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Immunology

A 100-kilodalton protein is associated with the murine interleukin 2 receptor: Biochemical evidence that p100 is distinct from the α and β chains

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ABSTRACT **Two proteins that specifically bind the T-cell growth factor interleukin 2 (IL-2) have been identified previously on the surface of T cells; these proteins have been designated IL-2Rα and IL-2Rβ for the α and β chains of the IL-2 receptor (IL-2R). The association of these independent binding proteins with each other on the surface of activated T cells correlates with the generation of high-affinity binding sites. These high-affinity sites transduce the major mitogenic signal of IL-2, yet the mechanisms of association of the α and β chains with each other as well as signal transduction in response to IL-2 are unknown. Cotransfection experiments of cDNAs encoding the α and β chains in T cells and fibroblasts have suggested functional requirements for other T cell-specific factor(s). We now provide biochemical evidence for a distinct 100-kDa protein that interacts with the α or β chains, or both, on the surface of the IL-2-dependent cell line CTLL-2 as well as activated murine splenocytes. This same 100-kDa protein is capable of being chemically cross-linked to ^{125}I-labeled IL-2.**

Interleukin 2 (IL-2) binds to intermediate-affinity ($K_d = 10^{-9}$ M), low-affinity ($K_d = 10^{-8}$ M), and high-affinity ($K_d = 10^{-11}$ M) IL-2 receptors (IL-2Rs) on human lymphocytes (1–8). Certain populations of resting peripheral blood mononuclear cells including large granular lymphocytes bind IL-2 with an intermediate affinity and express on their surfaces the 70-kDa IL-2-binding protein, denoted the IL-2R β chain (IL-2Rβ) or p70 (6–8), whereas activated T cells manifest a combination of low- and high-affinity binding patterns (2). Low-affinity sites, present in ≈10-fold excess of high-affinity sites on activated T cells, contain the 55-kDa IL-2 binding protein denoted p55 or the IL-2R α chain (IL-2Rα). High-affinity receptors contain both the α and β chains (3–5). Although functional correlates have been described for binding to intermediate- and high-affinity IL-2Rs (2, 7, 9) the critical mechanisms of signal transduction in response to IL-2 remain unknown. The biochemical composition of these three receptor classes on human cells has been largely assessed by using chemical cross-linking studies with radiolabeled IL-2 (affinity-labeling) (3–8). However, studies of affinity-labeled murine lymphocytes demonstrated a more complex pattern than that on human cells, allowing speculation about the possibility of the existence of other subunits (10, 11). The coprecipitation of additional bands with human and murine anti-IL-2Rα antibodies also has been reported (12, 13). In addition, the recent cloning and expression of a human IL-2Rβ cDNA has led to the hypothesis that additional T cell-specific factor(s) may be required for functional β-chain expression (14). We now present direct biochemical evidence for the existence of a 100-kDa surface glycoprotein that associates with the α or β chains, or both, and can be cross-linked to IL-2.

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MATERIALS AND METHODS

Cells and Cell Culture. Murine CTLL-2 cells were cultured in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum, glutamine, antibiotics, and 50 μM 2-mercaptoethanol. Murine splenocytes were obtained from BALB/c spleens, which were teased into a single-cell suspension and then centrifuged over Lympholyte M (Cedarlane Laboratories, Hornby, ON, Canada). Mononuclear cells were washed twice in phosphate-buffered saline. Where indicated, splenocytes were activated with 3 μg of Con A (Miles) per ml prior to affinity labeling.

Affinity Labeling. Radiolabeled IL-2 (at indicated concentrations) was bound and cross-linked to cells as described (3) prior to extraction and analysis on sodium dodecyl sulfate (SDS) gels. Cross-linking was performed with the noncleavable cross-linker disuccinimidyl suberate (Pierce).

Direct Identification of p100 with a Two-Dimensional Nonreducing/Reducing Gel. CTLL-2 cells were washed three times in methionine-free RPMI 1640 and resuspended in methionine-free RPMI 1640 containing 10% dialyzed fetal bovine serum (10^6 cells per ml) for 30 min at 37°C; then 200 μCi (1 μCi = 37 kBq) of Tran^{35}S-label (ICN) was added for 2 hr. Cells were washed twice in phosphate-buffered saline and resuspended in 1% bovine serum albumin in RPMI 1640 (pH 7.4) at 4°C, after which unlabeled 6 nM IL-2 was bound for 15 min. Cells then were cross-linked with the thiol-cleavable reagent dithiobis(succinimidyl proprionate) (DSP; 20 μg/ml). Detergent lysates of cells were immunoprecipitated with the anti-IL-2 monoclonal antibody (mAb) 17A1 or a control mAb, RPC5. Immunoprecipitates were boiled in Laemmli sample buffer and were electrophoresed nonreduced on a 7.5% tube gel, which then was treated with 60 mM dithiothreitol to cleave the cross-linker. The solubilized samples were electrophoresed into a 7.5% slab gel. Details are as described (8).

Peptide Mapping. Peptide mapping was performed by a modification of the method of Cleveland (3, 15) that used V8 protease at 10 μg/ml to overlay the gel slices. After treatment for 1 hr at room temperature, the samples were electrophoresed on a 15% SDS/polyacrylamide gel.

Cell Surface Iodination. CTLL-2 cells were washed twice in phosphate-buffered saline, resuspended at 1×10^8 cells per ml in a phosphate buffer, and labeled with Na^{125}I by using a standard lactoperoxidase-catalyzed method.

RESULTS

Affinity-labeling of the IL-2R on the IL-2-dependent murine cell line CTLL-2 and subsequent immunoprecipitation with an anti-IL-2 mAb, 17A1, yielded three prominent bands on SDS/polyacrylamide gels (Fig. 1*a*, lane 1). The cross-linking

Abbreviations: IL-2, interleukin 2; IL-2R, IL-2 receptor; IL-2Rα and IL-2Rβ, IL-2R α and β chains; DSP, dithiobis(succinimidyl proprionate); mAb, monoclonal antibody.

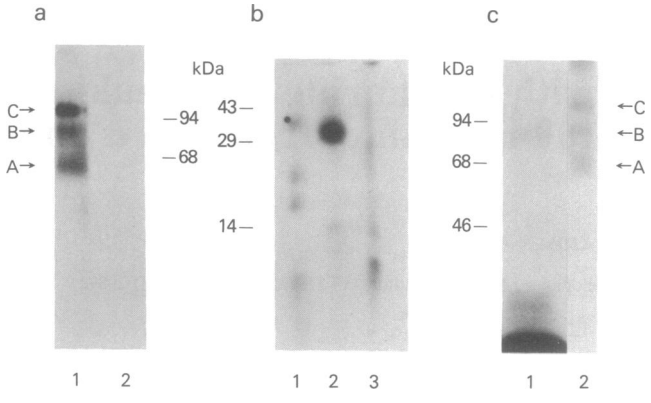

FIG. 1. Affinity-labeling of activated murine T cells yields three specific bands. (*a*) CTLL-2 cells were bound with 300 pM ^{125}I-labeled IL-2 (^{125}I-IL-2; NEN/DuPont) and cross-linked with disuccinimidyl suberate as described (3). Detergent lysates were immunoprecipitated with the anti-IL-2 mAb 17A1 and electrophoresed on a 7.5% SDS/polyacrylamide gel. Prior to IL-2 binding, cells were pretreated either with an anti-IL-2Rα blocking mAb, 3C7 (lane 2) or an anti-class I HLA control antibody, C1.2 (lane 1). (*b*) Affinity-labeled bands C, B, and A from *a* were excised, and their partial proteolytic peptide maps (see *Materials and Methods*) are compared in lanes 1, 2, and 3, respectively. (*c*) Affinity labeling of murine splenocytes from BALB/c spleens. Lanes: 1, affinity labeling of 200 × 10^6 unstimulated splenocytes at 3 nM ^{125}I-IL-2; 2, splenocytes (25 × 10^6 cells) stimulated for 72 hr with 3 μg of Con A (Miles) per ml and affinity-labeled with 300 pM ^{125}I-IL-2. Detergent lysates were immunoprecipitated with the anti-IL-2 antibody before electrophoresis. The increased intensity of band C in *a*, lane 1, and *c*, lane 2, relative to bands A and B does not permit any conclusions related to stoichiometry (see ref. 3). The intensity of each band is determined by a combination of prevalence and efficiency of cross-linking ^{125}I-IL-2.

of ^{125}I-IL-2 into each of these three complexes was specific for an IL-2R interaction because an anti-IL-2Rα mAb that blocks IL-2 binding, 3C7 (26), prevented the affinity-labeling of all three bands (Fig. 1*a*, lane 2), whereas preincubation with a control antibody had no effect (Fig. 1*a*, lane 1). Bands A and B most likely represent IL-2Rα (58 kDa in CTLL-2 cells) and IL-2Rβ (75 kDa in CTLL-2 cells) cross-linked to IL-2, respectively (10, 11, 13). Band C (migrating at 115 kDa) did not clearly correspond to any band identified in experiments using human cells (3–8). The existence of band C has previously been noted by others. Its composition has not been determined but has been hypothesized to represent several possibilities (10, 11, 13): (*i*) two 15.5-kDa IL-2 molecules cross-linked to one IL-2Rβ; (*ii*) one IL-2 molecule cross-linked to a dimerized IL-2Rα; (*iii*) one IL-2 molecule cross-linked in a ternary complex with a 30-kDa protein as well as IL-2Rβ; or (*iv*) one IL-2 molecule cross-linked to a 100-kDa protein. The purpose of this study was to resolve which of these possibilities was correct. Our data are most consistent with the last possibility.

We first excised the three affinity-labeled bands (A, B, and C) and exposed them to V8 protease by the method of Cleveland (3, 15). The partial proteolytic maps revealed distinct patterns for each of the three bands (Fig. 1*b*). Although this result is consistent with each band (in Fig. 1*a*) representing ^{125}I-IL-2 cross linked to a distinct protein, we cannot exclude that IL-2 might be differentially protected if cross-linked to a single protein in more than one way.

To directly identify the entity to which IL-2 was cross-linked to generate band C, we utilized two-dimensional, nonreducing/reducing diagonal gels. Unlabeled IL-2 was bound to [^{35}S]methionine-labeled CTLL-2 cells and cross-linked with the thiol-cleavable reagent DSP. Immunoprecipitations were performed by using an anti-IL-2 mAb and were analyzed by electrophoresis in the nonreduced state in the first dimension and then reduced in the second dimension. A 100-kDa protein (p100) migrated directly below the diagonal by a distance consistent with the cleavage of one 15.5-kDa IL-2 molecule in the second dimension (Fig. 2*A*). Although IL-2 is cleaved from the α chain, intrachain disulfide bonds (within the α chain itself) are also cleaved. The former results in faster mobility, the latter in slower mobility. Thus, the α chain is seen as a smear spanning the diagonal. The murine β chain is reproducibly not well resolved, possibly because it migrates on the diagonal or because it may have been labeled poorly under the conditions used. As expected, no bands migrated below the diagonal in a control immunoprecipitation (Fig. 2*B*).

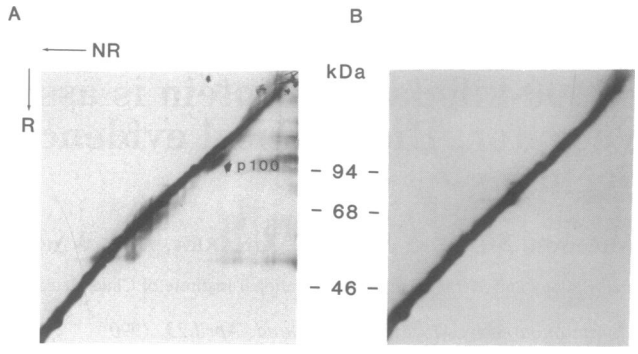

FIG. 2. Diagonal gel analysis of metabolically labeled proteins that interact with IL-2 on the cell surface. [^{35}S]Methionine-labeled CTLL-2 cells were bound with unlabeled IL-2 (6 nM) and cross-linked with DSP. Detergent lysates were immunoprecipitated with the anti-IL-2 mAb 17A1 (*A*) or a control mAb RPC5 (*B*), and analyzed on nonreducing/reducing diagonal gels as described.

Having demonstrated that p100, apparently distinct from IL-2Rα and IL-2Rβ, interacted with IL-2 on the surface of cells, we next evaluated whether a 100-kDa protein also could be detected in a complex with the α and β chains in the absence of added IL-2, as noted by Saragovi and Malek (13). CTLL-2 cells were first washed, surface-iodinated, and then exposed to DSP (in the absence of IL-2). Immunoprecipitations were then performed with either the anti-IL-2Rα mAb 7D4 (26) or a control antibody, Robaclone, followed by electrophoresis under reducing conditions (thereby cleaving the DSP) on two-dimensional, isoelectric-focusing (IEF) gels (Fig. 3 *A* and *B*). As is evident from these immunoprecipitations with the anti-IL-2Rα mAb, a 100-kDa protein (migrating with a pI of 5.3–5.7) was apparently capable of cross-linking to p58 (IL-2Rα). It was present in anti-IL-2Rα precipitates of cells that were exposed to DSP (Fig. 3*A*) but not in control immunoprecipitates of similarly treated cells (Fig. 3*B*). In the absence of DSP treatment of cells, anti-IL-2Rα immunoprecipitates did not demonstrate this 100-kDa protein (not shown).

We next excised the iodinated 100-kDa protein, IL-2Rβ, and IL-2Rα from the gel represented in Fig. 3*A* and generated partial proteolytic maps with V8 protease (Fig. 3*C*). The fragments were again different from each other, confirming the findings on affinity-labeled bands (Fig. 1*B*) and suggesting that the 100-kDa protein, IL-2Rβ, and IL-2Rα were distinct proteins. The appearance of Figs. 1*b* and 3*C* differed from each other as might be expected, since in one case the proteins were affinity-labeled and in the other case the proteins were labeled by surface iodination. Additional spots, whose identities are incompletely known, were identified at or above 100 kDa in Fig. 3*A*. However, we believe that the spot labeled "D" represents a dimer of IL-2Rα because (*i*) IL-2Rα has been shown to form homodimers, particularly under the oxidizing conditions of surface iodination (17, 18); (*ii*) spot D was generated after oxidation of cells with lacto-

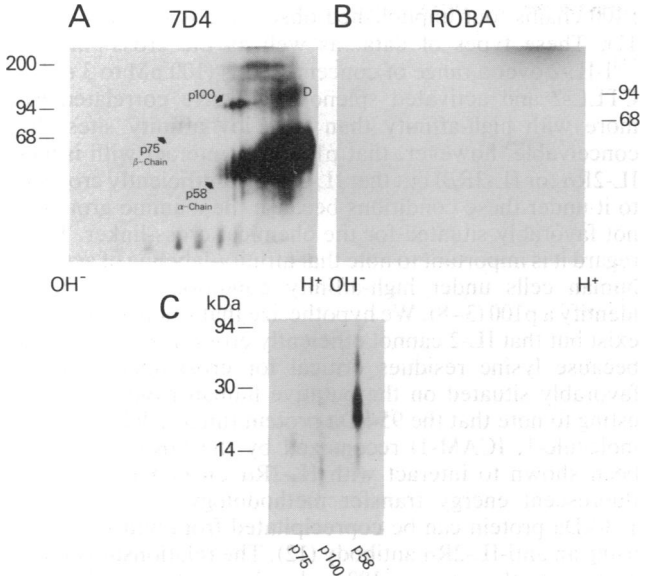

FIG. 3. A 100-kDa protein is closely associated with other IL-2 receptor chains. CTLL-2 cells were surface-iodinated, washed twice at 4°C in phosphate-buffered saline containing unlabeled NaI, and exposed to DSP for 10 min (in the absence of IL-2); detergent lysates were immunoprecipitated with the anti-IL-2Rα mAb 7D4 (*A*) or the rat anti-smooth muscle control antibody, Robaclone (Accurate Chemicals, Westbury, NY) (*B*). Immunoprecipitates were electrophoresed on two-dimensional O'Farrell gels (16). The first dimension was an isoelectric focusing tube gel (proteins electrophoresed from the basic to the acid end), and the second dimension was an SDS/polyacrylamide slab gel. The band D is believed to be dimerized IL-2Rα (see text). (*C*) Protease V8 partial proteolytic peptide map of p75 (IL-2Rβ), p100, and p58 (IL-2Rα) bands from *A* (3, 15).

peroxidase but not in the absence of these oxidizing conditions; and (*iii*) spot D was identified by the anti-IL-2Rα mAb even when the DSP cross-linker was not added to labeled cells (unpublished observations). As discussed above, the 100-kDa protein was not recognized by the anti-IL-2Rα mAb under such conditions.

An important question is whether the 100-kDa protein that interacts with the α and β chains (Fig. 3) is the same 100-kDa protein that interacts with IL-2 (as in Fig. 2). To evaluate this, we next metabolically labeled cells with [^{35}S]methionine and bound and cross-linked unlabeled IL-2 (400 pM) using DSP followed by: (*i*) precipitation with an anti-IL-2 mAb or (*ii*) precipitation with an anti-α chain mAb.

Immunoprecipitates were analyzed under reducing conditions on two-dimensional isoelectric focusing gels (not shown) and the 100-kDa spots were excised from each and evaluated by peptide mapping (Fig. 4). The p100 interacting with IL-2 migrated with an identical pI to the 100-kDa protein interacting with the α and/or β chains (pI of 5.3–5.7) and had an identical proteolytic peptide map (Fig. 4, compare lanes 1 and 2). Therefore, we believe that it represents the same protein.

To evaluate whether p100 could also be detected on normal cells, we cross-linked ^{125}I-IL-2 to unstimulated as well as Con A-stimulated murine splenocytes (Fig. 1*c*). IL-2Rβ was the principal subunit that was affinity-labeled on unstimulated splenocytes (Fig. 1*c*, lane 1, band B). p100 and IL-2Rα were not affinity-labeled in the unstimulated cells. The detection of only IL-2Rβ on resting splenocytes is analogous to the situation on unstimulated human peripheral blood mononuclear cells (7, 8), although a 3-fold higher concentration of human ^{125}I-IL-2 was needed to affinity-label IL-2Rβ on murine splenocytes. Because the unstimulated splenocytes are a mixture of cells, it is unclear whether T cells or large

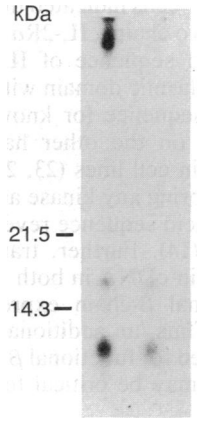

FIG. 4. The 100-kDa protein that interacts with the α or β chains (or both) is identical to the p100 that interacts with IL-2. Protease V8 peptide map is shown of the 100-kDa [^{35}S]methionine-labeled proteins that interact with IL-2Rα (lane 1) or with IL-2 (lane 2). CTLL-2 cells were labeled with [^{35}S]methionine as in Fig. 2, bound with IL-2 (400 pM), and cross-linked with DSP (20 μg/ml). Detergent lysates were immunoprecipitated either with an anti-IL-2Rα mAb (lane 1) or with an anti-IL-2 mAb (lane 2). Precipitates were run on isoelectric focusing gels under reducing conditions (not shown), and the 100-kDa spots were excised, treated with protease V8 (100 μg/ml), and electrophoresed on a SDS/15% polyacrylamide gel.

granular lymphocytes (or both) are principally responsible for yielding the band detected. As expected, IL-2Rα was induced within 72 hr after Con A stimulation (Fig. 1*c*, lane 2, band A). Of note, p100 was also detected on the splenocytes by 72 hr after exposure to Con A (Fig. 1*c*, lane 2, band C). Presumably either its expression was induced or it simply was more efficiently bound in the presence of IL-2Rα. In fact, the pattern of cross-linking to 72-hr Con A-stimulated splenocytes (at 300 pM ^{125}I-IL-2) (Fig. 1*c*, lane 2; ref. 11) resembled the pattern on CTLL cells (Fig. 1*a*, lane 1), with all three proteins (IL-2Rα, IL-2Rβ, and p100) available for cross-linking to IL-2. Partial proteolytic peptide maps of affinity-labeled IL-2Rα, IL-2Rβ, and p100 proteins from Con A-activated splenocytes (not shown) showed a similar pattern to that from respective CTLL-2 bands (see Fig. 1*b*). Higher and lower molecular weight bands were seen on SDS/PAGE of affinity-labeled splenocytes (see Fig. 1*c*). Although the exact content was unknown, the higher band (lane 2) precipitated with an anti-IL-2 mAb as well as an anti-IL-2Rα mAb and, therefore, may represent a complex of receptor subunits with ^{125}I-IL-2. The lower band (lane 1) precipitated only with an anti-IL-2 mAb, demonstrated a pI on isoelectric focusing gels identical to that of ^{125}I-IL-2, and, therefore, may represent dimerized ^{125}I-IL-2.

DISCUSSION

An initial interaction of IL-2 with specific surface membrane proteins on lymphocytes is critical for obtaining a biologic response to the cytokine. Although activated T cells express low-affinity sites in ≈10-fold excess of high-affinity sites, the IL-2-induced proliferative responses occur at concentrations consistent with titration of high-affinity sites with IL-2 (2). Although IL-2 rapidly activates a tyrosine kinase in the IL-2-dependent cell line CTLL (19), no kinase has yet been identified, and relatively little is known about the mechanisms mediating signal transduction after high-affinity binding (20, 21).

As a first step to further elucidation of the signal transduction in this system, we have emphasized studies of the structural aspects of the IL-2R. Affinity cross-linking studies

on human activated T cells indicated that the high-affinity site consists of at least two chains, IL-2Rα and IL-2Rβ (3–5). The deduced amino acid sequence of IL-2Rα reveals a short 13-amino acid cytoplasmic domain with no tyrosine residues and no consensus sequence for known enzymatic activity (22). The β chain, on the other hand, is tyrosine-phosphorylated on certain cell lines (23, 27, 28), yet there is no evidence for it harboring any kinase activity on its own, and its deduced amino acid sequence reveals no tyrosine kinase consensus domain (14). Further, transfection experiments with a human β-chain cDNA in both fibroblasts and T cells resulted in functional β-chain expression only in the T lymphocytes (14). Thus, an additional T cell-specific factor appears to be required for functional β-chain expression (14), and other subunits may be critical to IL-2-mediated signal transduction.

We have provided biochemical evidence for the existence of a 100-kDa cell surface protein that both interacts with IL-2 and appears to associate with the α and β chains on the surface of activated murine T cells. The cross-linking of p100 to ^{125}I-IL-2 is specific for an IL-2R interaction as determined by loss of cross-linked complexes after preincubation with blocking antibodies (Fig. 1*a*). Interestingly, the association of p100 with IL-2Rα or IL-2Rβ (or both) on the surface occurs in the presence or absence of added IL-2 (Figs. 3 and 4). This resolves conflicting hypotheses as to the composition of the 115-kDa cross-linked band (band C in Fig. 1*a* and refs. 10, 11, 13). The evidence that p100 is distinct from the α and β chains comes from peptide maps of these protein bands excised from one- and two-dimensional gels as well as the failure of p100 to be recognized by the anti-IL-2Rα antibody in the absence of a cross-linker. In addition, we have observed, based on pulse-chase experiments, that p100 is extremely rapidly synthesized and available for interaction with IL-2 on the surface of cells, thus distinguishing it from the α and β chains; and, based on digestion of p100 with glycosidases, that p100 is a glycoprotein containing N-linked carbohydrate and sialic acid residues (unpublished observations).

Our current model for the murine IL-2 receptor is represented in Fig. 5. Intermediate affinity sites (β chain) are present on resting splenocytes, whereas activated splenocytes have low-affinity (α chain) as well as high-affinity sites. Under high-affinity conditions, IL-2 interacts specifically with the three proteins, p58 (α), p75 (β), and p100. It is currently unclear if p100 can bind IL-2 on its own as do the α and β chains. We have identified p100 on the cell lines HT-2 and D-10, which express both high- and low-affinity IL-2Rs (not shown) as well as on CTLL-2 and activated splenocytes. However, the murine 5.1.2 cell line, which expresses only low-affinity binding sites, reveals the α chain without β or

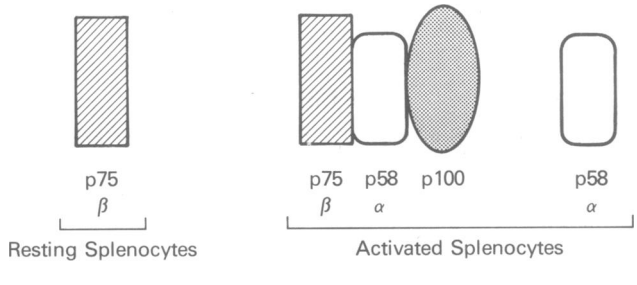

FIG. 5. Model of murine IL-2R subunits. Resting splenocytes express predominantly intermediate-affinity sites composed of the β chain. Activated splenocytes express low-affinity sites (α chain) in excess of high-affinity sites. These high-affinity sites appear to be composed of α chains, β chains, and p100. We have not excluded the possibility that p100 can exist by itself or in association with the isolated α or β chains. Other proteins also may be associated with these subunits. The stoichiometry of the chains on the surface of cells awaits further studies.

p100 chains (our unpublished observations and refs. 10 and 11). These types of data, as well as the cross-linking of ^{125}I-IL-2 over a range of concentrations (100 pM to 3 nM) on CTLL-2 and activated splenocytes, have correlated p100 more with high-affinity than with low-affinity sites. It is conceivable, however, that p100 may interact with isolated IL-2Rα (or IL-2Rβ) but that IL-2 cannot efficiently cross-link to it under these conditions because the ε amino groups are not favorably situated for the chemical cross-linker. In this regard it is important to note that affinity-labeling of activated human cells under high-affinity conditions has failed to identify a p100 (3–8). We hypothesize that a human p100 does exist but that IL-2 cannot efficiently cross-link to it, perhaps because lysine residues critical for cross-linking are not favorably situated on the putative human p100. It is interesting to note that the 95-kDa protein (intercellular adhesion molecule-1, ICAM-1) recognized by the OKT27 mAb has been shown to interact with IL-2Rα on human cells by a fluorescent energy transfer methodology (24) and that a 113-kDa protein can be coprecipitated from human cells by using an anti-IL-2Rα antibody (12). The relationship of these proteins to the murine p100 molecule is unknown. It should be noted that there is precedent for differences in the identification of associated proteins between human and murine systems. In particular, the ζ chain is readily detected as an associated protein of the T-cell antigen receptor in murine cells, but is difficult to detect in human cells except with an anti-ζ antibody (25). The potential role of p100 in IL-2-mediated signal transduction is an important area that awaits further investigation.

Note Added in Proof. Since submission of this manuscript, Saragovi and Malek (29) have reported the identification of additional proteins denoted p22 and p40 as being associated with the murine IL-2R. Because of differences in experimental purpose and design, we do not believe that our results and their results are mutually exclusive. It is therefore possible that the IL-2R is more complex than either paper alone hypothesizes; however, the role in IL-2 binding and signal transduction of all such candidate proteins awaits further investigation.

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