Regulation of interleukin 1 signalling through integrin binding and actin reorganization: disparate effects on NF-κB and stress kinase pathways

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Interleukin 1 (IL-1)-mediated gene regulation is dependent on cell–matrix interactions. Both IL-1-activated pathways, nuclear factor κB (NF-κB) and the stress-activated protein kinase (SAPK), can be regulated by cell adhesion and changes in the cytoskeleton, suggesting that cell–matrix effects on IL-1 responses are initiated in part though effects on signal transduction. Here we show that IL-1-induced transient alterations in cell shape and in the cytoskeleton in fibronectin attached cells are correlated with effects on peak activity of NF-κB and SAPK. Cells on fibronectin showed a 1.5–2-fold enhancement in IL-1-induced NF-κB activity compared with levels in cells on poly(L-lysine) or bare tissue culture plates. The effect was increased with increasing concentrations of fibronectin and was most prominent at lower concentrations of IL-1. In contrast, fibronectin attachment caused an approx. 50 % decrease in the IL-1 activation of SAPK, eliminating the peak activity after 15 min of stimulation with IL-1. IL-1-induced NF-κB activity showed a successive, substratum-independent increase during 4 h of attachment and spreading, whereas the inhibitory effect of fibronectin on the SAPK pathway was induced at the initial stages of attachment. Further, the addition of a peptide containing the motif RGD resulted in a 40 % decrease in NF-κB activity in cells on fibronectin, largely accounted for by an effect on the p50/p65 heterodimer. Similarly, blocking of integrin aggregation by RGD-containing peptide resulted in a total abrogation of the fibronectin effect on IL-1-induced SAPK activity. The results demonstrate disparate effects of cell adhesion on the activation by IL-1 of the NF-κB and SAPK pathways. Thus fibronectin attachment causes an up-regulation of NF-κB activity in the presence of IL-1, whereas in contrast it results in a pronounced decrease in IL-1-induced SAPK activity.

INTRODUCTION

Cell–matrix interaction influences cell behaviour as well as growth factor-induced biological responses [1]. The effects are mediated in part by the binding of integrins [2–4] and the induction of signal transduction [5–8] through the local activation of kinases at focal adhesions [9–11], and in part through effects on cytoskeleton-regulated second messengers [12,13] subsequently influencing downstream signal transduction pathways and gene activation.

Interleukin-1 (IL-1) is a central mediator of inflammation that exerts a pronounced effect on fibroblast cytoskeletal organization and cell–matrix interactions at focal adhesions, involving the phosphorylation of talin, a transmembrane linkage protein [14]. In addition, IL-1-mediated inflammatory responses are regulated by fibronectin attachment [15], suggesting an effect of integrin binding and/or organization of the cytoskeleton on transduction of the IL-1 signal. IL-1 activates the stress-activated protein kinases (SAPKs) and nuclear factor κB (NF-κB). SAPKs belong to the larger family of proline-directed mitogen-activated protein (MAP)-2 kinases, and regulate gene transcription in response to a number of cytokines and growth factors by phosphorylating the c-Jun component of activator protein-1 (AP-1). In addition, the c-Jun component of the pathway has been shown to be regulated through integrin aggregation and cytoskeletal organization [16,17]. NF-κB is a transcription factor involved in the regulation of multiple IL-1-responsive genes [18–20]. Activation of NF-κB can be induced independently of growth factor action through adhesion in monocytes [21], and in fibroblasts by engagement of the fibronectin receptor [8] as well as through stress-initiated events such as UV irradiation [22,23]. The best-characterized NF-κB complex, a heterodimer, consists of subunits p50/NF-κB1 and p65/RelA belonging to the Rel family [24,25]. However, both homodimers and heterodimeric NF-κB complexes containing these and other Rel family members and recognizing a similar consensus sequence (GGGAATTTCC) can influence gene transcription. The level of activation and the type of NF-κB/Rel dimer activated determine the extent and nature of the response induced [26,27].

Here we analyse the effects of cell–matrix interaction and the cytoskeleton IL-1-induced NF-κB and SAPK activities. The results show that integrin engagement and organization of the actin cytoskeleton, collectively, have distinct and opposite effects on these two pathways, and cause pronounced changes in the pattern of IL-1-induced signal transduction, correlating with the observed effects of fibronectin attachment on IL-1 gene regulation.

MATERIALS AND METHODS

Cell culture

Human dermal or gingival fibroblasts (six strains), transfer 9–14, were used. Cells were plated in 35 mm dishes (4×10^5 cells per

Abbreviations used: AP-1, activator protein-1; IL-1, interleukin 1; NF-κB, nuclear factor κB; SAPK, stress-activated protein kinase.

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dish) or in 10 cm dishes (3×10^6 cells per dish), on bare tissue culture plastic, or on tissue culture or untreated plastic dishes coated with fibronectin (0.1, 1, 5 or 10 μg/ml; Gibco, Gaithersburg, MD, U.S.A.) as described [28], or with poly(L-lysine) (0.1, 0.5 or 1.0 mg/ml; Sigma), in accordance with the manufacturer's instructions, for 2–4 h in 10% (v/v) fetal calf serum, unless stated otherwise. In some experiments, cells were incubated before plating (5 min), and plated in the presence of various concentrations of a peptide containing the motif RGD (GRGDSP; 10 nM to 100 μM; Gibco) or with the same concentrations of a non-specific RGE-containing peptide (GRGESP; Gibco) used as a control; or in the presence of heparin (1 nM to 10 μM) or chondroitin sulphate used as control. After attachment, cells were incubated with or without IL-1β at saturating levels (1 nM) or over a range of concentrations (1 fM to 100 nM) (a gift from Dr. Steven Dower of Immunex Corp.) for 30 min unless stated otherwise. Cells were examined by phase-contrast microscopy at various times during incubation and before harvest.

Immunocytochemistry and confocal microscopy

Human gingival fibroblasts were plated on tissue-culture-treated multiwell coverslip slides (Nunc) uncoated or coated with fibronectin (10 μg/ml; Sigma) for 2 h and were subsequently treated with IL-1β (1 nM) for various times (0, 5, 15, 30, 45 or 60 min). Cells were fixed with 3.7% (w/v) formaldehyde in PBS for 5 min at room temperature and permeabilized with Triton X-100 (0.5%, v/v) in Tris buffer (50 mM Tris/HCl containing 150 mM NaCl and 0.1% NaN_3, pH 7.5) for 5 min. Cells were incubated with FITC-conjugated phalloidin (50 μg/ml; Sigma) for 20 min at room temperature, rinsed three times in PBS and once in distilled water, then air-dried and mounted with glycerol (50%, v/v). Data were acquired with a Multiprobe 2010 Laser Scanning Confocal Microscope (Molecular Dynamics) interfaced to a Silicon Graphics workstation, with the 488 nm line of a krypton/argon mixed-gas laser and a 50 μm pinhole aperture, generating an optical section 0.5 μm thick. After acquisition, data files were exported to an Apple Macintosh computer and converted to 8-bit TIFF images for printing.

Gel-shift assay

Cells (3×10^6 per condition) were harvested with 10 mM Hepes buffer, pH 7.9, containing 1.5 mM $MgCl_2$ and 10 mM KCl (1 ml per 10^6 cells), centrifuged (190 ***g*** 2–3 min) and subsequently resuspended in the same buffer in the presence of Nonidet P40 (0.1%, v/v) (100 μl per 10^6 cells). The nuclear extracts were prepared by standard procedures [29] with modifications as described [8,19,30]. The pellet obtained after centrifugation (12000 ***g***, 5–10 min) was suspended in 20 mM Hepes buffer, pH 7.9, containing 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA and 25% (w/v) glycerol (20 μl per 10^6 cells), incubated for 10 min on ice and centrifuged (14000 ***g***, 5–10 min). Supernatant was diluted in 20 mM Hepes buffer, pH 7.9, containing 0.05 M KCl, 0.2 mM EDTA and 20% (v/v) glycerol (15 μl per 10^6 cells) and centrifuged as above. Supernatant from the fourth centrifugation, containing the nuclear fraction, was assayed for protein content with *N*-hydroxysuccinimido–biotin (20217X; Pierce, Rockford, IL, U.S.A.). Equal amounts of protein from each sample (15 μg) were mixed with a reaction buffer [1 mM EDTA/50 mM NaCl/10 mM Tris/HCl (pH 8.0)], containing poly(dI-dC) alternating copolymer (25%), Nonidet P40 (0.05%) and end-labelled oligonucleotide probe (5×10^5 c.p.m./μl). The NF-κB probe was a double-stranded synthetic oligonucleotide containing the κB motif (5′-TGACAGAGGGGACTTTCCGAGAGGA-3′). End-labelling was done with [γ-^{32}P]ATP (NEN, Boston, MA, U.S.A.) as described [8,19,30]. Samples equivalent to 5 μg protein were loaded on to a 4% (w/v) polyacrylamide gel and run at 180 V and 4 °C. Dried gels were allowed to expose film (X-Omat; Kodak) for 12–24 h at −70 °C with intensifying screens (Hypercassette; Amersham).

Quantification of NF-κB activity was done by determining the intensity of the NF-κB band, and the band containing free probe to ascertain equal loading, with a PhosphorImager (Molecular Dynamics model 400S; Sunnyvale, CA, U.S.A.). Alternatively, after autoradiography, fluorograms were scanned with a densitometer (model GS 300; Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) or analyses was performed on an Apple Macintosh (model IIsi) computer and scanner (ScanJet IIcx; Hewlett Packard, Boise, ID, U.S.A.) with the public-domain NIH IMAGE program (from Wayne Rasband at the U.S. NIH, and available from the internet by anonymous ftp from zippy.nimh.nih.gov). Control experiments for this type of analysis showed that the gel-shift analysis band intensity was linear with active extract concentration over a range from 0 to 100%, and independent of probe dose in the range 1–4 μl, verifying that the assay, as performed, was a quantitative measure for NF-κB activity.

Supershift gel analyses

Cells were plated as above and stimulated with or without IL-1β, then the nuclear fraction was extracted. Before incubation with the labelled peptide (see above), extracts were incubated with rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) against the various subunits of the NF-κB complex. Anti-p50 (22 μg/ml) or anti-p65 (12 or 22 μg/ml) antibodies were added to the reaction mixture at previously determined saturating concentrations. The relative amounts in shifted and non-shifted bands were determined for each condition by PhosphorImager analyses or densitometry, as above. The relative levels of dimers containing the various subunits were calculated for the different conditions.

Analyses of SAPK activity

Fibroblasts plated as above were incubated with IL-1 (1 nM) for various times (2, 5, 10, 15, 30 or 60 min) as indicated, and were extracted in cold 20 mM Hepes buffer, pH 7.4, containing 2 mM EDTA, in accordance with conventional procedures and prepared as described [31]. Cells were scraped into the extraction buffer, disrupted by shear stress and centrifuged (12000 ***g***, 30 min, 4 °C). The kinase activity was analysed with ST-1, a T669 peptide modified with a triplet of N-terminal arginine residues, as a substrate, by a method of Countaway et al. [32], with modifications as described by Bird et al. [33]. Cell extracts were mixed with reaction mixture containing 20 mM Hepes, pH 4, the ST-1 peptide and [γ-^{32}P]ATP and incubated for 20 min. Aliquots of triplicate supernatant samples obtained after centrifugation were spotted in duplicates on 2.5 cm diameter circles of phosphocellulose paper, air-dried and counted for radioactivity (Packard Tri-Carb, Model 1500). Radioactive counts in controls for each sample, incubated without substrate, were subtracted from the total.

RESULTS

A comparison of IL-1 effects on cell shape and cytosketetal organization in cells plated on different substrata showed pronounced alterations in the actin filament organization and in the appearance of cell–matrix attachment sites in cells on fibronectin, whereas cells on tissue culture plastic or on poly(L-lysine) seemed

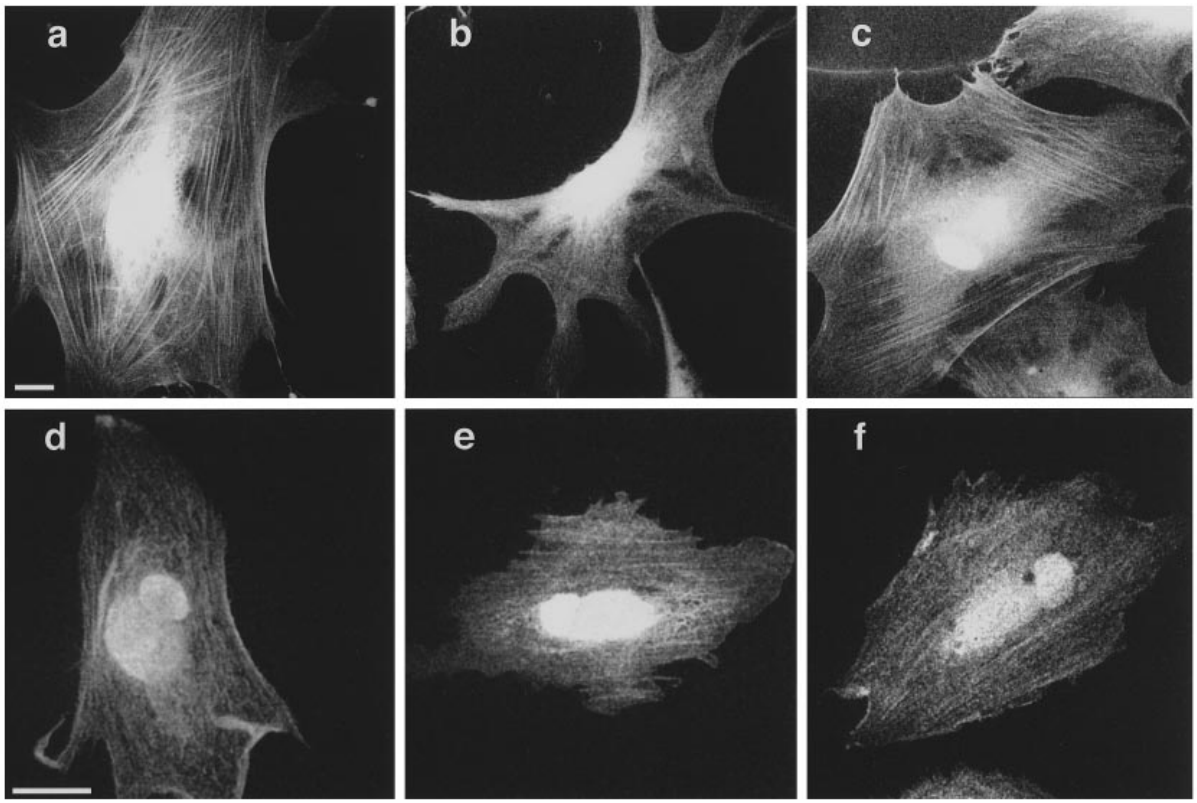

Figure 1 IL-1 induces transient effects on actin cytoskeleton and in appearance of cell–matrix attachment sites

Fibroblasts were plated on fibronectin-coated plates (10 μg/ml) (**a**, **b**, **c**) or on bare tissue culture plastic (**d**, **e**, **f**), then stimulated with IL-1 (1 nM) for 0 min (**a**, **d**), 30 min (**b**, **e**) or 60 min (**c**, **f**). Confocal microscopy after FITC–phalloidin staining of the actin cytoskeleton was performed as described in the Materials and methods section. The results shown are representative micrographs from one of three experiments. Scale bar, 10 μm.

less affected (Figure 1, and results not shown). Cells on fibronectin spread, showing a well-defined cytoskeleton (Figure 1a). Stimulation with IL-1 caused a pronounced contraction of the cells together with a disorganization of the cytoskeleton, resulting in sparse and faintly labelled actin filaments (Figure 1b). In addition, cells on fibronectin showed retractions at sites of focal adhesions and narrowing of the basal end of cell processes. The effect was transient, first observed at 15 min after the addition of IL-1, with the appearance returning to that in control cultures by 1 h (Figure 1c). In comparison, cells on bare tissue culture plastic were less well spread and also showed a less well organized and distinct actin cytoskeleton that was not affected by stimulation with IL-1 (Figures 1d–1f).

Analyses of NF-κB activity in these types of cultures revealed that both control and IL-1-stimulated levels in cells attached to fibronectin were significantly higher than those measured in cells on poly(L-lysine) and tissue culture plastic (Figure 2). Quantification showed an increase in fibronectin-attached cells of 4-fold and 14-fold compared with levels in cultures on tissue culture plastic and poly(L-lysine) respectively (Table 1). In contrast with the effect of fibronectin (Figure 2), the levels in cells on poly(L-lysine) were decreased with increasing concentrations of the substrate (Table 1) [8]. The effect of fibronectin attachment on IL-1-stimulated levels was less pronounced, averaging between 1.5-fold and 2-fold at the peak activity of 30 min (Table 1), with no effects on kinetics (results not shown).

The fibronectin-mediated activity induced during stimulation with IL-1 increased with increasing fibronectin concentration, reaching a plateau of between 1 and 10 μg/ml (Figure 3a) at saturating concentrations of IL-1. IL-1 dose–response curves revealed that the relative effect of fibronectin was most prominent at lower concentrations of IL-1, increasing the activity to approx. 250% (Figure 3b) and, further, suggested additive effects of matrix attachment and cytokine activation of this pathway. In contrast with the effect on the level of NF-κB activity, fibronectin attachment caused an approx. 50% decrease in IL-1-induced activity of the SAPK pathway (Figure 4). Kinetic experiments showed, as expected, a peak activity at 15 min [33] in cells plated on tissue culture plastic, whereas a much weaker and delayed (30 min) increase in activity occurred in cells attached to fibronectin (Figure 4).

The level of IL-1-induced NF-κB activity showed a successive increase of approx. 2–3-fold during 30 min to 4 h of attachment and spreading. This was seen both in cells plated on fibronectin and in those plated on bare plastic, reflecting to a significant extent a substrate-independent effect (Figure 5a). Similar experiments revealed, in contrast, a high level of IL-1-induced SAPK activity after 30 min of attachment, which was decreased by approx. 50% in the presence of fibronectin (Figure 5b). Further attachment and spreading resulted in a 25% decrease in activity in cells on bare plastic, whereas levels in cells on fibronectin were unaltered. Corresponding morphological studies showed that the effect on IL-1 signalling was correlated with an initial attachment at 0–60 min followed by extension of cell processes and spreading during 90–240 min of plating (results not shown).

The addition of an RGD-containing peptide, to interfere with

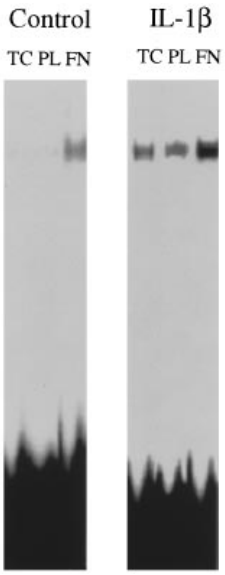

Figure 2 Up-regulation of the level of IL-1-induced NF-κB activity by fibronectin attachment

Human gingival fibroblasts were plated for short times (3–4 h) on tissue culture plastic (TC), or on untreated dishes coated with poly(L-lysine) (PL; 0.5 mg/ml) or fibronectin (FN; 10 μg/ml), then stimulated without (control) or with IL-1β (1 nM) for 30 min. Nuclear extracts were incubated with labelled oligonucleotide probe containing the NF-κB-binding site and separated on a polyacrylamide gel (4%, w/v). Shown are autoradiographs of a representative experiment from a total of four.

Table 1 Substratum regulation of NF-κB activity

Human gingival fibroblasts were plated on tissue culture plastic or on untreated dishes coated with poly(L-lysine) or fibronectin and stimulated without (control) or with IL-1β (1 nM) for 30 min. Nuclear extracts were incubated with labelled oligonucleotide probe containing the NF-κB-binding site. The results shown were obtained by scanning autoradiographs, after separation on polyacrylamide gels (4%, w/v). Results are expressed relative to levels in control cultures attached to poly(L-lysine) (1 mg/ml) and are means ± S.E.M. for four experiments.

	NF-κB activity (relative to control)	
	Control	IL-1-stimulated
Poly(L-lysine) (1 mg/ml)	1	38.14 ± 15.04
Poly(L-lysine) (0.5 mg/ml)	4.05 ± 2.53	41.19 ± 18.34
Tissue culture plastic	3.87 ± 1.10	50.25 ± 11.02
Fibronectin (10 μg/ml)	14.02 ± 1.39	73.68 ± 12.52

integrin aggregation and signalling, resulted in a concentration-dependent decrease in both control and IL-1-induced levels of NF-κB activity in cells on fibronectin (Figure 6a), whereas no effect was noted when a non-specific peptide (RGE) was added (results not shown). Further, the addition of heparin similarly resulted in a concentration-dependent decrease in both matrix and IL-1-induced NF-κB activity (Figure 6a), whereas no decrease was observed after the addition of chondroitin sulphate (results not shown). Quantification of such experiments showed that NF-κB activity induced by fibronectin attachment alone (control) was significantly inhibited (to approx. 20%) by the RGD-containing peptide. IL-1-mediated NF-κB activity was less sensitive to blocking of integrin–matrix interaction, resulting in a decrease of approx. 40% (Figure 6b), corresponding to the fibronectin contribution in IL-1-stimulated cells (see Table 1).

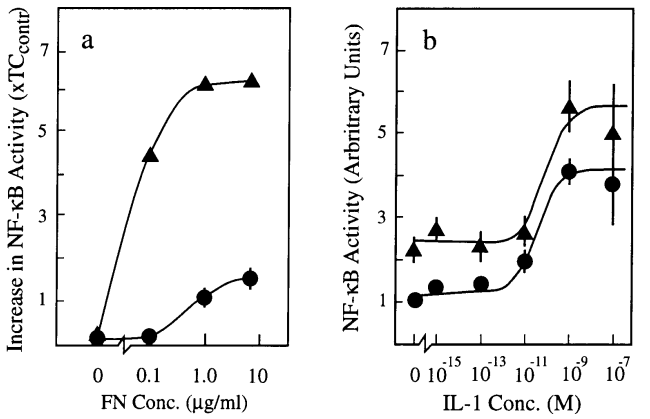

Figure 3 Co-regulation of NF-κB activity by IL-1 and fibronectin binding

(**a**) IL-1-induced NF-κB activity is dependent on fibronectin concentration. Cells were plated on various concentrations of fibronectin (FN; 0, 0.1, 1.0 or 10 μg/ml) and incubated without or with IL-1 (1 nM). Nuclear extracts were incubated with labelled probe and separated on polyacrylamide gels as described in the Materials and methods section. Quantification of the NF-κB band was done by scanning autoradiograms as described in the Materials and methods section; results are expressed relative to the level in unstimulated cells on bare tissue culture plastic. Background levels in unstimulated (1) or IL-1-treated (7.7) cells, without fibronectin attachment, have been subtracted from the data. Results are means ± S.E.M. for two experiments. Symbols: ●, non-IL-1 stimulated; ▲, IL-1-stimulated. (**b**) The relative effect of fibronectin attachment on NF-κB activity is dependent on IL-1 concentration. Cells were plated on fibronectin or on tissue culture plastic and subsequently incubated without or with increasing concentrations of IL-1β, as indicated. The level of NF-κB activity was determined as described above. Results are means ± S.E.M. for three experiments. Symbols: ●, tissue culture plastic; ▲, fibronectin.

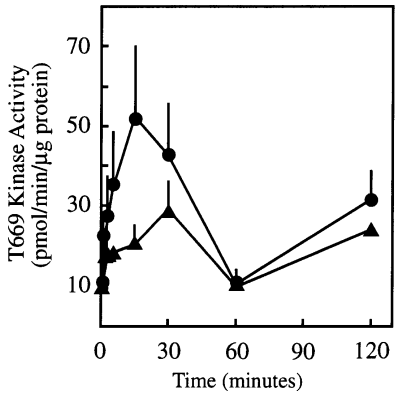

Figure 4 IL-1-induced activity of SAPK is inhibited by fibronectin attachment

Cells were plated on fibronectin (10 μg/ml)-coated plates or on tissue culture plates for 2 h and subsequently incubated with IL-1 for various times, as indicated. Activity of SAPK was measured with a synthetic peptide (T669), as described in the Materials and methods section. Results are means ± S.E.M. for three experiments. Symbols: ●, tissue culture plastic; ▲, fibronectin.

The addition of heparin, to inhibit non-$\alpha_5\beta_1$ interactions [34–37], had a pronounced impact on levels induced both in the absence and in the presence of IL-1, decreasing total activity to levels averaging 30% and 10% of the total respectively (Figure 6b). Further, the decrease in IL-1-stimulated activity of the SAPK pathway in fibronectin-attached cells, corresponding to 35–50%, was reversed in the presence of an RGD-containing peptide, resulting in levels similar to those in cells on tissue culture plastic.

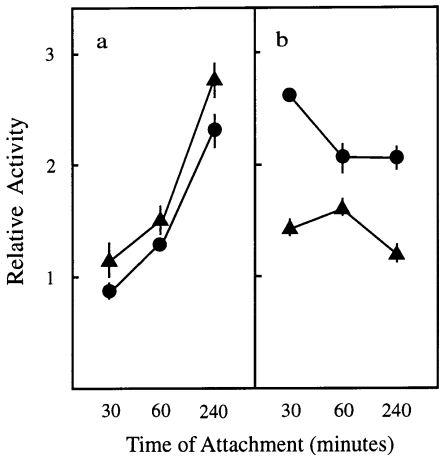

Figure 5 IL-1 induced NF-κB and SAPK activities are regulated differently by cell attachment and spreading

Cells were plated on fibronectin (10 μg/ml)-coated plates or tissue culture plates for between 30 min and 4 h, as indicated. Cultures were stimulated with IL-1 (1 nM) for the entire time (30 min) or for the last 30 min (60 and 240 min) of attachment for the assessment of NF-κB activity, and for the last 15 min for the assessment of SAPK activity. Subsequently the cells were harvested and NF-κB (**a**) and SAPK (**b**) activities were measured, as described in the Materials and methods section. Results are means ± S.E.M. for two experiments. Symbols: ●, tissue culture plastic; ▲, fibronectin.

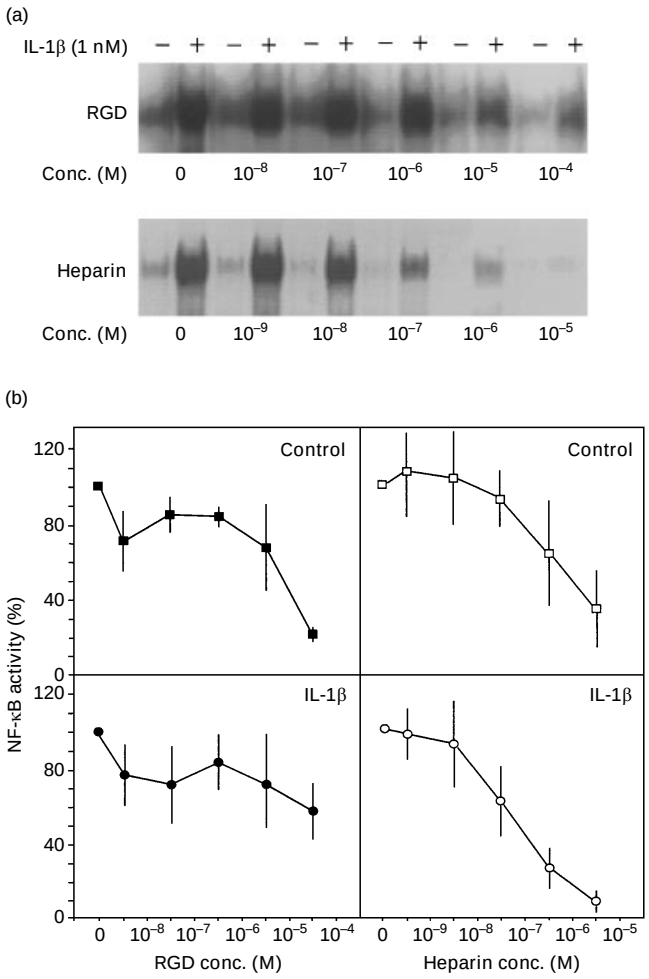

Figure 6 Attachment and IL-1-induced NF-κB activities are regulated through the RGD motif and the heparin-binding domain of fibronectin

(**a**) Gel-shift analyses of control and IL-1-induced NF-κB activities, inhibited by an RGD-containing peptide or heparin. Cells were plated on tissue culture plates coated with fibronectin (5 μg/ml) in the presence of various concentrations of an RGD-containing peptide (GRGDSP) or soluble heparin, as indicated, for 3 h, and incubated without (−) or with IL-1β (1 nM) (+) for 30 min. Nuclear extracts from attached cells were assayed for NF-κB activity by gel-shift analysis, as described in the Materials and methods section. Shown are autoradiographs of representative experiments from a total of three. (**b**) Quantification of control and IL-1-induced NF-κB activities after inhibition by RGD-containing peptide and heparin. Autoradiographs of shift gels obtained as above were analysed by scanning, as described in the Materials and methods section. Results represent the quantification of the NF-κB-specific band, expressed relative to the activity measured in fibronectin-attached cells in the presence of the same concentration of a non-specific peptide (GRGESP) or chondroitin sulphate respectively used as control. Results are means ± S.E.M. for three experiments.

The addition of heparin similarly enhanced the decreased levels of SAPK activity in IL-1-stimulated cells plated on fibronectin (results not shown). Phase-contrast microscopy revealed that cells plated under the conditions used and attached in the presence of inhibitor had a morphology similar to that of cells in control cultures, attached with or without non-specific inhibitors (results not shown).

Supershift analyses after the inhibition of NF-κB activity, as above, showed that the addition of RGD-containing peptide had a pronounced effect on all matrix-induced (control) dimers. The experiments further revealed that the partial decrease in IL-1-mediated activity, induced by the RGD-containing peptide, could be accounted for mainly by a decrease in the p50/p65 heterodimer, constituting the largest component of the NF-κB activity (Table 2). The addition of heparin caused a much more pronounced decrease, resulting in levels corresponding to between 0 and 15% of the total non-inhibited amount, in control and IL-1-stimulated cultures.

DISCUSSION

The results presented here show that IL-1 signal transduction is regulated by cell adhesion, correlating with integrin binding and actin filament organization. In addition, the results reveal disparate effects on NF-κB and SAPK activities.

The change in total NF-κB activity with increasing IL-1 concentrations shows additive effects of integrin and IL-1 receptor engagement. This is supported by ongoing studies with confocal microscopy, which show that NF-κB activity in these cells, induced through either receptor, primarily involves nuclear translocation of p65-containing dimers (K. Schooley, P. Zhu, S. K. Dower and E. E. Qwarnstrom, unpublished work). The higher levels of NF-κB activation in IL-1-stimulated cells, attached to fibronectin, are thus likely to result from increased levels of nuclear translocation induced by integrin-mediated signals. The large variation between experiments observed at the highest concentration could reflect near-chaotic behaviour of subunit translocation observed at more than saturating concentrations of IL-1 (E. E. Qwarnstrom and S. K. Dower, unpublished work). We have previously shown that integrins selectively activate the NF-κB p50/p65 heterodimer in adherent cells [8]. This agrees with the results reported here showing that a decrease in this dimer is predominantly responsible for the decrease in total activity induced by the addition of an RGD-containing peptide, suggesting that it is the main NF-κB element involved in integrin-mediated effects on IL-1-regulated genes [15].

A pronounced effect of integrin-mediated events in matrix regulation of the SAPK pathway is supported by the substrate-

Table 2 RGD inhibition of NF-κB dimers

Cells were plated on tissue culture plates coated with fibronectin (5 μg/ml) as described, in the presence of an RGD-containing peptide (GRGDSP; RGD; 100 μM) without (control) or with IL-1β (1 nM) for 30 min. Before addition of the oligonucleotide probe, nuclear extracts were incubated with anti-p65 or anti-p50 antibodies, as described in the Materials and methods section, and subsequently separated on polyacrylamide gels (4%, w/v). Quantitative analyses were done by determination of the intensity in the shifted and non-shifted bands as described in the Materials and methods section. The level of p50/p65 heterodimer (p50/p65 het) and the levels of p50 and p65 in other dimer combinations were expressed relative to total activity measured in unstimulated cells in the absence or presence of the same concentrations of a non-specific peptide (GRGESP) used as control (RGE). Results are means ± S.E.M. for two experiments.

	Band intensity (% of control total)				
Condition	Addition	p50/p65 het	p50	p65	Total
Control	RGE	69.0 ± 5.7	13.0 ± 5.0	18.0 ± 2.0	100.0 ± 12.7
	RGD	14.2 ± 6.6	0.9 ± 0.2	3.0 ± 1.6	18.1 ± 8.4
IL-1β	RGE	248.0 ± 24.2	56.6 ± 20.2	97.0 ± 4.0	401.6 ± 48.4
	RGD	146.3 ± 42.2	29.1 ± 2.0	54.6 ± 12.3	230.0 ± 56.5

specific decrease in activity observed at early times of attachment. In addition, the complete inhibition of the fibronectin effect on SAPK activity by addition of the RGD-containing peptide indicates that a major part of matrix regulation of this pathway involves integrin aggregation. It has been shown that cell–matrix binding through integrins in and of itself increases the activity of both extracellular-signal-regulated protein kinase (ERK) [38] and SAPK [17]. The observed decrease in the activation of SAPK by IL-1 in fibronectin-attached cells could thus reflect decreased integrin aggregation induced by the destabilization of focal contacts by IL-1 [14]. In addition, the lower level might be a consequence of desensitization due to prior activation of this pathway during the initial fibronectin attachment [17].

The results further suggest that the regulation of IL-1-induced signalling is in part mediated through the cytoskeleton. Such effects are probably related to the direct effects of IL-1 on cytoskeletal organization, as shown in Figure 1 [14], occurring during the peak activity of both pathways in fibronectin-attached cells. Further, the pronounced impact of inhibition of cell–matrix interaction could to some extent be due to secondary effects in the cytoskeleton. An effect of the cytoskeleton on the NF-κB pathway was indicated by the pronounced substrate-independent increase in activity during spreading (60–240 min after plating), coinciding with the organization of the cytoskeleton [39]. This type of effect on NF-κB activity could be induced by subunit interaction with actin filaments, through their ankyrin-like repeats [40]. This notion agrees with the observation that immunofluorescence with anti-(c-Rel) antibodies gives a cytoskeleton-like staining pattern (S. K. Dower and E. E. Qwarnstrom, unpublished work).

Integrin-mediated alterations of IL-1 signal transduction could involve the regulation of focal adhesion-associated kinases [10,12,41–43]. Further, the dependence of IL-1-induced downstream activities on cytoskeletal organization suggests an involvement of structurally regulated GTP-binding proteins [42,43]. This could be a result of the IL-1-induced alterations in talin, and the actin filaments [14], and/or be linked to the activation of GTP-ase(s) by IL-1 [44]. Distinct regulation through these mediators could involve differences in matrix-induced effects on Ras, upstream in the NF-κB pathway [23], and on Rac's regulating SAPK activity [16,45]. The disparate effects on these pathways could be a consequence of mutually exclusive involvement of signalling components such as mitogen-activated protein kinase kinases, which have been shown to link the activation of SAPK and NF-κB [46,47].

An effect of cell–matrix interaction initiated at the level of the IL-1 receptor is suggested by its localization at focal adhesions [48,49]. This could be independent of IL-1 binding and induced by IL-1 receptor interaction with other cell-surface molecules, through a mechanism similar to that mediating L1, N-Cam and N-cadherin activation of the fibroblast growth factor receptor [50,51]. Ongoing studies indicate that fibronectin attachment causes increased IL-1 receptor levels and changes in IL-1 binding kinetics involving heparin binding (C. Tsoi and E. E. Qwarnstrom, unpublished work), suggesting that its effects on signal transduction are, to a certain extent, initiated at the level of the receptor, possibly through a mechanism similar to that influencing the interaction of fibroblast growth factor and its receptor [52].

The influence of fibronectin attachment on IL-1 signal transduction pathways is correlated with its permissive effect on IL-1-induced biological responses, such as the regulation of IL-6 gene expression and of matrix proteins, collagen and versican [15]. The up to 5-fold change in relative activities of the two pathways (NF-κB and SAPK) resulting from the opposite effects induced by fibronectin attachment is likely to have a pronounced influence on promoters containing sites for both NF-κB and AP-1, such as IL-6 [53] and collagen I [54]. The pronounced influence of fibronectin attachment on the induction by IL-1 of IL-6 [15] suggests a strong regulatory effect by the NF-κB site in this gene, as has been shown for IL-8 [55]. The decreased collagen and versican expression, correlated with the decrease in SAPK activity, similarly, could indicate a significant influence of matrix attachment through the AP-1 site [54] and the C/EBP site [56,57] respectively in these genes. The involvement of both integrin binding and the actin cytoskeleton in the regulation of signalling suggests that their impact on the transcription of IL-1-induced genes is particularly pronounced during proliferation and migration at sites of inflammation.

In summary, the results show that IL-1-stimulated activities of SAPK and NF-κB are regulated by cell adhesion. Further, they suggest that the attachment regulation of IL-1-induced SAPK activity primarily involves integrin aggregation, whereas the effect on IL-1-mediated NF-κB activity is in addition regulated by mechanisms related to cytoskeletal organization. Finally, the pronounced change in the relative levels of these IL-1-activated pathways agrees with the notion that regulation through integrins and effects mediated through the cytoskeleton are potent factors in controlling responses during inflammation.

We thank Steven K. Dower (Immunex Corporation, Seattle, WA, U.S.A.) for the gift of IL-1β. Autoradiographic analyses were performed in part by the PhosphorImager Facility of the Markey Molecular Medicine Centre at the University of Washington, Seattle. The confocal microscopy analysis was carried out using the confocal microscope facility in the Laboratory of Molecular Immunology, University of Sheffield, co-funded by the Wellcome Trust and the Medical Research Council. The studies were supported by grants from the National Institutes of Health, U.S.A. (DE-10363 and DE-11251), the Arthritis and Rheumatism Council (Q0502) and the British Heart Foundation (PG97 112) to E.E.Q.

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Received 13 May 1997/3 October 1997; accepted 20 November 1997

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