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Integrin α and β subunit contribution to the kinetic properties of α2β1 collagen receptors on human keratinocytes analyzed under hydrodynamic conditions

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SUMMARY

The adhesion of keratinocytes to type I collagen or laminin 5 was studied in a laminar flow chamber. These experiments provided an insight into the binding kinetics of integrins in their natural environment and the effects of monoclonal antibodies specific for α and β chains. Cells driven by a force too low to alter the natural lifetime of a single bond displayed multiple arrests. Studying the frequency and duration of these arrests yielded fairly direct information on the rate of bond formation (on-rate) and dissociation (off-rate). Off-rate values obtained on collagen or laminin 5 (0.06 seconds^{-1}) were tenfold lower than values determined on selectins. Bond stability was strongly regulated by anti-β1 chain antibodies since the off-rate was decreased sixfold by activating antibody TS2/16 and increased fivefold by inhibitory antibodies Lia1/2 or P4C10, whereas neutral antibody K20 had no effect on this parameter. Binding frequencies were not significantly changed by all these antibodies. In contrast, both binding frequency and off-rate were altered by antibodies specific for the α2 chain, suggesting that these antibodies interfered with ligand recognition and also with the ligand-β1 chain interactions responsible for bond stabilization. The latter hypothesis was supported by the finding that the partial alteration of α2 chain function by inhibiting antibodies was corrected by anti-β1 chain antibody TS2/16. These results could not be ascribed to allosteric changes of the functional region of β1 integrin subunits regulated by TS2/16 since there was no competition between the binding of TS2/16 and anti-α2 chain antibodies.

Interpreted within the framework of current concepts of integrin-ligand binding topology, these data suggest that ligand-alpha chain interactions may be qualitatively important in ligand recognition and also influence the formation of the ligand-β1 subunit bonding involved in stabilization of the ligand-integrin complex by regulating its dissociation rate.

Key words: Keratinocyte, Integrin, Flow chamber, Kinetic parameter, Adhesion, Monoclonal antibody, Structure-function relationship

INTRODUCTION

Cell adhesion requires first the binding of plasma membrane receptors to molecules borne by the extracellular matrix or neighbouring cells, and second a link between bound receptors and the cell cytoskeleton. Adhesion may be regulated at both levels. The regulation of adhesion that takes place at the level of receptor-ligand interaction may involve a modulation of receptor density, distribution on the cell membrane or affinity. The regulation of adhesion by affinity modulation is of peculiar importance in the integrin superfamily (Hynes, 1987). Thus, leukocyte integrins are inactive under normal conditions and their activation may follow the triggering of various signal transduction pathways by growth factors or chemokines. This regulation is believed to involve an increase of receptor affinity through allosteric conformation changes (Loftus and Liddington, 1997). Indeed, when LFA-1 integrin (CD11a/CD18) was purified from T lymphocytes and assayed for ligand binding in vitro, receptor affinity exhibited a two hundredfold increase when cells were activated with phorbol ester (Lollo et al., 1993). Also, at least three different affinity states were found on the leukocyte α4β1 receptor for VCAM-1 when this integrin was probed with a VCAM-1/IgG fusion protein after activating cells with a divalent cation (Mn^{2+}) or an activating anti-β_1 monoclonal antibody (TS2/16) (Jakubowski et al., 1995). However, recent intriguing experimental evidence suggested that the leukocyte integrin Mac-1 (CR3, CD11b/CD18) might display increased association and dissociation rates when cells interacted with a suitable monoclonal antibody (Cai and Wright, 1995). This finding is important since it was recently emphasized that kinetic rates were as important as affinity constants in determining the functional behaviour of cell surface receptors (Williams, 1991).

Integrins are heterodimeric structures with α and β chains. Integrins sharing a β1 subunit are usually receptors for extracellular matrix components (Hynes, 1987). Ligand-

integrin interaction is known to involve the N-terminal regions of both α and β chains, the ligand and cations (Mould, 1996; Loftus and Liddington, 1997). The β1 α chain clearly contributes to the binding specificity since several integrins sharing a common β subunit may recognize different ligands. Alpha chain N-termini are made of seven repeats, and they were predicted to be folded into a so-called β propeller domain whose surface contains ligand binding sites and epitopes recognized by function-blocking antibodies (Springer, 1997). An experimental check of this model was provided by studies done on the α4 chain (Irie et al., 1997). Some integrin α chains contain an extra I domain that is inserted between the second and third repeat (Springer, 1997). These I domains usually display multiple ligand-binding specificities (Diamond et al., 1993; Kern et al., 1994; Calderwood et al., 1997) and the binding sites contained in these domains cooperate in a complex manner (Mesri et al., 1998). However, other sequences contained in the β propeller may also contribute ligand binding by integrins, even when they contain an I domain (Stanley et al., 1994). Bridges formed by coordinate links between a Mg^{2+} ion and oxygenated amino acids belonging to the integrin and its ligand seem to play an essential role in the binding process. These amino acid residues are displayed by CD or FG loops in the ligand (Casasnovas et al., 1997) and by metal ion dependent adhesion sites (MIDAS) within integrins (Lee et al., 1995). These sites are found in I domains, β chains, and possibly β propellers (Lee et al., 1995; Puzon-McLaughlin and Takada, 1996; Collins Tozer et al., 1996; Goodman and Bjat, 1996; Springer, 1997). In the β1 chain, these sequences lie near a regulatory region which is bound by activating and inactivating antibodies (Takada and Puzon, 1993). Ligand/receptor sequence complementarity and metal ion coordination seem to cooperate intimately since mutations or antibodies altering one of these parameters can abolish integrin-ligand association. Further, efficient binding requires an accurate spatial arrangement between ligand and integrin surfaces. Indeed, the regulation of integrin function, with concomitant affinity alteration, involves conformational changes of alpha and beta subunits. These structural changes of the integrin ectodomain can be demonstrated. Thus, epitopes recognized by specific monoclonal antibodies, such as ligand-induced or ligand-attenuated binding sites, may exhibit structural changes (Mould, 1996). These changes can be induced with divalent cations such as Mg^{2+}, Ca^{2+} or Mn^{2+} (Hu et al., 1996), function-activating or inhibiting monoclonal antibodies (Takada and Puzon, 1993), interaction with ligand molecules (Faull et al., 1993) or inside-out signalling (O'Toole et al., 1994). The latter process involves conformational changes propagated to the integrin ectodomain after engagement of cytoplasmic tail with specific cytosolic proteins and/or with the cytoskeleton. Thus, integrin receptors are highly flexible and there is much current interest in the relationship between their conformation and binding properties. However, the precise contributions of alpha and beta subunits to the sequence of events leading to bond formation remain poorly understood. As an example, underactivated receptors are often said to be cryptic, thus implying that activated receptors should be open, which suggests that the receptor ability to accomodate its ligand may be subject to regulation.

In the present paper, we attempted to gain new information on the respective contributions of alpha and beta integrin subunits to ligand binding. We studied binding kinetics, i.e. the rate of integrin-ligand bond formation and dissociation, and alterations induced by antibodies specific for alpha and beta subunits. These antibodies were expected to interfere with integrin function either by direct masking or by induction of conformational changes affecting the ligand binding sites (Mould, 1996; Irie et al., 1997; Loftus and Liddington, 1997). In order to get an insight into the function of integrins in a living cell environment, we studied binding kinetics by monitoring the adhesion of moving keratinocytes to type I collagen or laminin 5 in a flow chamber. Although this experimental approach is not relevant to keratinocyte biology, it was expected to yield valuable information on integrin binding properties. Indeed, this methodology was successfully applied to the study of selectin function (Kaplanski et al., 1993; Alon et al., 1995), antigen-antibody interaction (Pierres et al., 1994, 1995) and short term association between lymphocyte CD2 and CD48 ligand (Pierres et al., 1996), yielding a fairly direct information on the lifetime of bonds formed between surface-bound molecules. Results were consistent with data obtained on purified molecules with surface plasmon resonance technology.

Briefly, when keratinocytes, whose integrins are constitutively active, were driven along ligand-coated surfaces, they displayed numerous transient arrests that were specifically abolished by monoclonal antibodies known to block integrin function. Since cells were driven by a force that was insufficient to alter significantly the duration of single bonds, transient arrests should represent the formation of individual bonds. Therefore the arrest frequency is related to the rate of bond formation while the distribution of arrest durations may give direct information on the dissociation rate (Pierres et al., 1998a).

It is concluded that activating or inactivating conformational changes induced in β1 integrin subunits mainly affected bond dissociation rate, whereas both bond formation and dissociation were strongly hampered by function-blocking antibodies specific for alpha subunit. These data are consistent with a two-step model of integrin-ligand interaction, with ligand recognition by the alpha chain and bond stabilization by the β1 subunit.

MATERIALS AND METHODS

Cell culture

Human keratinocytes were obtained by dispase and trypsin dissociation of human foreskins, followed by culture in keratinocyte serum-free medium (KSFM) supplemented with growth factors. Cells were used at 60-80% confluency and between the second and fourth subculture.

Extracellular matrix proteins and antibodies

Type I collagen purified from rat tail was purchased from Sigma. Laminin 5 was purified from keratinocyte conditioned medium loaded on an affinity column made of an anti-laminin 5 mouse IgG1 monoclonal antibody (clone 81-8-9 produced in the laboratory) grafted to hydrazide-activated beads (Bio-Rad). Bound laminin 5 was eluted with 50 mM diethanolamine, then neutralized and dialysed against PBS. Purity was checked by SDS-polyacrylamide gel electrophoresis under reducing conditions, and early eluted fractions containing a fibronectin related molecule were discarded.

The following anti-integrin monoclonal antibodies (mAbs) were used: anti-β1 subunit mAbs were K20 (Immunotech, purified, used at 10 μg/ml), P4C10 (Gibco, ascite diluted 1/100). The following anti-β1 mAbs were kind gifts from Dr Sànchez-Madrid and Dr Cabanas (Madrid, Spain): TS2/16 (purified, used at 5 μg/ml), Lia1/2 (purified, 5 μg/ml), HUTS-21 (purified, 10 μg/ml). Anti-α2 subunit mAbs were Gi9 (Immunotech, purified, 10 μg/ml), P1E6 (Chemicon, ascite diluted 1/50). The anti-α3 subunit mAb was C3VLA3 (Immunotech, purified, 15 μg/ml). The anti-α1 subunit mAb was HP2B6 (Immunotech, purified, 15 μg/ml) and the anti-α6 subunit mAb was GOH3 (Immunotech, purified, 10 μg/ml). Control mouse IgG1, IgG2a and IgG2b were supplied by Immunotech and used at 15 μg/ml. Fluorescein-conjugated anti-mouse antibodies were purchased from Silenius and used at 1/10 dilution.

Procedure followed for antibody use

Cells were treated with antibodies after being detached from culture dishes and resuspended in KSFM. Incubation with anti-integrin antibodies was done for 45 minutes at 4°C. Cells were then warmed for 15 minutes at 37°C before injection into the flow chamber.

Flow cytometry was performed after incubating anti-integrin treated cells for another 30 minute period at 4°C with fluorescein-conjugated anti-mouse immunoglobulin or with preformed fluorescent complexes obtained by incubating a suitable anti-integrin antibody with the same molar amount of fluorescein-conjugated anti-mouse immunoglobulin for 60 minutes at 37°C, followed by reaction with a 50-fold molar excess of mouse immunoglobulin for 3 hours at 4°C.

Cells were then assayed with an Epics XL flow cytometer (Coulter).

Coating of adhesion surfaces with extracellular matrix proteins

Glass coverslips were washed with 20% H_2SO_4 in water for 2 hours, then derivatized with 3-aminopropyltriethoxysilane for 4 minutes and activated with 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 30 minutes. They were then dipped overnight at 4°C in a PBS solution containing 20 μg/ml type 1 collagen or 5 μg/ml laminin 5 so that only half the slide area was coated. Coverslips were then blocked with 0.1 M ethanolamine in PBS, pH 7.8, for 30 minutes and heat-denatured bovine serum albumin (BSA, Sigma, ref 7030) for 1 hour. All steps were separated by rinses with PBS.

Flow chamber

We used a modification of a previously described apparatus (Kaplanski et al., 1993). Briefly, the flow chamber (a kind gift from Prof. M. Delaage, Immunotech, Marseille) was assembled by glueing a collagen- or laminin-coated coverslip with silicon glue (Rubson) on the bottom of a plexiglas block bearing a cavity of $1\times17\times6$ mm^3 with a drilled inlet and outlet. The part of the coverslip that was coated with BSA alone was orientated towards the fluid inlet. The chamber was set on the stage of an inverted microscope (Olympus IM) bearing a CCD videocamera (Model SPT M208CE, Sony, Japan) connected to a timer/character generator (Sopro 600, Soprorep) and a videotape recorder. Observations were performed with a 10× objective. The flow was generated with a 2 ml plastic syringe mounted on an electric syringe holder (Razel Scientific instruments, Stamford, CT). The wall shear rate was set at 2 $second^{-1}$. The apparatus was maintained at 37°C in a plexiglas box supplied with warm air.

Monitoring of cell motion

In a typical experiment, a 0.2 ml aliquot of cell suspension (about 0.5 million per ml of KSFM supplemented with 0.5% BSA) was driven into the chamber and the motion was recorded on a videotape for about 10 minutes for delayed analysis. The microscope was focused on the cells rolling on the chamber floor. The examination field was fixed in a region of the slide that was coated with matrix protein and close to the non-adherent area and fluid inlet, in order that most cells might reach the field before adhering to the surface.

The recorder output was connected to a PCvision+ digitizer (Imaging Technology, Bedford, MA) mounted on an IBM-compatible desk computer. This allowed real time digitization with 8-bit accuracy, yielding 512×512 pixel images. A custom-made software allowed manual superimposition of a cursor driven by the computer mouse on live images. Individual cell trajectories were thus obtained by following the front edge of moving cells with the cursor and recording time and position with a frequency of about 20 points per second.

Each videotape was replayed a sufficient number of times to monitor the motion of every cell passing through the microscope field. Cells were found to display numerous arrests whose duration ranged from a fraction of a second to several minutes or more. Arrests were defined as *durable* when they lasted 15 seconds or more and *transient* when their duration was lower than 15 seconds. Three series of measurements were performed for all tested experimental conditions:
(i) First, the proportion of cells exhibiting at least one durable arrest during their passage through the microscope field was determined. This parameter was expected to give a qualitative account of adhesiveness.
(ii) Second, a number of cells were followed for simultaneous determination of total trajectory length and number of detectable arrests. Data were used to determine the *binding frequency* as explained below.
(iii) Third, the durations of a number of cell arrests were determined by individual monitoring of every arrested cell for at least 15 seconds. Data were used to calculate the *dissociation rate* as explained below.

Data analysis

A custom made software was used to process trajectories as previously described (Kaplanski et al., 1993), yielding two different pieces of information.

Binding frequency

The binding frequency P was calculated as the ratio between the number N of (transient or durable) arrests and the total displacement length measured on a given cell sample:

$$P = N/L \ . \qquad (1)$$

This parameter was expressed as μm^{-1}. The accuracy of determination of this parameter was calculated by using known properties of Poisson distribution, yielding $N^{1/2}/L$ as an estimate of the standard deviation.

Dissociation rate

The durations of individual arrests measured under given experimental conditions were ordered, yielding a sequence such as:

$$t_1 < t_2 < .... < t_i < .... < t_N \ , \qquad (2)$$

where t_i is the duration of the ith arrest out of a total of N (typically about 50) values.

The fraction F(t) of cells remaining bound at time t_i after arrest is thus simply i/N. As previously discussed, if arrests are mediated by the formation of single bonds with a *dissociation rate* (or *off-rate*) k_-, F is expected to follow an exponential law:

$$F(t) = \exp(-k_- t) \ . \qquad (3)$$

Therefore, the off-rate is simply the initial slope of the curve obtained by plotting F(t) versus time with semi-logarithmic coordinates. As previously shown, at least two different mechanisms might make experimental curves different from straight lines (with an upward concavity):
(i) Adhesion might be strengthened by the formation of additional interactions (Pierres et al., 1994, 1996).
(ii) Bond formation might be a multiphasic process, with initial formation of a transient intermediate state (Pierres et al., 1995).

The only way to perform a detailed analysis of these possibilities

would be to use artificial systems with low lateral diffusion of binding molecules and vary the receptor density over a wide range (Pierres et al., 1995, 1996). Since this was not feasible in the present case, only the slope of dissociation curves at time zero could be used to determine the dissociation rate of the initial bound state. This slope was calculated by determining the regression line of experimental points on a limited time interval (Fig. 1B). It was important to estimate the error involved in this procedure. This was achieved by computer simulation as follows: for the sake of simplicity, we chose $1/k_-$ as unit of time. F(t), as given in equation (3), is thus simply exp(−t). Then we select an arbitrary number of arrests N and a time limit t_{max} corresponding to the maximum duration of transient arrests. We generate N random numbers (x_1... x_N) ranging between 0 and 1, and we associate an arrest duration t_i with each random number x_i according to the following formula:

$$t_i = -\ln(x_i)/k_- = -\ln(x_i)\ . \qquad (4)$$

It is easily shown that the distribution of random variables t_i is identical to the expected distribution of the durations of arrests generated by a single molecular event. The N values of t_i could thus be used to obtain an estimate of the dissociation rate by determining the regression line of ln(F(t) over the time interval [0, t_{max}]. This procedure was repeated a thousand times for different values of parameters N and t_{max}. It was thus possible to obtain a mean and standard deviation for the accuracy of the estimate of the slope of detachment curves. Results are presented below.

RESULTS

α2β1 integrin receptors mediate specific attachment of flowing cells to collagen-coated surfaces

In a first series of experiments, cells were driven along collagen-coated glass surfaces in the flow chamber. A typical trajectory is shown in Fig. 1A: two transient arrests and a durable one are clearly visible. About 50% of cells flowing on collagen-coated surfaces displayed durable arrests (i.e. more than 15 second duration). They were considered as having adhered to the surface.

It was then checked that the binding of flowing cells to collagen involved α2β1 integrins as expected (Ando and Jensen, 1993). This was done by studying the effect on adhesion of antibodies known to interfere with integrin function. It was found that the frequency of durable arrests was decreased by 60 and 80%, respectively, when cells were treated with function-blocking antibodies specific for the β1 and α2 subunits. Further, durable arrest frequency displayed 30% increase when cells were treated by function-activating anti-β1 subunit antibodies (Table 1). It was surprising that the inhibition of adhesion by anti-β1 antibodies Lia1/2 or P4C10 was significantly lower than the inhibition (almost 100%) found in conventional adhesion assays (data not shown). However, this apparent discrepancy was explained when it was found that more than 90% of cells that bound collagen after anti-β1 antibody treatment were detached when the wall shear rate was increased from 2 to 20 seconds^{-1}, whereas more than 90% of control collagen-bound cells resisted the higher shear stress (not shown). Indeed, this higher shear stress might mimick the washing conditions used in conventional adhesion assays. In other experiments, it was found that adhesion was not significantly altered when two other potential collagen receptors, i.e. α1β1 or α3β1 integrins, were blocked with anti-alpha chain antibodies (data not shown). Thus, α2β1 integrins were indeed dominantly involved in cell adhesion to collagen-coated surfaces.

Accuracy of dissociation rate determination

The values of transient arrest durations were used to build

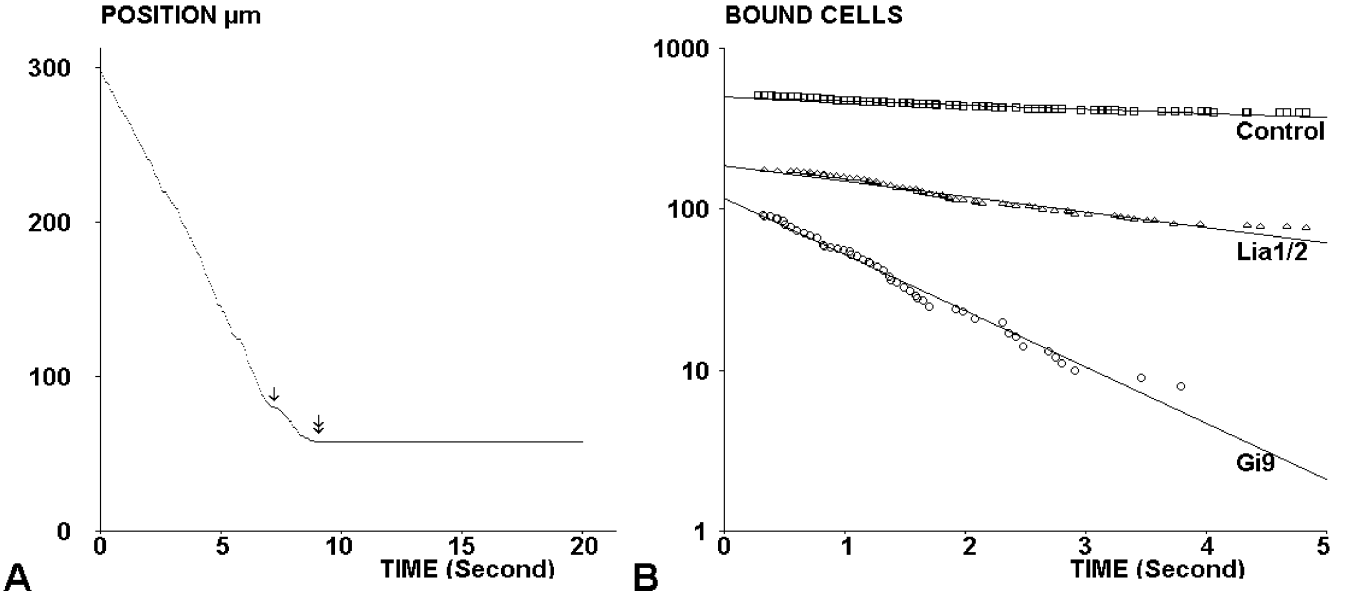

Fig. 1. Analysis of cell motion in a flow chamber. (A) Representative cell trajectory. Keratinocytes were driven along collagen-coated surfaces under microscopic control. A typical trajectory is shown in A. The abscissa of the leading edge was plotted versus time. Each point represents an instantaneous position. Periods of uniform displacement are interrupted by transient (arrow) or durable (double arrow) arrests appearing as horizontal lines. (B) Determination of initial detachment rate. Arrest durations were ordered, and the number of cells remaining bound at a time t after arrest was plotted versus time. The initial detachment rate was calculated as the slope of the regression line on the interval [0s, 5s]. Curves obtained with control cells, or cells treated with function-blocking anti-β1 (Lia1/2) or anti-α2 (Gi9) monoclonal antibodies are shown. Correlation coefficients are 0.966, 0.991 and 0.979, and slopes are –0.06 seconds^{-1}, –0.22 seconds^{-1} and –0.81 seconds^{-1}, respectively.

Table 1. Effect of anti-integrin antibodies on the interaction between flowing keratinocytes and collagen surfaces: raw data

	Study of durable arrests (>15 seconds)		Study of arrest frequency			Time values used for dissociaton rate study	
Antibody	Cell number	% Durable arrests	Cell number	Arrest number	Total path (mm)	Arrest number	Arrest <5 seconds
None	1455	42	580	500	384	1016	199
TS2/16 (anti-β1)	676	63.3	100	76	55	377	23
Lia1/2 (anti-β1)	580	21.3	272	274	238	174	63
P4C10 (anti-β1)	60	20	40	46	46	46	25
HUTS-21 (anti-β1)	186	47.3	150	99	96	135	20
K20 (anti-β1)	407	39.8	284	177	167	235	39
Gi9 (anti-α2)	218	0.92	177	34	138	92	47
P1E6 (anti-α2)	89	10.1	40	12	35	40	21
Gi9+TS2/16	52	48	34	52	35	45	12
P1E6+TS2/16	92	31.5	98	38	70	49	5
Gi9+P1E6	129	0	–	–	–	–	–
Gi9+P1E6+TS2/16	110	7.3	50	10	43	43	24

Cells were treated with different combinations of anti-integrin antibodies before being driven along collagen-coated surfaces under microscopic observation and video-recording. Three sequential studies were performed in order to determine (i) the percentage of cells exhibiting a durable arrest, lasting 15 seconds or more (2nd and 3rd columns), (ii) the total length of trajectories spanned by a given cell sample together with the total number of arrests (transient or durable): 4th, 5th and 6th column, and (iii) the durations of arrests were recorded. The total number of detected arrests (7th column) and number of arrest lasting 5 seconds or less are shown.

dissociation curves as exemplified in Fig. 1B. Curves were fairly linear during the first five seconds on semi-log axes (the correlation coefficient between time and logarithm of the fraction of bound cells was generally higher than 0.97). The slope of the regression line could thus be used as an estimate of the dissociation rate of the bond mediating observed arrests (i.e. the off-rate). The off-rate measured on cells flowing on collagen (199 time values lower than 5 seconds out of 1,016 values) was 0.06 seconds^{-1} (Table 2), corresponding to a theoretical average binding time of 1/0.06 = 16.7 seconds. Since most arrests lasted more than 1 minute, some strengthening phenomenon must occur during the first tens of seconds following the formation of the first bond. This may correspond to the formation of more than one bond as well as further strengthening by receptor connection to the cytoskeleton.

However, the estimate of the slope was somewhat dependent on the time interval selected to determine the regression line. Thus, the respective slopes obtained for the intervals [0s, 1s], [0s, 2s] and [0s, 5s] with data shown in Fig. 1B (control) were, respectively, 0.056 (n=17 values, correlation coefficient r=0.985), 0.083 (n=38, r=0.979) and 0.065 (n=83, r=0.991). It was thus felt necessary to perform a theoretical study of the reliability of our method in order to determine the minimal number of time values required to achieve sufficiently accurate determination of dissociation rates. This information was obtained by numerical simulation and results are displayed in Fig. 2. Results were expressed as dimensionless values in order to allow their application to various situations. Conclusions may be summarized as follows:

- First, as shown in Fig. 2A, the use of a time limit t_{max} results in a systematic overestimate of the dissociation rate.

- Second, as shown in Fig. 2B, the systematic bias in the estimated value of the off-rate k_- is essentially dependent on the number of time values retained to determine the regression line *whatever the total number of arrests*. When more than 6 time values are used, the systematic error is expected to be less than about 20%, which seems quite reasonable.

- Third, the coefficient of variation (i.e. ratio between the standard deviation and mean) of estimated slope values is also strongly dependent on the number of points used (Fig. 2C). Indeed, the coefficient of variation is about 50% when 7 values are used, and it is about 39%, 25%, 17% and 8.5% when 10, 25, 50 or 200 time values are used to determine the slope.

In conclusion, our determination of the dissociation rate is only semi-quantitative (i.e. the coefficient of variation is higher than 30%) when the number of time values is lower than about 15.

The dissociation rate of cell-collagen bonds, not the binding frequency, is strongly influenced by function-modulating anti-β1 subunit antibodies

Since the adhesion of flowing keratinocytes to collagen was shown to involve α2β1 integrins and a quantitative

Table 2. Effect of anti-integrin antibodies on the kinetic parameters of interaction between flowing keratinocytes and collagen surfaces

Antibody	Binding frequency (mm^{-1})	Dissociation rate (second^{-1})	Mean fluorescence intensity
None	1.30±0.06	0.06±0.005	0.2
TS2/16 (anti-β1)	1.38±0.16	0.01±0.002	52
Lia1/2 (anti-β1)	1.15±0.07	0.22±0.035	59
P4C10 (anti-β1)	1.00±0.15	0.41±0.09	ND
HUTS-21 (anti-β1)	1.03±0.10	0.05±0.013	2
K20 (anti-β1)	1.06±0.08	0.05±0.01	62
Gi9 (anti-α2)	0.25±0.04	0.81±0.14	29
P1E6 (anti-α2)	0.34±0.10	0.56±0.13	29
Gi9+TS2/16	1.49±0.21	0.02±0.007	73
P1E6+TS2/16	0.54±0.09	0.03±0.05	69
Gi9+P1E6	0	–	45
Gi9+P1E6+TS2/16	0.23±0.07	0.3±0.07	97

Cells were treated with different combinations of anti-integrin antibodies before being driven along collagen-coated surfaces in a flow chamber. The binding frequency and bond dissociation rate were calculated and results are shown ± standard deviation as explained. Some samples were also labeled with fluorescent anti-immunoglobulin and mean fluorescence was determined with a flow cytometer. ND, not determined.

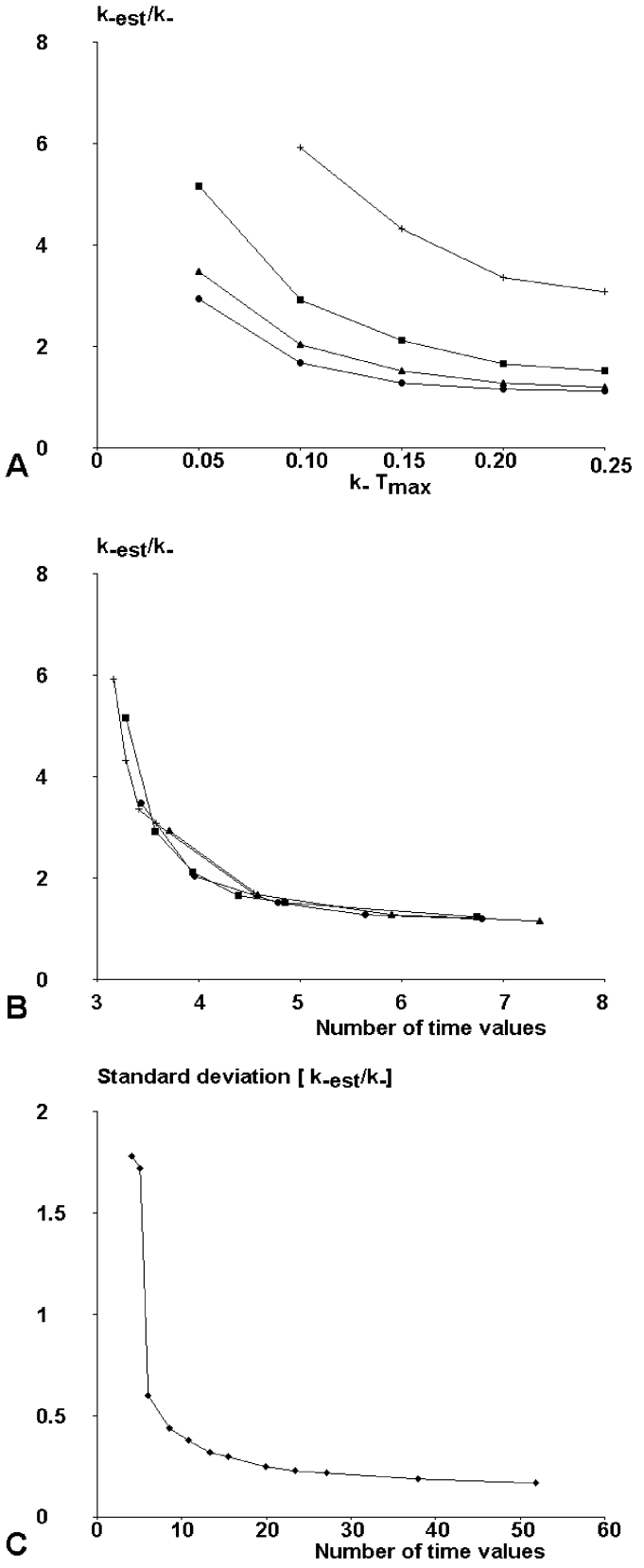

Fig. 2. Accuracy of dissociation rate determination. In several series of simulations, two values N and t_{max} were chosen, and 1,000 random sequences of N arrest durations were generated with a distribution corresponding to a theoretical dissociation rate k_- of 1 second^{-1}. For each sequence, the n numerical values of durations that were lower than t_{max} were used to estimate the dissociation rate. The dimensionless ratio k_{-est}/k_- between the estimated and actual (i.e. 1 second^{-1}) dissociation rates was calculated, and the mean and standard error for this ratio were determined for each couple (N, t_{max}). Results were used to plot k_{-est}/k_- versus dimensionless quantity k_-t_{max} (A), k_{-est}/k_- versus number n of retained time values (B), and the standard error of k_{-est}/k_- (i.e. the coefficient of variation of k_{-est}) versus the number n of retained values (C). The values of N were 10 (crosses,), 20 (squares), 30 (triangles), 40 (circles) or 60 (diamonds).

determination of binding frequency and dissociation rate was feasible, it was warranted to look at the influence of function-modulating anti-β1 subunit antibodies on the kinetic parameters of cell-to-collagen interaction. We used function-inhibiting antibodies Lia1/2 and P4C10, and function-activating TS2/16 antibody. Also, we tested K20, a neutral antibody that was not expected to affect integrin function, and HUTS-21, an antibody interacting with an epitope induced by ligand binding. It was checked with flow cytometry that cell binding of function-modulating antibodies was competitive as expected, since these antibodies were supposed to bind to the same regulatory region in the β1 subunit (Takada and Puzon, 1993). Indeed, as shown in Fig. 3A, the binding of Lia1/2 displayed 100% competition with TS2/16 or P4C10, not with neutral antibody K20 which is known to interact with an epitope located out of the regulatory loop.

The effects of anti-β1 antibodies on integrin binding kinetics are shown in Tables 1 and 2. None of the studied antibodies interfered with the frequency of cell arrests. In contrast, the initial detachment rate was decreased sixfold by TS2/16 and increased about fivefold by Lia1/2 or P4C10, amounting to a 30-fold variation of the off-rate depending on the state of integrin activation. K20 or HUTS-21 did not modify the dissociation rate of bonds formed between cells and collagen. Although it is known that HUTS-21 does not change integrin binding affinity (Gomez et al., 1997), the lack of significant effect of this antibody in our experiments might be due to a poor binding to keratinocytes (Table 2).

Thus, these data suggest that bond stability rather than bond formation was affected by antibodies binding to the β1 subunit and interfering with integrin function.

The inhibitory effect of two anti-α2 antibodies is somewhat reversed by activating anti-β1 antibody

The next step consisted of studying the modulation of keratinocyte-collagen binding by two anti-α2 chain antibodies. We used P1E6 and Gi9 that are known to block α2β1 receptor function after binding to the I domain, which is a major collagen binding region in the α2 chain (Kamata et al., 1994; Calderwood et al., 1997). Flow cytometric analysis revealed partially additive binding of these antibodies to keratinocytes, suggesting that they recognized non-identical epitopes. Indeed, a mean fluorescence intensity of 29 was obtained after incubating cells with each antibody at the concentration used in adhesion assays (Table 2), and this value was not

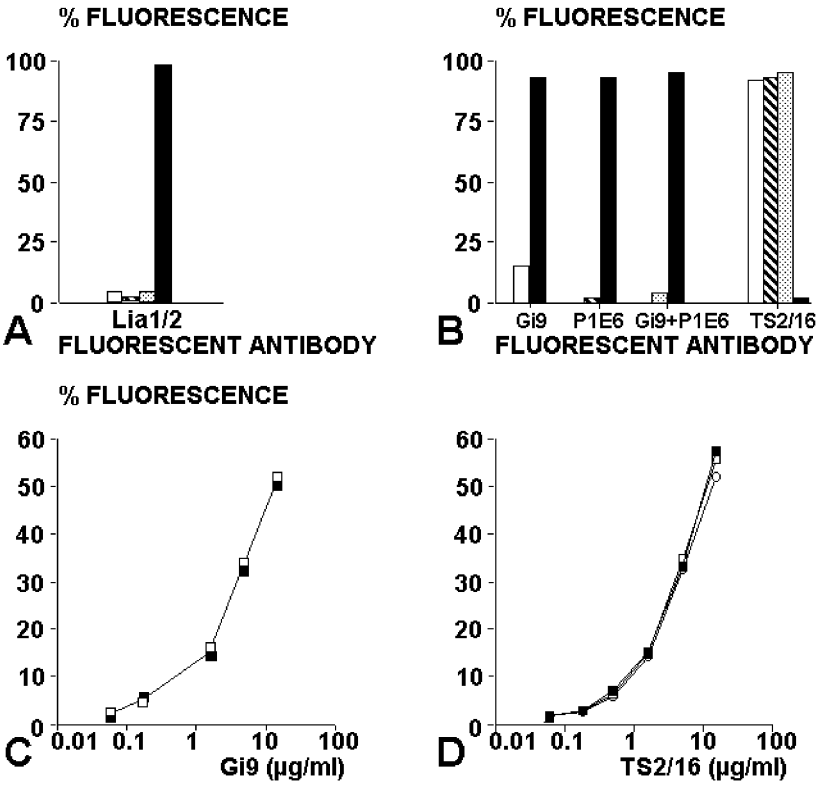

Fig. 3. Competitive cell binding for inhibiting and activating anti-β1 monoclonal antibodies but not for TS2/16 and anti-α2 antibodies. (A) Competition between function-modifying anti-β1 antibodies. A complex between Lia1/2 and fluorescent anti-mouse immunoglobulin was allowed to bind to cells preincubated with a control IgG1 (black area) or with Lia1/2 (white area), P4C10 (hatched area) or TS2/16 (dotted area). The mean fluorescent intensity was then determined with flow cytometry and expressed as a percentage of control. Duplicate determinations were performed and standard deviations were less than 5%. Mean fluorescence obtained with a complexe made of a non-immune IgG1 instead of Lia1/2 was less than 0.4. (B) Absence of competition between TS2/16 and anti-α2 antibodies. Complexes between antibodies Gi9, P1E6, Gi9+P1E6 or TS2/16 and fluorescent anti-mouse immunoglobulin were allowed to bind to cells preincubated with a control IgG1, or with Gi9 (white areas), P1E6 (dotted areas), Gi9 and P1E6 (hatched areas) or TS2/16 (black areas). Mean fluorescence intensities were expressed as percentage of controls. Duplicate determinations were performed and standard deviations were less than 5%. Mean fluorescence values for non-immune complexes were less than 0.4 in all tested conditions. (C) Cell binding of Gi9 is not modified by TS2/16. Cells were incubated with different concentrations of a complex formed between Gi9 and fluorescent anti-mouse immunoglobulin after a first incubation with control IgG1 (open squares) or TS2/16 (closed squares) at 20 µg/ml. Duplicate determinations were performed and standard deviations were less than 5%. (D) Cell binding of TS2/16 is not modified by Gi9 or Gi9 + P1E6. Cells were incubated with different concentrations of a complex formed between Gi9 and fluorescent anti-mouse immunoglobulin after a first incubation with control IgG1 (open squares), Gi9 (closed squares) or (Gi9+P1E6) (open circles). Each antibody was used at 20 µg/ml. Duplicate determinations were performed and standard deviations were less than 7%.

significantly increased when the antibody concentration was increased by a factor of 2 or 10 (not shown). In contrast, the mean fluorescence intensity was increased up to 45 when cells were incubated with both Gi9 and P1E6 antibodies (Table 2).

Cell treatment with Gi9 or P1E6 resulted in a drastic alteration of both binding frequency and dissociation rate (Table 2). Surprisingly, the alteration of the binding frequency and dissociation rate by Gi9 was completely reversed by the function-activating anti-β1 antibody TS2/16. The dissociation rate increase induced by P1E6 was also reversed by TS2/16. In contrast with Gi9, however, the drop of the binding frequency induced by P1E6 (down to 0.34 mm^{-1}) was poorly compensated by TS2/16 (0.54 mm^{-1} versus 1.30 mm^{-1} in controls: Table 2). Thus, the sensitivity of integrin-collagen binding to anti-α2 chain antibodies Gi9 and P1E6 was drastically reduced by TS2/16-mediated activation. These data might be explained if Gi9 or P1E6 and TS2/16 were allosteric competitors, i.e. if the binding of anti-α2 antibodies was blocked by a conformational change stimulated by TS2/16. However, this hypothesis is not supported by the finding that the binding of Gi9 or P1E6 and TS2/16 was additive (Table 2), and the binding parameters for Gi9 remained unchanged by an excess of TS2/16 (Fig. 3B,C). Similar results were obtained when P1E6 uptake was studied (data not shown).

Experiments done in steady state adhesion assays with function-blocking antibodies to integrin α1 and α3 chains indicated that the adhesion to collagen of cells treated with Gi9 (or P1E6) and TS2/16 could not be due to the activation of α1β1 or α3β1 integrins (not shown).

Further, when cells were treated with both Gi9 and P1E6, binding parameters were dramatically affected, as expected. Indeed, the binding frequency was equal to zero. Accordingly, the dissociation rate could not be measured (Table 2). However, this inhibition was hardly reversed by TS2/16 since binding parameters were still highly altered when cells were treated with a combination of Gi9, P1E6 and TS2/16. Antibody binding analysis indicated that the inability of TS2/16 to restore the dissociation rate was not due to an allosteric inhibition of TS2/16 binding to keratinocytes after cell treatment with a combination of Gi9 and P1E6 (Fig. 3B,D).

Taken together, these data are consistent with the hypothesis that the integrin α2 chain might contain two distinct binding sites for function-blocking antibodies. TS2/16, an activating anti-β1 antibody, reversed the binding frequency and dissociation rate alteration produced by either of anti-α2 antibodies, not the inhibition induced by a combination of both. A remaining problem is to know whether these antibodies interfered with the function of independent integrin-binding sequences or cooperated to alter a unique recognition site.

The inhibition of cell-laminin 5 adhesion induced by blocking anti-α3 antibodies is not reversed by activating anti-β1 antibody

Many integrin alpha chains lack the I domain contained in the

Table 3. Effect of anti-integrin antibodies on the interaction between flowing keratinocytes and laminin surfaces: raw data

	Study of durable arrests (>15 seconds)		Study of arrest frequency			Time values used for dissociaton rate study	
Antibody	Cell number	% Durable arrests	Cell number	Arrest number	Total path (mm)	Arrest number	Arrest <5 seconds
GOH3 (anti-α6)	49	41	30	26	20	48	12
GOH3+Lia1/2	49	16	49	57	57	38	19
GOH3+C3VLA3 (anti-α3)	220	0	–	–	–	–	–
GOH3+TS2/16	44	64	33	27	19	22	3
GOH3+C3VLA3+ TS2/16	160	0	–	–	–	–	–

Cells were treated with different combinations of anti-integrin antibodies before being driven along laminin 5-coated surfaces under microscopic observation and video-recording. Three sequential studies were performed in order to determine (i) the percentage of cells exhibiting a durable arrest, lasting 15 seconds or more (2nd and 3rd columns), (ii) the total length of trajectories spanned by a given cell sample together with the total number of arrests (transient or durable): 4th, 5th and 6th column, and (iii) the duration of arrests were recorded. The total number of detected arrests (7th column) and number of arrest lasting 5 seconds or less are shown.

α2 chain. This I domain is a 200 amino acid long structure inserted between the 2nd and 3rd repeats composing the N-terminal moiety of integrin alpha chains. It was of interest to know whether our results were specific for integrins containing an I domain. The properties of α3β1 integrins, which lack an I domain, was explored by studying the interaction between keratinocytes and laminin-5. Experiments were conducted with cells treated with a blocking anti-α6 antibody, to focus on α3β1 integrin function. Indeed, keratinocyte-laminin 5 interaction is mediated by α3β1 and α6β4 integrins (Rousselle and Aumailley, 1994). As shown in Table 3, 41% of cells treated with anti-α6 displayed durable arrests while flowing on laminin 5-coated surfaces. Binding frequencies and dissociation rates were quite similar to the parameters obtained by studying cell-collagen interaction (Table 4). Adhesion was abolished by function-blocking anti-α3 chain antibodies, and this inhibition was not reversed by treatment with TS2/16 (Tables 3, 4). The difference between α2 and α3 integrins might be due to the multivalent binding properties of I domains.

DISCUSSION

The purpose of this work was to obtain quantitative information on the kinetic parameters of integrin receptors maintained in a cellular environment. Further, we studied the modulation of these parameters by antibodies known to affect integrin-mediated cell adhesion. Our method consisted of studying the motion of keratinocytes driven along collagen or laminin 5-coated surfaces by a hydrodynamic force insufficient to influence the natural lifetime of a single ligand-receptor bond. Cells were thus expected to display multiple arrests that should allow direct visualization of the formation of single molecular bonds. Binding frequency should be dependent on many parameters such as receptor density on interacting surfaces, distance between the cell surface and chamber floor, contact area between interacting surfaces, and intrinsic kinetic constant of receptor-ligand association (Bell, 1978; Hammer and Lauffenburger, 1987; Pierres et al., 1997, 1998a,b). Assuming that only the latter parameter was affected by function-modulating anti-integrin antibodies, the variations of binding frequencies observed in our study might thus reflect conformation-dependent changes of the kinetic rate of integrin-ligand association.

Also, values of arrest durations could be used to build detachment curves which displayed initial exponential decrease. Only the initial detachment rate could be safely interpreted, since later events might be dependent on many phenomena such as strengthening of initial bonds (Pierres et al., 1995), formation of additional integrin-ligand bonds (Pierres et al., 1996), or formation of additional poorly defined ‘nonspecific’ cell-surface interactions (Pierres et al., 1994). Indeed, accounting for all of these phenomena would require the introduction of multiple unknown parameters that could not be determined unequivocally by fitting theoretical and experimental curves. The interactions we studied specifically involved collagen or laminin 5 and integrin molecules, since observed arrests were essentially abolished by cell treatment with anti-α2 and anti-α3 antibodies as expected. Thus, two possible interpretations for the initial detachment rate may be considered. On the one hand, if all cell surface integrins shared identical properties, the initial detachment rate would be equal to the dissociation rate (k_-) of the integrin-ligand bond. On the other hand, if multiple conformational states of an integrin receptor were expressed on studied cells, the experimental dissociation rate, $k_-(exp)$, would be equal to:

Table 4. Effect of anti-integrin antibodies on the kinetic parameters of interaction between flowing keratinocytes and laminin surfaces

Antibody	Binding frequency (mm^{-1})	Dissociation rate ($second^{-1}$)	Mean fluorescence intensity
GOH3 (anti-α6)	1.3±0.29	0.05±0.02	4
GOH3+Lia1/2	1.0±0.13	0.27±0.07	ND
GOH3+C3VLA3 (anti-α3)	0	–	46
GOH3+TS2/16	1.4±0.27	0.06±0.036	61
GOH3+C3VLA3+TS2/16	0	–	84

Cells were treated with different combinations of anti-integrin antibodies before being driven along laminin 5-coated surfaces in a flow chamber. The binding frequency and bond dissociation rate were calculated and results are shown ± standard deviation as explained. Some samples were also labeled with fluorescent anti-immunoglobulin and mean fluorescence was determined with a flow cytometer.

$$k_{-}(\exp) = \sum_{i} p_i k_{i-}, \quad (5)$$

where p_i is the probability that an arrest is due to an integrin receptor in state (i), with a corresponding dissociation rate k_{i-}. Thus, additional experiments would be required to discriminate between the possibilities that cells might bear several populations of integrin receptors with different dissociation rates or a single homogeneous population of receptors with an intermediate dissociation rate.

The experimental lifetime of cell-collagen bonds observed under our experimental conditions was found to be between 10- and 100-fold higher than measured in previous studies made on selectins (Kaplanski et al., 1993; Alon et al., 1995) or CD2 receptors (Pierres et al., 1996). This result seems quite reasonable in view of previous comparisons done between the properties of selectin and integrin molecules (Lawrence and Springer, 1991).

Ligand-integrin binding is known to result from multivalent interactions between the ligand and sequences in the alpha and beta chain of the receptor (Mould, 1996; Loftus and Liddington, 1997). Here we studied the respective contributions of alpha and beta chains to integrin binding parameters by looking at the changes of binding frequency and dissociation rate induced by function-modulating antibodies specific for the alpha and beta integrin subunits.

Function-modifying anti-β1 subunit antibodies essentially altered bond dissociation rate since the binding frequency was unaffected by all tested antibodies while dissociation rates measured on control cells, respectively, exhibited a 6-fold decrease and a 4-fold increase when cells were treated with activating antibody TS2/16 or inhibiting antibody Lia1/2. These results might be consistent with the existence of three affinity states for integrin receptors, depending on the β1 chain conformation, with a respective dissociation rate comprised between 0.22 and 0.41 in the presence of Lia1/2 or P4C10 antibodies (note that these experimental values were not significantly different according to Student's *t*-test), 0.06 in controls, or 0.01 in presence of TS2/16 antibody. The 20- to 40-fold difference between the dissociation rates measured on antibody-activated and antibody-inhibited cells is quite comparable to the 20-fold affinity difference reported for α5β1 integrins expressed by resting erythroleukemic cells, whose integrins are underactive, and cells treated with an activating monoclonal antibody (Faull et al., 1993). These data are consistent with the hypothesis that the bond stabilization function of the β1 chain might depend on the spatial positioning of β1 chain ligand-binding sequences such as those mapped on oxygenated amino acid residues lying at a distance upstream (130-132) or in a MIDAS-like sequence (residues 224-295) immediately downstream of the regulatory loop (residues 207-218) where antibodies Lia1/2 and TS2/16 are binding (Takada and Puzon, 1993; Puzon-McLaughlin and Takada, 1996).

The dissociation rate of cell-collagen bonds obtained with inactivating antibody Lia1/2 was high but measurable. The residual cohesiveness of the bonds measured on cells treated with this antibody may still be somehow contributed by β1 chains. However, it might also depend on a ligand-alpha subunit interaction since it has been shown that the I domain, which is a major binding site for collagen and the α2 chain, displayed significant affinity for collagen (Kern et al., 1993; Calderwood et al., 1997).

Both binding frequency and dissociation rate were strongly affected by function-blocking anti-α chain antibodies. Indeed, bond formation was completely blocked by anti-α3 antibodies, preventing quantitative determination of bond dissociation rate with keratinocytes flowing on laminin 5 after antibody treatment. Two function-blocking antibodies, Gi9 and P1E6, which bind additively to the α2 chain I domain, were needed to abrogate cell-collagen interaction. This suggests that multiple sites are recognized on collagen and/or that cooperative conformational changes are required to inactivate integrin receptors, in accordance with known ligand-binding properties of I domains. Further, it was observed that activating anti-β1 antibody TS2/16 could compensate the alteration of bond formation and dissociation resulting from partial modification of α2 chain function. This would suggest the occurrence of a complex cooperation between alpha and beta chain subunits in ligand recognition and binding.

The finding that ligand-binding parameters were so profoundly altered by inhibitory anti-alpha chain antibodies was surprising. Indeed, the intrinsic properties of ligand-β1 chain interactions might have been expected to account for detectable residual binding in view of their importance in the regulation of bond stability, provided (i) they were not perturbed by anti-alpha chain antibodies, and (ii) ligand binding to the alpha and beta chains were independent events. However, our data might be explained if anti-alpha chain antibodies interfered with β1 chain binding properties. This interference might result from the induction of an inactivating allosteric conformational change in these β1 chain subunits. This change should affect the conformation of the structure that is controlled by TS2/16, since this monoclonal antibody reversed the dissociation rate change induced by anti-α2 antibodies. However, this hypothesis is unlikely because the binding parameters of cells treated with any combination of anti-α2 chain antibodies were not modified by TS2/16. On the contrary, if anti-α2 antibodies had a competitive effect on the regulation of bond dissociation by inducing allosteric changes of a same functional region of β1 integrin subunits, a strong inhibition of Gi9 or P1E6 cellular uptake by TS2/16, and an inhitibion of TS2/16 binding by Gi9 and P1E6 would be expected. Indeed, the increase in bond dissociation rate was corrected by TS2/16 only when it was produced by either of the anti-α2 antibodies, not when they were combined.

It was recently found that fibronectin-α5β1 association involved interactions between the synergy region in fibronectin and the β propeller domain of α5, and also between the RGD sequence and β1 subunit (Mould et al., 1997). In line with the concept that ligand-integrin association is multivalent, these findings emphasized the relative contribution to the binding process of interactions between integrin subunits and corresponding sequences in the ligand. Since this may be a general paradigm of ligand-integrin interaction, we should consider the possibility that the alteration of ligand-β1 chain association by anti-alpha chain antibodies might be a direct consequence of the blockade of alpha chain interaction with putative accessory regions in collagen or laminin 5. Indeed, these interactions should be important for ligand recognition since they seemed able to support normal bond formation when the binding function of β1 subunit was altered by specific

antibodies. Further, the finding that the blockade of ligand-alpha chain interaction by inhibitory antibodies also abrogated ligand-β1 chain association may be understood if the former interaction was a prerequisite for the latter, i.e. if the ligand binding by β1 chain could not happen in the absence of a prior interaction with alpha chain. This concept is consistent with recent findings concerning the α5β1-fibronectin model: indeed, recombinant fibronectin molecules bearing inactivating mutations in the synergy region were poor adhesion substrates and bound with low affinity to purified α5β1 integrins, suggesting that the interaction between RGD-containing sequences and β1 subunits might be strongly facilitated by alpha chain-ligand interactions (Mould et al., 1997). The hypothesis that this mechanism might also be relevant to collagen-integrin interactions is consistent with studies reported by Depraetere et al. (1997): while avoiding the use of antibodies, these authors showed that the masking of potential alpha chain-specific sequences in collagen with recombinant α I domain abrogated α2β1-mediated adhesion. Further, it was also found by Mould et al. (1997) that activating anti-β1 monoclonal antibodies compensated the binding defect resulting from inactivation of the fibronectin synergy region. This result suggested that the integrin ligand could bind directly to the interaction sites in the β1 subunits when they were made apparent by activating conformational changes.

In our experiments, the binding parameter alterations triggered by inhibition of alpha chain-ligand interactions were also reversed by activating antibodies. However, reversion was only found when α2 I domain binding activity was partially altered, not when complete blockade was achieved nor when α3β1 integrins were inactivated with anti-α3 antibodies. This suggested that the ligand could not interact directly with the β1 chain unless it also bound alpha chains. Although the difference might be due to the antibodies used, it is possible that the ligand interaction with α2β1 and α3β1 integrins was more dependent on alpha chain-mediated binding facilitation than interaction with α5β1 integrin. Thus, in cells treated with anti-α2 antibody P1E6 and activating anti-β1 antibody TS2/16, bond formation was markedly impaired whereas the lifetime of bonds that were formed was comparable to the lifetime of bonds formed with control cells. This result suggested that the rate limiting step of bond formation might be the ligand association with alpha chains. In recent models of the topology of integrin-ligand interaction, the ligand binding sites in the alpha and beta subunits were located ten angströms apart, and their relative position was subjected to stringent constraints liable to regulation by shape changes of the I domain and beta subunit (Mould, 1996; Loftus and Liddington, 1997; Springer, 1997). The alpha chain-ligand interaction might thus be qualitatively important in preluding the formation of a bond between the ligand and the β1 subunit. The latter bond would stabilize ligand-receptor interaction and decrease the dissociation rate. The alpha chain engagement might result in the positioning of the sequences containing oxygenated amino acid residues in the ligand and the β1 subunit, so that they are close enough to allow their bridging through metal ion coordination (Lee et al., 1995). It should be emphasized that the formation of this bond was the actual condition for triggering the outside-in signalling events leading to adhesion. This is in line with the concept that adhesion involves receptor oligomerization (which may be favoured by low dissociation rate) and conformational changes of the β1 subunit (Hynes, 1992). The partial alteration of the multivalent binding facilities offered by α2 chains might disturb the ligand-β1 subunit interface, thus leading to unstable β1-ligand bonds which may be strengthened by activating topological changes of the β1 subunit. The nature of these changes remains elusive. However, they might involve a loop immediately contiguous to the regulatory region, the sequence of which exhibits important variations between different beta subunits. This loop has been shown to participate in ligand binding specificity, possibly by restricting ligand engagement by the receptor (Takagi et al., 1997).

In conclusion, we used a methodology allowing quantitative study of the rate of bond formation and dissociation between integrin receptors on intact cells and solid phase extracellular matrix proteins. Interpreted within the framework of the current concept that integrin function involves multiple interactions between the ligand and several sequences in the alpha and beta subunit, our data bring some insight into the hierarchy of events leading to integrin-ligand binding.

The main findings are that bond formation was mainly affected by interference with alpha chain function while bond dissociation was regulated by the β1 subunit conformation. In addition, results suggested that ligand-alpha subunit interaction was a prerequisite for ligand-β1 chain binding. Our data are consistent with a two-step model for integrin-ligand binding: ligand recognition and bond stabilization might result from separate but sequential and interwoven events. This model is phenomenologically consistent with recent studies on integrin-ligand binding topology (Mould et al., 1997). More extensive comparison of binding frequencies and dissociation rates measured with integrins and ligands bearing inactivating mutations at predicted interaction sites should allow a more refined topological model of integrin-ligand interactions and adhesion.

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