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22 February 1993; accepted 3 May 1993

Proliferation of Human Smooth Muscle Cells Promoted by Lipoprotein(a)

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Elevated blood concentrations of lipoprotein(a) [Lp(a)] and its constituent, apolipoprotein(a) [apo(a)], constitute a major risk factor for atherosclerosis, but their physiological activities remain obscure. Lp(a) and purified apo(a) stimulated the growth of human smooth muscle cells in culture. This effect resulted from inhibition of plasminogen activation, and consequently the activation by plasmin of latent transforming growth factor–β, which is an inhibitor of smooth muscle cell growth. Because smooth muscle proliferation is one of the hallmarks of atherosclerotic lesions, these results point to a plausible mechanism for the atherogenic activity of Lp(a).

A high concentration of Lp(a) in blood constitutes a major risk factor for atherosclerosis, coronary heart disease, and stroke (*1*). Lp(a) differs from low density lipoprotein (LDL) by the presence of the glycoprotein apo(a). Because the amino acid sequence of apo(a) is approximately 80% identical to that of plasminogen (*2*), it is possible that the pathophysiology of Lp(a), including effects on fibrinolysis, is attributable to apo(a). Lp(a) binds to endothelial and macrophage cells and to extracellular components such as fibrin and inhibits cell-associated plasminogen activation (*3, 4*). To date, no direct effect on cell proliferation has been demonstrated. Abnormal proliferation and migration of vascular smooth muscle cells is a major component of vascular disease, including atherosclerosis and restenosis after angioplasty. Elevated plasma Lp(a) concentration is one of the most important risk factors for both of these conditions (*1, 5*).

To investigate the effects of Lp(a) on smooth muscle cells, we subjected cultured human and rat smooth muscle cells to plasma-derived Lp(a) and to its constituent parts, LDL and apo(a). Human aortic vascular smooth muscle cells (VSMCs) derived from healthy donor tissue were cultured in Dulbecco's modified essential medium (DMEM) plus 10% fetal calf serum (FCS) as described (*6*). Addition of Lp(a) to subconfluent human VSMCs stimulated their proliferation in a dose-dependent manner (Fig. 1A). Apo(a) had a similar effect, although a higher concentration was required for half-maximal stimulation. This disparity could be due to conformational differences between free apo(a) and its lipoprotein-associated form, Lp(a), or to size variants of apo(a) that differ in the number of repeated kringle domains. The recombinant apo(a) is a smaller isoform [relative molecular mass (M_r) ~ 500,000] than those present in the donor's plasma (M_r ~ 650,000 and 800,000). Addition of 500 nM Lp(a) to human VSMCs caused a reduction of the time taken for cells to double in number from 82 ± 4 hours to 47 ± 4 hours (Fig. 1B). LDL had no effect on cell proliferation at all concentrations tested, up to 1 μM. In contrast to the effect seen on human cells, neither Lp(a), apo(a), nor LDL affected the proliferation of cultured rat VSMCs (Fig. 1C).

Further studies were performed to elucidate the nature of the stimulation of proliferation by Lp(a) and apo(a). It is possible that apo(a) could act as a mitogen because it shares global homology and 38% amino acid identity with hepatocyte growth factor, which is a mitogen for hepatocytes and several other cell types (*7*). Alternatively, apo(a) could act by competitive inhibition of surface-associated plasminogen activation and the subsequent activation of transforming growth factor–β (TGF-β) by plasmin. The TGF-β family consists of a number of related cytokines of diverse function. TGF-β is a potent inhibitor of cell proliferation for a number of anchorage-dependent cells, including smooth muscle cells, and may be a physiological modulator of smooth muscle cell proliferation during wound healing and atherosclerosis (*8*). Latent TGF-β is a homodimer in which the active moiety is noncovalently linked to the NH_2-terminal portions of the propeptide (*9*). Although activation of TGF-β may be achieved in vitro by acid treatment, plasmin can activate the latent molecule by cleavage within the propeptide region and is a likely candidate for a physiological regulator of TGF-β activity (*10*). Owing to the antiproliferative effect of TGF-β on smooth muscle cells, we hypothesized that apo(a) could act on cultured human VSMCs by interfering with the activation of latent TGF-β. Such an effect might not be expected for rat cells because they synthesize little TGF-β in culture (Table 1), whereas addition of active TGF-β to rat VSMCs suppresses their proliferation (Fig. 1C). Thus, Lp(a) could act in a species-specific manner on cultured human VSMCs by interfering with the activation of plasminogen and, therefore, TGF-β.

Plasmin activity associated with the cells was reduced sevenfold by Lp(a) and fivefold by apo(a) in both human and rat VSMC cultures (Fig. 2A). The plasmin activity in the conditioned medium was also reduced by the Lp(a) and apo(a) by almost twofold, but was much lower than cell-associated plasmin activity in both VSMC cultures (Fig. 2B). This is consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than fluid phase, plasminogen activation (*3*).

To exclude the possibility that Lp(a) was affecting the synthesis of plasminogen activators (PAs), we measured PA levels in the human cell cultures in the absence and

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presence of the lipoproteins. PA activity in the conditioned medium was measured in the presence of a large excess of plasminogen so that the lipoproteins present would not act significantly as competitive inhibitors. The total PA activity was not affected by the presence of the lipoproteins in either human or rat VSMC cultures: PA activity in conditioned medium remained at 0.7 ± 0.06 mU/ml with up to 500 nM Lp(a).

The amount of active TGF-β in the medium was measured by the mink lung epithelial cell bioassay (*11*). Lp(a) and apo(a) both reduced the amount of active TGF-β more than 100-fold as compared with control or LDL-treated cultures (Table 1). However, the amount of total TGF-β (latent plus active) measured by enzyme-linked immunosorbent assay (ELISA) was unaffected by the presence of Lp(a) or apo(a). In contrast, medium conditioned on rat VSMCs contained undetectable amounts of active TGF-β and low amounts of latent TGF-β under all conditions tested (Table 1). We conclude that Lp(a) stimulates proliferation of human VSMCs by inhibiting the conversion of latent TGF-β to active TGF-β by plasmin.

To test this conclusion further and exclude the possibility that Lp(a) was acting by binding active TGF-β as well as reducing plasmin activity, we cultured human VSMCs in the presence of Lp(a). These cells had a population doubling time of 47 ± 3 hours. However, addition of plasmin was able to overcome the effect of the Lp(a) and reduce the growth rate to control levels, with the population doubling time increased to 97 ± 4 hours (Fig. 3A). The role of plasmin was confirmed by studies in which inhibitors of plasmin activity were added to human VSMC cultures. Like Lp(a), these protease inhibitors increased the rate of cell proliferation: Aprotinin decreased the population doubling time from 82 ± 4 hours in control cultures to 48 ± 5 hours, and α2-antiplasmin decreased the population doubling time to 45 ± 2 hours, whereas addition of 500 nM Lp(a) to the aprotinin resulted in only a slight additional stimulation of proliferation [population doubling time in the presence of both Lp(a) and aprotinin was 45 ± 6 hours]. In summary, Lp(a), two plasmin inhibitors, and neutralizing antibody to TGF-β stimulate proliferation, whereas plasmin nullifies the growth stimulation of Lp(a) (Fig. 3A).

In contrast to the human VSMCs, rat VSMCs do not produce significant amounts of active TGF-β in culture; consequently Lp(a) did not stimulate their proliferation. However, when treated with tamoxifen, rat VSMCs produce substantial TGF-β (Table 1) (*12*). Addition of tamoxifen therefore slowed the proliferation of rat VSMCs, increasing the population doubling time from 35 ± 2 hours in control cultures to 55 ± 2 hours (Fig. 3B) (*12*). Addition of Lp(a) or apo(a) reduced the growth-inhibitory activity of tamoxifen [population doubling time in the presence of tamoxifen and Lp(a) was 42 ± 2 hours]. Furthermore, the presence of Lp(a) reduced the amount of active TGF-β produced in response to ad-

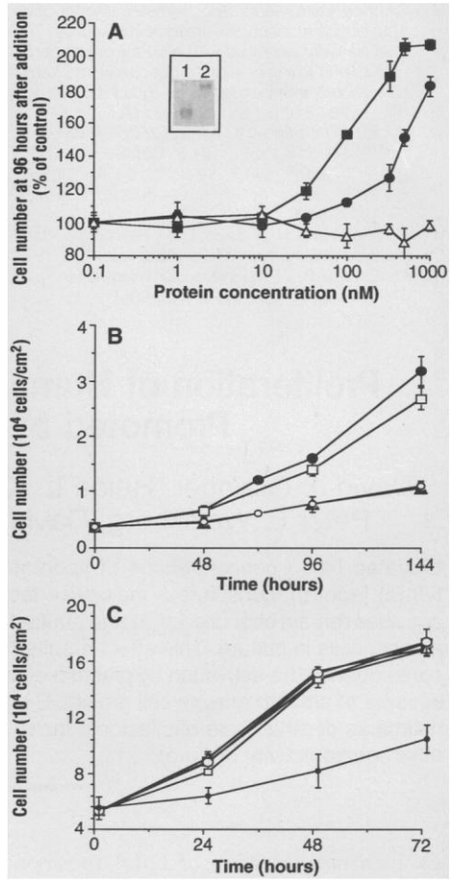

Fig. 1. Effects of lipoproteins on the proliferation of human and rat VSMCs in culture. (**A**) Dose-response curve for the effect of various concentrations of Lp(a) and apo(a) on the proliferation of human VSMCs. Cell number was determined 96 hours after addition of Lp(a) (■) or apo(a) (●) and expressed relative to the number of cells in the control population (1.6×10^4 cells per square centimeter). LDL (△) had no effect on the number of cells at 96 hours. Inset shows the purity of LDL (1) and Lp(a) (2) preparations, respectively, by gel electrophoresis. (**B**) Proliferation of human VSMCs in DMEM + 10% FCS and 500 nM of either Lp(a) (●), apo(a) (□), LDL (▲), or no addition (○). (**C**) Proliferation of rat VSMCs in DMEM + 10% FCS and 500 nM Lp(a) (□), apo(a) (△), LDL (○), or no addition (■). The effect of addition of recombinant, active TGF-β (10 ng/ml; Amersham International) (●) is shown for comparison. Values represent the mean ± SEM of three experiments. Human and rat VSMCs were cultured from enzyme-dispersed preparations of aortic media as described (*6*). After 24 hours, subconfluent cells were supplied with fresh DMEM plus 10% FCS and lipoproteins. Lp(a) and LDL were isolated from the plasma of an individual donor (with informed consent) by density gradient ultracentrifugation and dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4) as described (*17*). Purity of Lp(a) and LDL preparations was determined by nondenaturing 2.5 to 8% gradient polyacrylamide gel electrophoresis (PAGE) and immunoblotting with antibodies to apo(a) and apolipoprotein B. Lp(a) isoforms from the donor were larger than apolipoprotein B-100, migrating at 61 and 78% of the mobility of apolipoprotein B-100 in 6% SDS-PAGE under reducing conditions (*17*). Recombinant apo(a) was purified from mammalian tissue culture as described (*18*). Cells were counted at each time point by haemocytometer after release by trypsin-EDTA.

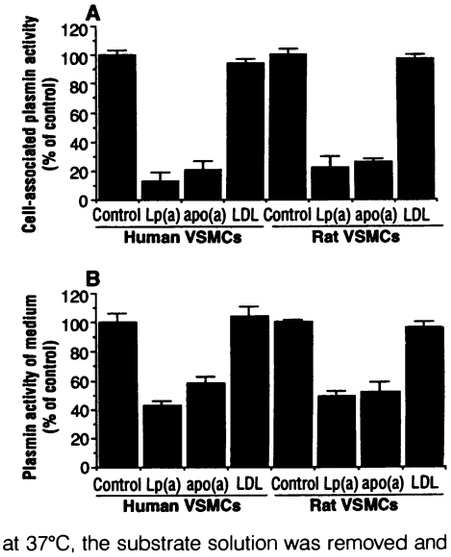

Fig. 2. Effect of lipoproteins on plasmin activity. (**A**) Plasmin activity associated with the cell surface is expressed as a percentage of the activity in the control wells (23 ± 5 mU/ml). (**B**) Plasmin activity in the conditioned medium is expressed as a percentage of the activity in the control wells (5 ± 2 mU/ml). All activities are corrected for the number of cells present. Human and rat VSMCs were subcultured as in Fig. 1. Lipoproteins were added to 500 nM final concentration. The conditioned medium was collected after 96 hours for determination of plasmin activity. Conditioned medium (100 μl) was added to 850 μl of phosphate-buffered saline (PBS) and 50 μl of the chromogenic plasmin substrate Phe-Leu-Lys-*p*-nitroanilide (1 μM; Sigma) and incubated at 37°C for 30 min. Released *p*-nitroanilide was measured spectrophotometrically and compared with a standard curve. Cell-associated plasmin activity was measured similarly except that the cells were washed three times for 5 min with PBS at 37°C before the chromogenic substrate was added. After 30 min at 37°C, the substrate solution was removed and the release of *p*-nitroanilide was measured spectrophotometrically. The plasmin activity in unconditioned DMEM + 10% FCS was <1 mU/ml.

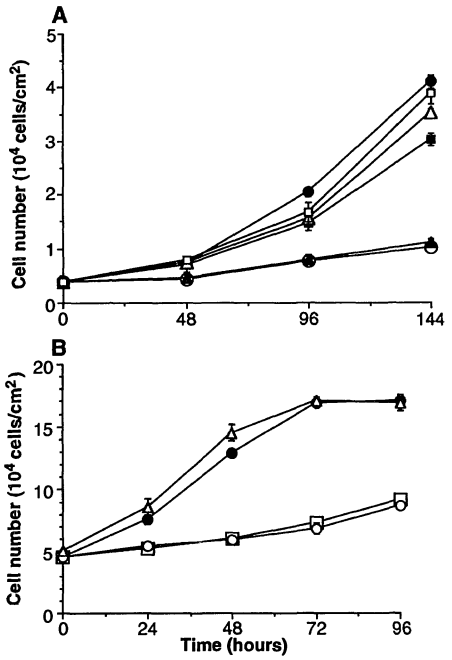

Fig. 3. Modulation of plasmin activity and TGF-β production. (**A**) Effects of Lp(a), plasmin, and inhibitors on human VSMC proliferation. Graph shows the time course of proliferation of human VSMCs in DMEM + 10% FCS with no addition (○) or addition of aprotinin (1 μg/ml) (■), α2-antiplasmin (1 μg/ml) (△), aprotinin (1 μg/ml) plus 500 nM Lp(a) (●), 500 nM Lp(a) plus plasmin (0.1 U/ml) (▲), or neutralizing antibody to TGF-β (10 μg/ml) (□). To ensure continued activity, we repeated the dose of α2-antiplasmin and TGF-β antibody every 24 hours. (**B**) Effects of Lp(a), tamoxifen, and plasmin on rat VSMC proliferation. Graph shows the time course of rat VSMCs in DMEM plus 10% FCS and 0.1% ethanol with no addition (△) or addition of 10 μM tamoxifen (□), 10 μM tamoxifen plus 500 nM Lp(a) (●), or 10 μM tamoxifen plus 500 nM Lp(a) plus plasmin (0.1 U/ml) (○). Cell culture and counting were done as in Fig. 1; components were added simultaneously at the zero time point of the graph, which was 24 hours after subculturing.

Table 1. Effect of lipoproteins on the activation of TGF-β. Results were obtained on at least three separate cultures. VSMCs were subcultured into DMEM + 10% FCS and grown for 24 hours. The medium was replaced, and the lipoproteins were added at 500 nM final concentration. Tamoxifen (Aldrich) was added to 10 μM final concentration from a 10 mM stock in 0.1% ethanol. Plasmin (Sigma) was added to 0.1 U/ml final concentration. After 96 hours the medium was removed, stored at −20°C, and assayed within 1 week. The amount of active TGF-β in the medium was determined by a modification of the mink lung epithelial cell assay (*11*). The conditioned media were diluted 1:100 in serum-free DMEM supplemented with epidermal growth factor (100 ng/ml) and insulin (50 ng/ml; Bachem, Inc.). The TGF-β standards were diluted in the same medium. DNA synthesis was determined by [^{3}H]thymidine (1 μCi/ml) incorporation during a 1-hour pulse 23 hours after the addition of the conditioned media with or without neutralizing antiserum to TGF-β (R & D Systems). TGF-β activity was calculated as the proportion of the inhibition of DNA synthesis that was reversed in the presence of the neutralizing antibody. The TGF-β samples and conditioned media both contained 10% FCS. The amount of latent and active TGF-β was determined by immunoassay (ELISA). Maxisorb 96-well ELISA plates (Gibco) were coated with neutralizing antiserum against TGF-β at 2 μg/cm^2 in PBS overnight. The plates were then incubated with the samples (2 hours), with detection antibody to TGF-β (1 hour), with antibody to rabbit immunoglobulin G conjugated to peroxidase (Sigma; 1 hour), and then with the chromogenic substrate *o*-phenylenediamine (Sigma; 15 min). Absorbances at 492 nm were converted into quantities with a standard curve. Both conditioned media and standards were assayed in the presence of 10% FCS. The TGF-β antibodies recognize both latent and active TGF-β.

Treatment	TGF-β (ng/ml)	
	Active	Latent plus active
	Human VSMCs	
Control	9.8 ± 1.8	15.2 ± 1.6
Lp(a)	<0.1	13.8 ± 3.8
Apo(a)	<0.1	16.9 ± 4.2
LDL	10.1 ± 3.2	14.8 ± 0.9
	Rat VSMCs	
Control	<0.1	2.1 ± 1.8
Lp(a)	<0.1	3.1 ± 1.2
Apo(a)	<0.1	1.8 ± 0.7
LDL	<0.1	2.7 ± 2.2
Ethanol vehicle	<0.1	4.1 ± 2.8
Tamoxifen	4.8 ± 0.9	13.7 ± 2.2
Tamoxifen + Lp(a)	<0.1	12.9 ± 3.5
Tamoxifen + Lp(a) + plasmin	12.6 ± 0.4	14.1 ± 2.6
Unconditioned medium	<0.1	1.1 ± 0.4

dition of tamoxifen by at least 50-fold (Table 1). Addition of plasmin to rat VSMCs treated with tamoxifen and Lp(a) resulted in the activation of most of the latent TGF-β (Table 1), and proliferation was again slowed (population doubling time of 57 ± 3 hours; Fig. 3B). These observations are consistent with our conclusions that Lp(a) acts by inhibiting TGF-β activation.

We have shown that the proliferation of human smooth muscle cells in culture is accelerated by Lp(a) and apo(a), but not by LDL, in a dose-dependent manner. The response is most likely due to the competitive inhibition of surface binding and activation of plasminogen by its homolog, apo(a). This, in turn, reduces the activation of TGF-β, a potent inhibitor of smooth muscle cell proliferation under the conditions of serum stimulation reported here. [Only under low serum conditions, when the cells are not maximally stimulated to divide, has TGF-β been reported to enhance DNA synthesis (*13*).] It should be noted that the highest concentrations of Lp(a) and apo(a) used here exceed those found in many human blood samples. The plasma concentration of Lp(a) varies in the population from about 1 nM to 1 μM, whereas that of plasminogen is about 1 μM. However, localized concentrations of Lp(a) may be considerably greater. The repeated kringle domains of apo(a) may enhance its binding to substrates shared with plasminogen or to unique substrates. Several reports indicate that Lp(a) is more highly concentrated in the arterial wall than in plasma (*14*).

Approximately one-quarter of premature myocardial infarctions in males can be accounted for by elevated amounts of plasma Lp(a) (*15*), yet the pathogenic mechanism of Lp(a) remains obscure. The unexpected sequence homology between apo(a) and plasminogen suggested a link between lipoproteins, atherosclerosis, and thrombosis. Lp(a) binds to fibrin and endothelial cells and thus decreases the surface-associated activation of plasminogen (*3*). Lp(a) increases the amount of smooth muscle cell migration in cocultures of bovine endothelial and smooth muscle cells by inhibition of TGF-β activation (*16*). Both inhibition of clot lysis and enhancement of cell migration could contribute to the process of atherogenesis. We suggest that Lp(a) may contribute to the growth of the arterial lesions of atherosclerosis by promoting the proliferation of vascular smooth muscle cells.

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7 December 1992; accepted 4 March 1993

Complexes of Ras·GTP with Raf-1 and Mitogen-Activated Protein Kinase Kinase

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The guanosine triphosphate (GTP)–binding protein Ras functions in regulating growth and differentiation; however, little is known about the protein interactions that bring about its biological activity. Wild-type Ras or mutant forms of Ras were covalently attached to an insoluble matrix and then used to examine the interaction of signaling proteins with Ras. Forms of Ras activated either by mutation (Gly12Val) or by binding of the GTP analog, guanylyl-imidodiphosphate (GMP-PNP) interacted specifically with Raf-1 whereas an effector domain mutant, Ile36Ala, failed to interact with Raf-1. Mitogen-activated protein kinase (MAP kinase) activity was only associated with activated forms of Ras. The specific interaction of activated Ras with active MAP kinase kinase (MAPKK) was confirmed by direct assays. Thus the forming of complexes containing MAPKK activity and Raf-1 protein are dependent upon the activity of Ras.

The majority of cellular Ras in quiescent cells is in the inactive guanosine diphosphate (GDP)–bound conformation. Stimulation of cells with mitogens or differentiation factors increases the abundance of the active GTP-bound form of Ras (*1*). The activity of Ras is required for regulatory signaling by plasma membrane tyrosine kinase oncogenes and receptors (*2*). Blocking the action of Ras, either by microinjection of antibodies or through the expression of a dominant negative mutant, prevents the activation of the cytosolic serine-threonine protein kinases Raf-1 and MAP kinase (*3, 4*). The expression of activated Ras, either in whole cells or in vitro activates these cytosolic serine-threonine protein kinases (*5–7*). These cytosolic enzymes can function as part of a kinase cascade, in which MAP kinases are activated by a MAPKK (*8*), which in turn can be phosphorylated and activated by the Raf-1 protein kinase (*9*). The components of this pathway may not always be coupled to each other, however, because neither Ras or Raf-1 activates MAP kinases in Rat1a cells (*10*).

Though it is clear that Ras participates in signaling between the cell membrane and the cytosol, the biochemical interactions that mediate the action of Ras are poorly understood. To determine if Ras might physically interact with cytosolic signaling components in a GTP-dependent manner, we covalently bound recombinant c-Ha-Ras (referred to as Ras) to silica beads activated by an *N*-hydroxyl succinimide group (*11, 12*). The coupled Ras was active in binding guanine nucleotides and has been used to examine interactions between Ras and neurofibromin, a regulator of Ras function (*12*). The immobilized Ras was used to probe rat brain cytosol for signaling proteins that specifically interacted with Ras·GTP.

Immobilized Ras bound to GMP-PNP (a nonhydrolyzable GTP analog) formed a stable complex with Raf-1 (Fig. 1A). Approximately 15% of the total cytosolic Raf-1 associated with immobilized Ras·GMP-PNP. The transforming Ras mutant, Ras^{Val12} bound to GMP-PNP, was about 10% as effective as wild-type Ras in forming stable complexes with Raf-1 (Fig. 1A). An even smaller amount of Raf-1 associated with the GDP-bound form of Ras^{Val12}. A form of Ras with a mutation in the effector domain, Ras^{Ala36}, bound to either GDP or GMP-PNP failed to bind detectable amounts of Raf-1 (Fig. 1B). Thus, the stable association of Raf-1 with immobilized Ras correlated well with the formation of the active GTP-bound form of Ras.

We investigated the GTP-dependent association of Ras with MAPKK and MAP kinase, which have been reported to function downstream of Raf-1. Immunoprecipitates of MAP kinase from the rat brain lysate did not catalyze the phosphorylation of myelin basic protein (MBP) in the presence of [γ-^{32}P]adenosine triphosphate (ATP) (*13*). This enabled us to use the activation of endogenous MAP kinase activity as an assay for MAPKK. Incubation of cytosolic proteins bound to immobilized Ras·GMP-PNP with ATP produced soluble kinase activity with a high substrate specificity for MBP as compared with either histone or casein (Fig. 2A) (*14*). The soluble kinase activity was also detected with

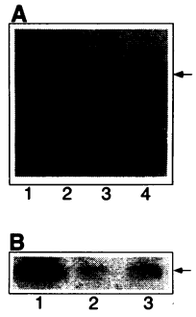

Fig. 1. Association of Raf-1 with immobilized Ras. (**A**) Lysate was incubated with immobilized Ras·GDP (lane 1), Ras·GMP-PNP (lane 2), Ras^{Val12}·GDP (lane 3), or Ras^{Val12}·GMP-PNP (lane 4) for 60 min at 4°C (*15*); 2× Laemmli sample buffer was added to the sedimented material and the eluted proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred, and immunoblotted with a rabbit polyclonal antibody to Raf-1. The position of Raf-1 is denoted by the arrow. (**B**) As in (A) except that lysate was incubated with immobilized Ras·GMP-PNP (lane 1), Ras^{Ala36}·GDP (lane 2), or Ras^{Ala36}·GMP-PNP (lane 3). Control immunoprecipitation of lysate with the antibody revealed a single band of 68 kD whereas the rabbit non-immune serum did not react with this protein. Quantitation of Raf-1 bound to immobilized Ras was done on a phosphorimager and expressed as the percentage of Raf-1 protein immunoprecipitated from identical amounts of crude lysate. Comparable amounts of antibody and lysate were used to those in the experiment described in Table 1.

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