed to give a full-length chimeric protein containing the extracellular and transmembrane region of CD19\(^H\)S and the cytoplasmic tail of CD22a.

As described above, the CD22a molecule is a minor isoform of CD22 that may not be expressed in normal B-cells. Because of its truncated cytoplasmic domain and reduced number of tyrosines, it is likely to have a number of signaling properties that are different from those of the predominant CD22a isoform. For the purposes of this study, the membrane proximal regions of the molecule that control internalization (see under “Results”) are identical in CD22a and CD22β.

Both CD19\(^H\)S and CD22a cDNA clones were subcloned into pcDNA3 (Invitrogen, San Diego, CA) using the HindIII–NotI sites for CD19\(^H\)S and the XhoI site for CD22a. Deletion of the CD22a cytoplasmic tail was accomplished by PCR using primer pairs 22\(^F\) (TTGAGATCCAGACCGGGAACACGGCTTCGACACG) and 22\(^A\)ctyR (TCGCTAGATCTAGACCCACAGATTGGCAGAAG). To delete the whole cytoplasmic tail, a stop codon (TAG) was introduced after Leu510 in the transmembrane region. PCR was performed in a Protocol Thermal Cycler (AMS Biotechnology Ltd.) using CD22a full-length cDNA as template. All reagents were from the Repli-Pack kit (Boehringer Mannheim). The PCR construct generated contained both the extracellular and transmembrane domains and was then subcloned into pGEM-T vector (Promega, Madison, WI) for sequencing and into expression vector pcDNA3 for transfection.

To replace the cytoplasmic region of CD19\(^H\)S with that of CD22a, a two-step recombinant PCR strategy was used (28). In this method, a pair of chimeric PCR primers was designed to span the chimeric junction (5' end: CGCTGGCACGTTCCTAAGTAGTGAAGAATGCCCA, 3' end: GAGCTTAAGATGAAGAATGCCCA), bound to the 3' end of the transmembrane region of the antisense strand of CD19\(^H\)S DNA between base pairs 939 and 954, whereas the 5' end of the primer bound to the 5' end of the transmembrane region of the antisense strand of CD22a cDNA (base pairs 1622–1638). A second chimeric primer, 19/22\(^R\) (TGGGCAGCATTTCTTCATCTTAAGCTCCAGCGACGTTG), the complimentary oligonucleotide to CD22a, was made with 200 μl of leucine-free RPMI medium containing 1 mg/ml Geneticin (Life Technologies, Inc.) and 5% fetal bovine serum. Each pair of reagents was selected to bind to the cell via a single epitope and to respectively replace the corresponding segments of CD19 and CD22, or CD38 and to the saporin via two different epitopes (29, 30). Cells (10⁷/ml) were incubated with 125I-labeled mAb (2⁹⁹Ci/ml) at 4 °C for 1 h to allow binding and then to warmed to 37 °C to allow internalization. Aliquots were taken at various intervals, washed twice in 10% supplemented RPMI medium before centrifugation at 13,000 rpm for 45 s through a cold solution of 5% bovine serum albumin (in phosphate-buffered saline) and counted (36). For each construct, the internalization count, the medium in the second wash was replaced by a HCl-titrated buffered RPMI medium (pH 2.0), which removes surface bound activity and thus allows the internalized 125I-antibodies to be measured and surface binding estimated by subtraction of the internalization counts from the total counts.

**H[3H]Leucine Uptake by Cells Cultured with BsAb-Saporin Complexes**—To measure the incorporation of [3H]leucine into protein, Jurkat T-cells were cultured with human CD19 BS and CD22a—We decided to investigate the molecular basis of CD22a internalization and in particular the structural features of the cytoplasmic tail that control delivery into the cell. Our strategy was to construct a panel of CD22a cDNA containing vectors in which the cytoplasmic tail of CD22 was selectively altered. These vectors were then used to make stable transfec-
molecules in which the cytoplasmic tail of CD22α was spliced onto the extracellular and transmembrane region of CD19 (CD19Δcyt/22αcyt), together with a range of CD22α mutants containing truncated cytoplasmic tails and molecules in which certain amino acids were changed.

The Jurkat T-cell leukemia line was chosen as a suitable negative lymphoid cell line in which to express these molecules. Jurkat cells were stably transfected and then screened by flow cytometry to isolate clones that expressed the various mutated CD22α molecules. As shown in Fig. 2, both wild type CD19BS (Fig. 2A) and CD22α (Fig. 2C) were expressed at easily detectable levels, showing that neither surface B-cell marker required associated B-cell restricted molecules for their expression. The CD22α clone was employed as a positive control for assessing CD22α internalization throughout the remainder of this investigation, whereas CD19BS Jurkat cells provided our negative controls (see below). Fig. 2 also shows the levels of mutated CD22α expression for the other transfectants. With the exception of one clone, CD22αΔcyt (Fig. 2I), which gave only weak immunofluorescence staining, relative to other transfectants, all cells expressed levels of surface CD22α that were as high as or even higher than that of CD2β generally expressed on normal B-cells and B-cell lines (data not shown). The expression of all the constructs was also confirmed by immunoprecipitation of Nonidet P-40 lysates of the radioiodinated transfectants (results not shown).

| Clone      | Extracellular | Cytoplasmic | IC50 (M) |
|------------|---------------|-------------|----------|
| CD19α      | CD19α         |             | 2.6±0.4×10^-10 |
| CD19Δcyt22αcyt | CD19Δcyt22αcyt |             | <1×10^-11   |
| CD22α      | CD22α         |             | 2.0±2.0×10^-10 |
| CD22αΔcyt  | CD22αΔcyt     |             | 1.2±0.8×10^-10 |
| CD22αΔcyt/600 | CD22αΔcyt/600 |             | 2.5±0.5×10^-10 |
| CD22αΔcyt/656 | CD22αΔcyt/656 |             | 6.0±0.2×10^-10 |
| CD22αΔcyt/527 | CD22αΔcyt/527 |             | 8.5±0.4×10^-10 |
| CD22αΔcyt/524 | CD22αΔcyt/524 |             | 5.0±1.2×10^-10 |
| CD22αΔcyt/523 | CD22αΔcyt/523 |             | 3.5±0.5×10^-10 |
| CD22αΔcyt/522 | CD22αΔcyt/522 |             | 8.0±1.0×10^-10 |
| CD22αΔcyt/521 | CD22αΔcyt/521 |             | 1.2±1.1×10^-10 |
| CD22αΔcyt/520 | CD22αΔcyt/520 |             | 3.8±0.4×10^-10 |

Cells were incubated with radiolabeled anti-CD22 or anti-CD19 mAb for 1 h at 4 °C and then warmed to 37 °C for various intervals. The cells were then acid-washed to remove surface bound mAb, and therefore, any increase in the cell-associated radioactivity must be due to the endocytosis of the antigen-antibody complex. The results show that both Daudi cells (CD22β+) and CD22α-expressing Jurkat cells accumulated 125I-labeled anti-CD22 mAb at a similar rate (Fig. 3A). There was a sharp initial uptake of mAb in the first 2 h, after which the level remained quite steady. The accumulation of intracellular 125I-labeled mAb by these cells was accompanied by a corresponding loss of surface-associated mAb. In contrast, the Jurkat cells expressing the CD22αΔcyt, in which the cytoplasmic region of the molecule had been removed (Fig. 1), were unable to accumulate significant levels of intracellular radiolabel, and the mAb remained on the surface of the cells. This result is consistent with the cytoplasmic tail playing a central role in CD22α turnover at the cell surface and with immunoelectron microscopy data that showed that CD22α is strongly clustered around coated pits in transfected Jurkat cells but that following removal of the cytoplasmic tail the truncated molecules are evenly distributed along the plasma membrane (data not shown).

The importance of the CD22α cytoplasmic tail in mediating internalization was confirmed using cells expressing the chimeric CD19Δcyt/22αcyt molecule in which the CD22α cytoplasmic tail had been grafted onto the extracellular and transmembrane domains of CD19BS (Fig. 3B). Whereas CD19 (endogenous expression on Daudi cells) and CD19BS (transfected Jurkat) were relatively poor at taking up radioiodinated anti-CD19 mAb, once the cytoplasmic tail of CD19BS was replaced by that of CD22α, it behaved like the intact CD22α and internalized mAb very effectively. In fact, for reasons that are not understood, uptake of 125I-labeled mAb by this chimeric molecule was quicker and more extensive than by the transfected CD22α. These results suggest that there is an internalization signal within the cytoplasmic region of CD22α.

The Cytoplasmic Tail of CD22α Controls Effective Delivery of Toxin into Target Cells—For the next stage of this investigation we used the Jurkat transfectants as target cells in an assay which measures the cytotoxicity of a toxin, saporin, when delivered to the cells via CD19BS or CD22α. In the assay, cells are exposed to a BsAb ([anti-CD22 × anti-saporin] or [anti-CD19 × anti-saporin]) at 1 μg/ml together with saporin at various concentrations for 24 h at 37 °C, during which period the BsAb-saporin complexes are taken up by the transfectants according to the readiness of the target antigen (CD22α or CD19BS) to internalize. The cultures were then pulsed for a further 12 h with [3H]leucine to establish the level of protein synthesis in any surviving cells. As a positive control to show that all Jurkat cells, transfected or not, were sensitive to killing with BsAbs-saporin complexes, we used a BsAb directed at CD38 ([anti-CD38 × anti-saporin]), which is expressed constitutively by these T-cells.

Fig. 4 shows a typical set of results with transfected Jurkat cells. The CD22α molecule is highly effective at delivering BsAbs-saporin complexes, giving an IC50 for saporin of approximately 2 × 10^-10 μ, a value close to that achieved with the anti-CD19 BS Ab. This showed that saporin delivered by both anti-CD22 and anti-CD38 BsAbs was approximately 2500-fold more toxic than saporin alone (5 × 10^-7 μ). The increase in the toxicity of CD22-specific BsAbs on the CD22α-transfected Jurkat cells was similar to that observed when B-cell lines were treated with this reagent (20), suggesting that the transfected CD22α molecules behave similarly to native CD22β on B-cells. In contrast, when the tail of CD22α was removed and the
truncated molecule expressed in the CD22αD cyt-transfected cells, then the same anti-CD22 BsAb was completely unable to increase saporin toxicity. This result is important because it is consistent with the internalization data shown in Fig. 3 and shows that the rate at which a molecule is internalized from the cell surface is a key feature in determining whether it will make an effective target for antibody delivery of toxins.

In Fig. 4, we also see that the cytoplasmic tail of CD22 will transform CD19BS into a useful vehicle for delivering BsAb-zsaporin complexes for cytotoxicity. Thus, whereas Jurkat cells expressing CD19BS were not susceptible to anti-CD19-specific antibodies, the same antibodies directed against CD22 can deliver saporin effectively. This result is significant because it demonstrates the potential of using the cytoplasmic tail of CD22 to target toxins to cells that express CD19.

FIG. 2. Levels of CD22α and CD19BS expression on transfected Jurkat cells. The histograms show staining patterns for Jurkat cells transfected with the various molecular constructs (see Fig. 1). Each clone was labeled with a nonstaining control fluorescein isothiocyanate-IgG mAb (open histogram) and either fluorescein isothiocyanate-anti-CD22 or fluorescein isothiocyanate-anti-CD19 mAb, as appropriate for the transfected molecule (filled histogram).

FIG. 3. Internalization of 125I-labeled anti-CD19 and anti-CD22 mAb by Daudi and transfected Jurkat cells. 125I-mAb (A, anti-CD22; B, anti-CD19) was incubated with cells for 1 h at 4 °C to allow binding. The temperature was then increased to 37 °C, and duplicate aliquots were taken at the time intervals shown. The total radioactivity associated with each aliquot of cells, and that remaining inside the cells after washing in acidic RPMI medium (pH 2) was determined as described under “Materials and Methods.” The levels of intracellular (solid symbols) and surface (total minus intracellular) (open symbols) 125I-mAb were calculated as the average 125I-mAb molecules/cell. The levels of intracellular and surface 125I-mAb are expressed as a percentage of the surface bound 125I-mAb at time 0. The target cell lines are as indicated (A, Jurkat cells transfected with CD22αD cyt, Jurkat cells transfected with CD22α, or Daudi cells; B, Jurkat cells transfected with CD19BS, Jurkat cells transfected with CD19Acyt/22acyt, or Daudi cells). This is one of three similar experiments.

FIG. 4. Cytotoxicity assay testing BsAb-saporin complexes on Jurkat cells transfected and selected for expression of CD22α, cytoplasmic tail deletion mutant (CD22αD cyt), CD19BS, and a chimeric molecule containing the extracellular and transmembrane region of CD19BS and the cytoplasmic region of CD22α (CD19BSΔcyt/22acyt). Transfected cells (10⁵ cells/well) were cultured for 18 h with BsAb at 1 μg/ml and saporin at the concentrations shown before pulsing with [3H]leucine and harvesting of cells to assess the level of incorporated radioactivity. Triplicate samples were assayed for each concentration of saporin investigated, and the mean values are shown. BsAbs used include saporin alone (●), [anti-CD19 × anti-saporin] (○), [anti-CD38 × anti-saporin] (□), and [anti-CD22 × anti-saporin] (▲). This is one set of three similar experiments.
Internalization Motif for CD22

BsAb-saporin complexes, those carrying CD19Δcyt/22Δcyt were hypersensitive, with an IC$_{50}$ for saporin that was below 10$^{-11}$ M. A Turn Structure Motif Is Essential for Internalization—To investigate whether the cytoplasmic tail tyrosine residues of CD22a are involved in the internalization, CD22a-mutants were constructed in which the cytoplasmic tail was truncated fractionally by introducing a stop codon at the tyrosine residues (CD22aΔY556, CD22aΔY566, and CD22aΔY600) using PCR-directed mutagenesis. The three constructs were expressed at a high level in Jurkat cells (Fig. 1). Cytotoxicity assays showed that these truncated CD22a molecules were all equally as good as the unmutated CD22a at delivering BsAb-saporin complexes into the cells (Fig. 1), showing that the carboxyl-terminal end of the cytoplasmic tail is not directly involved in the internalization motif.

The results using tyrosine mutants described above suggested that the region of cytoplasmic tail nearer to the cell membrane might be critical in the endocytosis process. Crystallographic work (31) and NMR studies (32) suggest that a tight-turn structural motif is involved in the clustering signals for surface molecules, and individual specificity is conferred by side-chain differences. It is thus interesting to note that two tight-turn structures are predicted near to the membrane within the CD22a cytoplasmic tail (Gln$_{513}$–Gln$_{523}$ and Asn$_{528}$–Gln$_{532}$). To determine whether a turn motif is involved in the endocytosis process, truncated CD22a mutants were constructed, using PCR-directed mutagenesis, in which a stop codon was introduced at positions Gln$_{513}$ (CD22aΔQ513), Gly$_{524}$ (CD22aΔG524), and Gln$_{527}$ (CD22aΔQ527) to yield mutants with two amino acids in the cytoplasm or just one of the turns (Fig. 1). These mutants were transfected and selected in Jurkat cells (Fig. 2), and the resulting clones were tested in cytotoxicity assays. The results (Fig. 5) showed that with two amino acids remaining in the cytoplasmic tail (CD22aΔQ513), saporin toxicity was not enhanced by the presence of CD22-specific BsAb, demonstrating that the surface CD22 molecule was no longer active for delivery. This result was the same as that obtained using the cytoplasmic deleted mutant, CD22aΔcyt, even though the expression levels were different (Fig. 2). This indicated that the motif for internalization lies further to the COOH-terminal end of the molecule. Interestingly, the deletion mutants CD22aΔG524 and CD22aΔE527, which each contain one predicted turn in the cytoplasm, were fully active in this assay. Thus, in the presence of BsAb, the IC$_{50}$ values for saporin toxicity were 1.5 × 10$^{-10}$ and 5.5 × 10$^{-10}$ M, respectively, with these two transfectants (Fig. 5.), comparable to those seen with the CD22aΔtransfectant (Fig. 4) or Daudi cells (20) and indicate quite clearly that the internalization motif of CD22 lies between residues 512 and 523, just under the membrane.

Mutagenesis of the Internalization Motif—The 11-amino acid turn structure motif in CD22a is unusual in that, unlike most other internalization signals described, it contains no tyrosine residues (33). Examination of the sequence (QRWKRiQTGQQ), however, showed the presence of a serine bearing a hydroxyl group at position 521. It is possible that serine replaces a tyrosine residue in this motif and that the hydroxyl group of both amino acids may play a similar role in the internalization signal. To investigate this, a mutant was constructed in which Ser$_{521}$ was mutated to Ala$_{521}$ (Fig. 1), and its ability to endocytose was determined in the cytotoxicity assay. Surprisingly, this substitution did not affect delivery of BsAb-saporin complexes (Fig. 6), indicating that although serine may have replaced tyrosine in the position of the internalization motif in CD22a, the mechanism of internalization/ endocytosis of CD22a involves interaction with structures other than the side group of a determinant amino acid.

It has been proposed that charged or polar amino acids surrounding the crucial tyrosine residue in an internalization motif may also be important for function (34, 35). Of the 11 amino acid residues in the CD22a motif, 8 are polar or positively charged (Fig. 7). Such a cluster of polar amino acids clearly suggests that a charged interaction (hydrogen bonding or electrostatic interaction) is likely to be important for internalization. Comparison with tyrosine-containing motifs has shown that amino acids either side of the tyrosine tend to be more prominently polar and also important for function. It was
thus decided to make a CD22α mutant with amino acid changes on either side of the serine. This mutant, CD22Q520L/Q522L (Fig. 2), had a cytoplasmic tail truncated at amino acid 526 with Gln520 and Gln522 mutated to leucine residues. Cytotoxicity assay results with this mutant showed that it had a greatly reduced ability to deliver saporin in the presence of BsAb (Fig. 6). The IC50 value for saporin toxicity was only $3.3 \times 10^{-9}$ M in the presence of BsAb, compared with $2.1 \times 10^{-10}$ M for the CD22α transfectants, confirming the previous proposal that polar amino acids are important.

**DISCUSSION**

CD22 plays a key role in the regulation of B-cell responses to antigen (1, 2). One relatively unexplored mechanism of controlling CD22 function and ensuring that it provides accessory signals at the appropriate time is by restricting its cellular localization, i.e. making sure that CD22 is at the right place at the right time. Recently, Sherbina et al. (36) have shown that following stimulation of the B-cell antigen receptor, intracellular CD22 moves rapidly from the cytoplasm to the plasma membrane, resulting in a 50–100% increase in surface expression within 5 min of stimulation. In addition to membrane delivery, it is clear that CD22 also undergoes rapid and constitutive internalization from the plasma membrane (17). Shan and Press (17) have reported that CD22 is turning over at the cell surface with a t½ of less than 1 h, that internalization occurs regardless of anti-CD22 mAB binding, and that the CD22, once internalized, is transported to lysosomes for degradation rather than being recycled back to the plasma membrane as occurs with many lymphocyte receptors (17). Although it is unclear why CD22 transport is controlled in this way, it obviously has enormous potential for regulating its function.

In the current study, we have investigated the structural basis of CD22α internalization using chimeric and mutated constructs expressed in Jurkat T-cells. The results show that the cytoplasmic tail of CD22α controls its endocytosis into the cells. CD22α molecules without a cytoplasmic tail were not internalized and hence failed to deliver radioactive mAb or BsAbs-saporin complexes inside the cell. Furthermore, when the cytoplasmic region of a second B-cell marker, CD19, was replaced by that of CD22α, the resulting chimeric molecule behaved like CD22α and internalized efficiently (Fig. 1). Stepwise deletion located the endocytosis motif to an 11-amino acid sequence (QRRWKRTQSQ) proximal to the plasma membrane; provided this short peptide remained on the truncated CD22α, it performed perfectly well in delivering toxin BsAbs-saporin complexes into the transfected cells. A complete list of all the constructs, together with data on deliver BsAbs-saporin complexes into transfected cells for cell killing, is summarized in Fig. 1. Because the predominant CD22β isoform has the same membrane proximal 11-amino acid sequence, it seems reasonable to assume that this motif controls internalization of both CD22α and CD22β isoforms. Interestingly, the mouse CD22 molecule shows a very similar sequence in this region (37), with just four conserved amino acid differences, and consequently may perform the same function.

Typically plasma membrane receptors are moved into early endosomes via clathrin-coated pits and vesicles (38). Our immunoelectron microscopy data confirm that CD22α is strongly clustered around coated-pits. Identified signal sequences for entry into or formation of clathrin-coated pits are degenerate and quite variable, making it difficult to define precise motifs (33, 36, 38). However, generally they fall into two classes. The most common are tetra- or hexapeptides containing an aromatic residue, usually tyrosine or phenylalanine (33), placed in the context of one or more amino acids with largely hydrophobic side chains. Typical examples would include EDNPNY for low-density lipoprotein receptor (39), YKSYSKY for CD-manose 6-phosphate receptor (40), and YXRF for the transferrin receptor (31). The second general class of control signals involves adjacent leucine-type residues, which include dileucine or leucine plus a small hydrophobic residue near the carboxyl terminus of the cytoplasmic domain. The current list of receptors using these motifs is quite short and tends to include leukocyte receptors, such as Fc receptor and CD74 (invariant chain of MHC class II) (41). Clearly the CD22 control sequence, QRRWKRTQSQ, does not fall easily into either group and may represent a novel class, or more likely a modified member of the tyrosine-based signaling motifs. A comprehensive investigation by Ktistakis et al. (34) has defined two salient features required for function of these structures: first, they contain residues that show a preference to “break” regular structure in protein domains; and second, they have a high concentration of polar or positively charged residues on both sides of the tyrosine. A schematic representation of the endocytic tyrosine-based signals is shown in Fig. 7A. This generic internalization signal consists of 8–10 cytoplasmic amino acids with the tyrosine residue present in the membrane distal portion of the sequence. There appears to be a strong preference for certain types of amino acid at specific positions, suggesting that the orientation of the motif with respect to the membrane is critical and that it is independent of polarity of the protein, and, for both type I and II transmembrane proteins, extends 6 amino acids membrane proximal and 2 residues membrane distal to the tyrosine. The most critical amino acids appear to be located at positions +2, +1, −1, −4, and −6 with respect to the tyrosine. It is thought that the amino acids that disturb regular structure may be required to disrupt the polypeptide chain after it leaves the transmembrane sequence and provide a fairly extended structure immediately proximal to the membrane. Structural predictions on these internalization motifs suggest that the polar groups either side of the tyrosine generate hydrogen-bonded tight turns, membrane proximal to the tyrosine. It is thus predicted that the recognition sequence for the clathrin adapter protein complex-2 is simply a small surface loop (34).

Using the Garnier-Gibrat-Robson prediction algorithm (42), we found that the CD22 motif (Gln513–Gln523) showed a preference for a turn structure that was strikingly similar to that for a known tyrosine-based signal (Fig. 7B). It is highly polar and basic and differs from the schematic tyrosine-based motif only in a switch of serine for tyrosine. Mutagenesis studies on
tyrosine-based motifs have shown that the tyrosine residue can be substituted with phenylalanine or tryptophan, suggesting that any aromatic residue could suffice for the internalization signaling (43). Surprisingly, in the present study, investigation to determine whether the serine was critical to the CD22 motif showed that it was not, and substituting the serine with alanine did not influence internalization of BsAb-saporin complexes in cytotoxicity assays (Fig. 6). However, in contrast, mutating the polar groups either side of the serine completely destroyed function and prevented saporin toxicity, suggesting that polarity was critical for internalization. Numerous questions remain about this newly defined internalization motif and its interaction with the endocytic pathway. However, it appears that internalization is more dependent on charge and secondary structure than on linear sequence, and this is consistent with CD22 associating with the clathrin-associated adapter, clathrin adapter protein complex-2, or an clathrin secondary structure than on linear sequence, and this is consistent with CD22 associating with the clathrin-associated adapter, clathrin adapter protein complex-2, or an clathrin adapter protein complex-2-like molecule that recognizes the membrane proximal, charged loop for constituent delivery of CD22 into the cell.

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