The integrins can be expressed on the surface of cells in a latent form, which is activated by a variety of stimuli. As an approach to examining the transition to an active receptor, a panel of stimulatory antibodies to $\beta_1$ were produced and characterized. These antibodies induced adherence of the T-leukemic cell line J urkat to collagen and fibronectin. Competitive antibody binding assays indicated the existence of at least three distinct epitope clusters A (B3B11, J B18, 21C8), B (B44, 13B9), and C (N29) defined by the indicated antibodies. Two antibodies to the A site, J B18 and B3B11, were shown to localize to positions 671–703 and 657–670, respectively, of the $\beta_1$. This region is located in an area encompassing a predicted disulfide bond between linearly distant cysteines in $\beta_1$ (Cys452-Cys672). The homologous region of the $\beta_1$ integrin (490–690 and 602–690) has been shown to be one of the sites recognized by stimulatory antibodies to ligand-induced binding sites. The present results indicate the existence of multiple stimulatory regulatory regions and suggest considerable homology between the locations of $\beta_1$ and $\beta_3$ regulatory sites.

Members of the integrin family mediate cellular adherence to a variety of extracellular proteins (e.g. collagen, fibronectin, vitronectin, laminin) or to cell surface-associated molecules through homotypic or heterotypic (1–3) interactions. Several cell types (e.g. platelets, leukocytes) express some of the integrins on their surfaces in latent forms. It is only subsequent to cell activation that these molecules display binding potential for their cognates (4–7).

The mechanisms responsible for the transition to an activated state are unknown. However, it is apparent that receptor conformational charges are associated with the generation of a functional complex (8–10). A number of antibodies that can activate receptors have been described for the integrins (11–18). In the case of $\alpha_{IIb}\beta_3$, a number of the stimulatory antibodies (anti-LIBS1, D3GP3(14), anti-LIBS2 (15), anti-LIBS3, anti-LIBS6 (16), and AP5 (17)) recognize epitopes that are expressed on ligand occupancy of the receptor. Some of these anti-LIBS reagents have been particularly useful in probing aspects of cation requirements for receptor function and for the localization of possible regulatory regions of the molecule (10, 17). However, there is considerably less information about regulatory sites on other members of the integrin family.

The $\beta_1$ integrins play a critical role in the functional activity of lymphocytes by influencing cellular distribution patterns and by functioning as costimuli for differentiation or proliferation induction (20, 21). Similar to the $\alpha_{IIb}\beta_3$ system, the functional status of the $\beta_1$ integrins is stringently controlled with activation being induced by a variety of stimuli including antibodies to the $\beta_1$ chain (11–13).

Two groups have reported on the locations of some of the regulatory regions of the $\beta_1$ integrins using monoclonal antibodies to the $\beta_1$ chain (22, 23). The result of one study suggested that there may be multiple regulatory regions (23), while a second indicated that the majority of stimulatory and inhibitory antibodies reacted with a very restricted region of the $\beta_1$ molecule (22). Studies from the $\beta_1$ integrin system would seem to support the existence of multiple distinct regions defined by regulatory antibodies.

As an approach to defining the number and location of potential regulatory sites on the $\beta_1$ chain, we have identified and characterized six stimulatory antibodies. The relative positions of the epitopes recognized by these antibodies were determined by competitive binding studies. Furthermore, the location of one set of such epitopes in the $\beta_1$ molecule was determined.

**EXPERIMENTAL PROCEDURES**

Materials—Unless otherwise indicated, all chemicals were purchased from Sigma. Media, fetal bovine serum, and fibronectin were obtained from Life Technologies, Inc. Custom synthesized peptides were purchased from Chiron Mimotopes Peptide Systems.

Antibodies—Mice were immunized with J urkat-derived $\beta_1$ integrins, which had been affinity purified with the anti-human $\beta_1$ monoclonal antibody J B1 (24). Hybridomas were produced and enzyme-linked immunosorbent assay screened for reactivity with purified $\beta_1$ (25, 26). Positive clones were screened for reactivity with electrophoresed proteins of SDS, 7% polyacrylamide gel electrophoresis-separated J urkat proteins and their abilities to immunoprecipitate $\beta_1$ integrins from 125I surface-labeled J urkat cells (7). Antibodies were purified from the 30–50% ammonium sulfate fractions of ascitic fluid on a protein A-Sepharose column (26).

Biotinylation—Protein A-purified antibodies, 10 mg/ml, were suspended in 50 mM sodium bicarbonate, pH 8.5, and reacted with N-succinyl LC biotin, 400 $\mu$g/ml (Pierce), for 30 min at room temperature. The buffer was exchanged with PBS, 0.1% sodium azide using Centricon-30 centrifuge column (Amicon). The final sample was adjusted to 0.5–1.0 mg/ml.

Cells and Culture—The human T cell leukemia J urkat was grown in RPMI 1640 supplemented with 10% fetal bovine serum and 50 $\mu$g/ml 2-mercaptoethanol.

CHO cells transfected with human $\alpha_{II}$, provided by Dr. R. J.uliano (27), were cotransfected by electroporation with pFnR$\beta_1$, a construct containing the full-length human $\beta_1$ gene provided by Dr. E. Ruoslahti (28), and pREP4, a vector carrying the hygromycin reductase marker, provided by Dr. M. Tykocinski (29). The cells were cultured for 48 h in the presence of G418.

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II To whom correspondence should be addressed: RDU Research Laboratory, Departments of Immunology, Medical Microbiology, University of Manitoba, Winnipeg MB R3A 1M4, Canada.

Fax: 204-787-2420; E-mail: jwilkin@ccu.umanitoba.ca.

The abbreviations used are: PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; BSA, bovine serum albumin; bp, base pair(s).
RPMI with fetal bovine serum containing G418 (80 μg/ml), after which hygromycin B was added (300 units/ml). Transfectants reacting with the anti-human β1 monoclonal, J B1 (24), were selected by fluorescence activated cell sorter and cultured in the selection media. This cycle was repeated three times to achieve stable lines. It was found to be necessary to use the CHO α2 chain as the host cell for β1 expression, as in our hands the wild type CHO cells did not efficiently express the human β1 gene product. Specificity of the transfectants were confirmed using a panel of monoclonal antibodies to β1 including J B1A (30), 353 (31), and J B1 (24). Cell Binding Assay—Binding assays were performed as described previously (12). Non-tissue culture microtiter wells were coated with purified plasma fibronectin (5 μg/ml) or type I collagen (20 μg/ml). The wells were washed and blocked with 1% BSA in RPMI. J urkat cells prelabeled with 51Cr were preincubated with the indicated antibodies for 30 min and then added (100/well) to fibronectin, collagen, or BSA-coated wells and incubated for 1 h at 37°C. The nonadherent cells were removed by three washes with PBS, 1% BSA. The bound cells were solubilized in 1% SDS, and the levels of radioactivity were quantitated. Each assay was performed in triplicate, and the experiments were repeated at least three times. The S.E. of the replicates was less than 15%.

Competitive Binding Assay—Cells were suspended (5 × 10^6) in PBS, 1% BSA in a V-bottom 96-well microtiter tray and incubated in the presence of the indicated concentration of unlabeled antibody for 1 h at 4°C. The cells were washed with PBS-BSA, after which biotinylated reporter antibody (0.2 μg/ml) was added for 30 min at 4°C. The cells were washed, reacted with a horseradish peroxidase-conjugated avidin complex, washed again, and the color was developed with substrate 400 μM O-phenylenediamine. The reaction was stopped by the addition of an equal volume of 10 M H₂SO₄ and quantitated at 492 nm.

Epitope Library Production and Screening—Libraries were constructed using the NovaTope system (Novagen Inc.) according to the supplier's instructions. The method based on the use of modified pET expression vectors consisted of digesting the full-length pFnR with DNase I in the presence of Mn²⁺ and size fractionating the random fragments by electrophoresis in 1.2% agarose gels. The 50–150-bp or 150–300-bp fragments were then added to T4 DNA polymerase, single-dA-tailed, and ligated into the EcoRV site of the pTope-1B (+) plasmid. Novablue (DE3) cells were transformed with the plasmid, and colonies were immunoscreened with a panel of anti-β1 monoclonal antibodies and an alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (26). Positive colonies were subcloned and examined for reactivity with the antibodies. The inserts from individual clones were sequenced using a T7 gene 10 primer.

Enzyme-linked Immunosorbent Assays and Blocking Assays—Peptide were used at 10 μg/ml in 0.1% dimethyl formamide in water and allowed to dry overnight, 0.5 μg/well, in Nunc Maxisorp plates. The plates were washed three times with 0.5% Tween 20 in PBS and blocked for 2 h at room temperature with 2% BSA in PBS. The indicated antibodies (5 μg/ml) were added to the wells, and the binding was quantitated using a horseradish peroxidase rabbit anti-mouse immunoglobulin and developed with 400 μM O-phenylenediamine as substrate.

Blocking assays were performed by preincubating antibodies (150 ng/ml) with the indicated peptide concentrations for 2 h. The antibodies were then added to Nunc plates coated with affinity-purified placental β1 integrin (32). The color was developed after reaction of the wells with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin in substrate.

RESULTS

Anti-β1 Antibodies—The characteristics of the antibodies used in this study are listed in Table I. The specificity of the antibodies for the β1 integrin chain were demonstrated by several methods. The antibodies specifically stained CHO cells stably cotransfected with human α2 and β1 cDNAs (Fig. 1). In contrast, neither the wild type cells nor those transfected with only the α2 chain showed any binding with these antibodies. Each of the antibodies was also shown to immunoprecipitate all of the β1 species (i.e., α2, α2, and α₃) on J urkat cells (Refs. 8 and 19 and data not shown).

Previous studies had demonstrated that J urkat cells display a low level of adherence to immobilized collagen (7). However, following stimulation with phorbol 12-myristate 13-acetate (7, 12) or some antibodies to the α2 or β1 chains (12), there was a marked increase in adherence. When a panel of anti-β1 reagents was analyzed for the effects on J urkat adherence, six stimulatory antibodies were identified (Fig. 2). This stimulation was detectable within 30 min of exposure and lasted for several hours (Ref. 12 and data not shown). This effect was not a property of all antibodies to β1, as 353 (31) and J B1 (24) did not induce adherence. In the case of 353, there was inhibition of the low level of spontaneous binding of these cells to collagen.

Competitive Binding of Stimulatory Antibodies to J urkat Cells—The identification of several monoclonal antibodies with similar biological activities raised the possibility that they were reacting with a common region of the β1 chain. To address this question, competitive blocking studies were performed in which cells were preincubated with an unlabeled competitor antibody. A biotinylated reporter antibody was then added, and the level of binding was assessed relative to that in the absence of competitor.

Fig. 3 gives an example of the type of results obtained using J B1B. There was a dose-dependent inhibition by the unlabeled J B1B. There was also competition by B3B11 and 2IC8. In contrast, another stimulatory antibody (N29) and an inhibitory antibody (353) failed to influence J B1B binding. Similar experiments were performed with each of the antibodies, and a summary of the results is given in Table II. Based on these results, it appears that there are at least three regions of the β1 integrin that can act as targets for antibodies which activate integrin function. These are designated group A (B3B11, J B1B, 2IC8), group B (B44, 13B9, and group C (N29).

Inhibition by anti-β1 antibodies was not influenced by antibody binding to β1 integrins on immunoblot. In contrast, the 2IC8 epitope was most probably a discontinuous one, as it was reduction sensitive. To increase the likelihood of identifying the locations of both continuous and discontinuous epitopes, a random β1 epitope fusion protein library containing large β1 inserts (150–300 bp) was constructed.

The library was screened with a pool of J B1B, B3B11, and 2IC8, and five reactive colonies were identified. Assay of the individual clones with each of the antibodies indicated that the epitopes recognized by J B1B and B3B11 were likely to be linear sequences of the β1 chain since reduction did not influence antibody binding to β1 integrins on immunoblot. In contrast, the 2IC8 epitope was most probably a discontinuous one, as it was reduction sensitive. To increase the likelihood of identifying the locations of both continuous and discontinuous epitopes, a random β1 epitope fusion protein library containing large β1 inserts (150–300 bp) was constructed.

| Antibody | Specificity | Blot | Activity | Reference |
|----------|-------------|------|----------|-----------|
| J B1     | β1          | +    | -        | NULL 24   |
| J B1A    | β1          | +    | +        | Inhibitory 30 |
| 353      | β1          | +    | +        | Stimulatory 31 |
| B3B11    | β1          | +    | +        | Stimulatory This study |
| N29      | β1          | +    | +        | Stimulatory This study |
| J B1B    | β1          | +    | +        | Stimulatory This study |
| 2IC8     | β1          | +    | +        | Stimulatory This study |
| 13B9     | β1          | +    | +        | Stimulatory This study |
| B44      | β1          | +    | +        | Stimulatory This study |

* Antibody is active in immunoprecipitation.

** Antibody is active in immunoblot of reduced antigen.

† Effect of antibody on adherence to fibronectin and collagen.

TABLE I

Activity: in Table I .
reaction. It was noteworthy that 21C8 gave a very weak reaction with reduced proteins from this clone (Fig. 4B, lane 3) but not with unreduced fusion protein (data not shown).

The screening of a second epitope library containing smaller inserts (50–150 bp) identified a single colony, which reacted with the antibody pool. The product of this clone, B3, was found to react with B3B11 but not 21C8 or JB1B (Fig. 4A). Based on these results, it is clear that these antibodies recognize distinct epitopes. Furthermore, as predicted by the competitive binding studies, the JB1B and B3B11 epitopes are in close proximity to one another on the β1 chain.

The analysis of the DNA sequences of the inserts in those clones containing both the B3B11 and JB1B epitopes (A, B, D, G, E) indicated that these epitopes were located in a peptide containing a predicted amino acid sequence (33) corresponding to residues 636–705 of the mature β1 chain (Fig. 5). The B3 insert sequence further served to localize the B3B11 epitope to amino acids 648–670. Based on the overlap of the clone sequences, the JB1B epitope would appear to be contained in the peptide spanning residues 671–703.

The sites of reactivity of B3B11 were further examined using

FIG. 1. Specific binding of antibodies to CHO cells transfected with human β1 integrin. The binding of β1 antibodies to CHO cells coexpressing human α5 and β1 (solid line) but not to CHO wild type or those expressing only human α5 (dotted lines) indicates the specificity of these antibodies for human β1.

FIG. 2. The induction of Jurkat cell adherence to type I collagen. A, Jurkat cells were pretreated with the indicated anti-β1 monoclonal antibodies for 1 hour, after which adherence to immobilized collagen was determined. B, a comparison of JB1B, B3B11, and 21C8 induced adherence. Values represent the mean of sextuplicate assays. All values were within 10% of the mean.

FIG. 3. Competitive blocking of JB1B binding to Jurkat cells by monoclonal antibodies to β1 integrin chain. Blocking studies were performed as described under “Experimental Procedures.” The stimulatory antibodies JB1B, B3B11, and 21C8 inhibit JB1B binding. Another stimulatory anti-β1, N29, and an inhibitory antibody, 3S3, do not affect JB1B binding. Results are expressed as the percent inhibition of JB1B binding relative to that of JB1B to untreated cells.
three overlapping peptides, which spanned the predicted sequence of the B3 fusion protein. B3B11 reacted specifically with the peptide P3, which corresponded to residues 657–670 of the β1 sequence (Fig. 5). In contrast, neither of the other two peptides, P1 and P2, spanning the remainder of the B3 insert was bound by B3B11. There was also no reactivity of the other two competing antibodies, 21C8 or JB1B, with any of the peptides.

Further support for P3 being the location of the B3B11 epitope was obtained with the demonstration that only this peptide inhibited the binding of B3B11 to purified β1 integrin (Fig. 7). Neither P1 or P2 peptides blocked B3B11 binding. Furthermore, the P3 effect appeared to be specific as it did not influence the binding of an unrelated antibody to β1 JB1A.

These results indicate that the B3B11 epitope is contained in residues 657–670 and that it lies in close proximity to the JB1B epitope.

DISCUSSION

The results of the present study provide several new pieces of information: 1) the characterization of a panel of antibodies to β1 that induce Jurkat adherence to collagen; 2) the demonstration of the presence of at least three distinct β1 sites, which stimulatory antibodies can react with, i.e. group A (B3B11, JB1B, 21C8), group B (B44, 13B9), and group C (N29); 3) the localization of the epitopes detected by some of group A antibodies (JB1B, B3B11) to a membrane proximal region (648–705) of the β1 integrin. The biological activities of the antibodies described in this study are not unique as a number of stimulatory antibodies to both β1 (11–13), β2 (18), and β3 (14–17) integrins have been described. However, it was possible through the use of this panel to begin to address the location and existence of multiple regions of the integrin, which could act as targets for stimulatory antibodies.

In two previous studies relating to this point, there were somewhat divergent views presented on the question of the number of regulatory sites (22, 23). Takada and Puzon (22) observed that the region 207–218 of the β1 appeared to be critical for the generation of epitopes, which were recognized by a number of inhibitory and stimulatory antibodies. These re-

| A          | B          | C          | D          | E          | F          |
|------------|------------|------------|------------|------------|------------|
| JB1B       | +          | +          | +          |            |            |
| B3B11      | +          | +          | +          |            |            |
| 21C8       | +          | +          | +          |            |            |
| B44        | -          | -          | -          |            |            |
| 13B9       | -          | -          | -          | +          | +          |
| N29        | -          | -          | -          | +          | +          |
| JB1        | -          | -          | -          | -          | -          |
| 3S3        | -          | -          | -          | -          | -          |

**TABLE II**

Summary of competitive antibody blocking studies

Each experiment was performed three times with triplicate samples in each assay. +, >80% and −, <10% inhibition of binding of labelled antibody by 20 μg/ml of competitor antibody.

**FIG. 4.** Localization of JB1B and B3B11 epitopes on β1 fusion proteins. Bacterial lysates of clones expressing β1 integrin fusion proteins were screened with 1) JB1B, 2) B3B11, 3) 21C8, 4) JB1A, and 5) no first antibody. Molecular mass markers indicated by the arrows are 30, 46, 69, and 98, respectively. A, reactivity of clone B3 with B3B11; B, reactivity of clone B with JB1B and B3B11. Weak reactivity with 21C8 was also noted under reducing conditions.

**FIG. 5.** Predicted amino acid location of the inserts in the mature β1 chain. Fusion proteins from clones G, A, B, D, and E react with both JB1B and B3B11. The product of clone B3 reacts with B3B11 but not JB1B. The sequence of positions 636–705 of the β1 chain is given in the lower portion of the figure. This corresponds to the region spanned by the insert in clone G.

**FIG. 6.** The reaction of anti β1 antibodies with synthetic peptides corresponding to the B3B11 epitope of β1. Three overlapping peptides corresponding to residues 636–649 (P1), 646–659 (P2), and 657–670 (P3) were examined for their reactivities with JB1B, B3B11, and 21C8. These fragments spanned the entire sequence of the B3 clone isolated from the epitope fusion library (Fig. 5).
results were based on the observations that the expression of human regulatory epitopes on interspecies \( \beta_1 \) chimeras required inclusion of this region of the human \( \beta_1 \) integrin. It was also possible to demonstrate that the introduction of multiple point mutations (S207R, E208K, N210E, K211V) into the highly conserved homologous region of the chicken integrin resulted in the generation of epitopes recognized by anti-human \( \beta_1 \) monoclonal antibodies. Thus, it appeared that a single region of the \( \beta_1 \) was capable of modulating \( \beta_1 \) function in either a positive or a negative fashion. The authors did, however, note that there might be other regulatory regions, as it was not possible to localize the epitopes of all regulatory antibodies.

Shih et al. (23) used a similar approach but found that the expression of regulatory epitopes mapped to two distinct regions using chicken mouse chimeric \( \beta_1 \) molecules. Epitopes related to the interference with ligand binding appeared to localize to the first 260 residues of the molecule. In contrast, those antibodies that altered ligand specificities or interfered with \( \alpha_\beta_1 \) association were dependent upon the more membrane proximal part of the integrin. Their results were interpreted to indicate the presence of multiple regulatory regions with the inhibitory and stimulatory epitopes mapping to different sites on the \( \beta_1 \) chain.

Two antibodies of the group A cluster, J3B1 B and B3B11, localize the membrane proximal region of the \( \beta_1 \) (648–705). The third antibody in this cluster, 21C8, reacts with a discontinuous epitope, which is not detectable on Western blot. The B3B11 (657–670) and J B1 B (671–705) flank Cys571, which is predicted to be involved in a disulfide bond with Cys34 (34). Such a bond would bring sequentially distant regions of the \( \beta_1 \) chain into close spatial proximity. The predicted \( \beta_1 \) disulfide bonding pattern is based upon those of the \( \beta_3 \) chain, as both integrins have an identical number of cysteines, and a homologous pairing pattern has been proposed (33, 35). However, the sequences in this region do not show significant homology. Thus, while the disulfide bond locations may be similar, the intervening sequences are completely different.

It is noteworthy that several anti-LIBS to \( \beta_3 \) chain, which activate adhesion, have been suggested to map in the 490–690 using interspecies \( \beta_3 \) chimeras (17). In the case of anti-LIBS2, the reduction-sensitive epitope has been localized to 602–690 (10). Thus, a number of stimulatory monoclonal antibodies appear to bind to the sterically constricted region of \( \beta_3 \), which is homologous to the A region on \( \beta_1 \).

The results of our competitive binding assays support the existence of at least three \( \beta_1 \) regions, which are the targets of stimulatory antibodies. The presence of multiple sites is consistent with the results of the \( \beta_3 \) chain, where three regions have been identified as sites of stimulatory anti-LIBS activity (14–17). It has been suggested that these sites may be in close proximity in the intact molecule as a result of long range disulfide bonds (17). By analogy, it would appear that the A region of \( \beta_1 \) corresponds to the LIBS2, LIBS3, LIBS6, P41 site of \( \beta_3 \) (10, 17). A second site in \( \beta_3 \) (1–6) recognized by AP5 encompassed a Cys6 involved in a long range disulfide bond (17). Preliminary data suggest that N29 reacts with an epitope within the first 100 amino acids of \( \beta_1 \). It will be of interest to determine the relationship between the N29 epitope and that of AP5 on \( \beta_3 \). The group B epitopes have not been localized to date.

In summary, it would appear that there is homology between the sites recognized by the group A antibodies and some of the \( \beta_3 \) stimulatory epitopes. It remains to be determined if these antibodies are similar to the LIBS-type reagents of the \( \beta_3 \) system. Preliminary data do indicate differences in expression levels on adhesion-competent and latent cells. However, the stringency of expression on the lymphoid cells does not appear to be as great as for the LIBS.

We have previously demonstrated that several of the antibodies in this panel can have a number of effects on adhesion (12), intracellular signaling (36), and the association of cytosolic proteins (37). It will be of interest to determine whether interactions with the different stimulatory regions will differentially influence these other parameters of integrin activation.

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