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Ashwani K. Khanna; ... et. al

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Recombinant p21 Protein Inhibits Lymphocyte Proliferation and Transcription Factors

Ashwani K. Khanna,[1] Matthew Plummer, Vani Nilakantan, and Galen M. Pieper

Cellular proliferation determines the events leading to the initiation and development of inflammation, immune activation, cancer, atherogenesis, and other disorders associated with aberrant cell proliferation. Cyclin inhibitor p21 plays a unique role in limiting cell cycle progression. However, its effectiveness can only be demonstrated with direct in vitro and in vivo delivery to control aberrant proliferation. We demonstrate that using a protein-transducing domain p21 protein a) localizes within the nuclear compartments of cells, b) interacts with transcription factors, NF-κB, and NFATs (NFATc and NFATp), and c) inhibits lymphocyte proliferation and expression of proinflammatory cytokines. This study using lymphocyte proliferation as a model suggests that the recombinant p21 protein can directly be delivered as a therapeutic protein to provide a novel, viable, and powerful strategy to limit proliferation, inflammation, alloimmune activation, cancer, and vascular proliferative disorders such as atherosclerosis. *The Journal of Immunology,* **2005, 174: 7610–7617.**

Regulation of cell cycle is a complex process, which is reflected in numerous events related to aberrant cell proliferation such as activation of the immune system and inflammation. These events are the integral components of the pathogenesis of the majority of diseases, e.g., cancer, atherosclerosis, and allograft rejection in organ transplantation. Cyclins/cyclin-dependent kinases control cell cycle progression and the ensuing events of immune activation, inflammation, carcinogenesis, atherogenesis, and other related disorders. Therefore, the negative regulators of cellular proliferation can be potential targets to inhibit the events leading to initiation and the progression of various diseases associated with cellular proliferation. This concept has stimulated the search for the role of cyclin inhibitors as therapeutic tools. The strategies to modulate directly or indirectly cyclin activity could result in the development of novel therapeutics. The indirect approach can be through the agents capable of inducing the expression of cyclin inhibitors or via peptides mimicking the effects of these inhibitors. In a number of studies, the indirect effect of cyclin inhibitor p21 has been demonstrated, either overexpression via viral and nonviral vectors (1–6) or through the induction of p21 by different therapeutic agents (7–10). We have demonstrated previously that anti-inflammatory/immunosuppressive drugs, e.g., cyclosporine (CsA)[2], tacrolimus (TAC), and sirolimus (SRL) (11–13), induce the expression of cyclin inhibitor p21. Also, in vitro and in vivo overexpression of p21 in lymphocytes results in decreased response to mitogenic stimuli and greater sensitivity to the effects of CsA (14) and prolongation of graft survival in a rat heart transplant model (15). These studies demonstrated an indirect effect of p21 on various biological effects; however, a purified recombinant protein can accomplish the direct effect. In this study, we studied the direct effect of the recombinant p21 protein on cellular proliferation. Using lymphocyte proliferation as a model, we evaluated the effect of the recombinant p21 protein on lymphocyte proliferation, cytokine expression, and the mechanism of antiproliferative effects of p21 in activated lymphocytes. We also analyzed how the recombinant p21 protein enters the cell without a carrier protein. We compared these results with those obtained by immunosuppressive agents currently used in clinical organ transplantation.

Materials and Methods

Preparation of a p21 protein

A purified p21 protein was prepared by the method described by Mayrose et al. (16). Briefly, p21 cDNA cloned into Topo vector (14) was excised and overexpressed in plasmid pET-3d and named as pET-p21, which was transformed into *Escherichia coli* strain BL21 (DE3). The transformed cells were grown in Luria-Bertani culture medium at 37°C overnight. The control protein was isolated and purified from empty vector, transformed into *E. coli* strain BL21 (DE3), grown, and harvested cells similar to the p21 gene-containing vector, and was termed as the control protein. Using Western blot analysis and ELISA, a purified recombinant p21 protein was characterized.

Preparation of lymphocytes and experimental protocol

PBMCs from the blood obtained from the blood center were separated by Ficoll-Hypaque, and proliferation with mitogens was performed as described previously (11). The lymphocytes were activated with PHA with and without CsA (100 ng/ml), TAC (10 ng/ml), SRL (10 ng/ml), p21 (20 U/ml), and a similar amount of the control protein. These agents were added 30 min before the treatment with mitogen.

Detection of mRNA by RT-PCR

Total RNA was isolated from lymphocytes using TRIzol (Invitrogen Life Sciences), and quality of RNA was verified by a 260:280-nm ratio. One microgram of RNA was reverse transcribed to cDNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). The amplification by PCR was conducted using 1 μl of cDNA and 2 μl each of 2.5 mM coding and noncoding oligonucleotide primers and Platinum PCR Supermix (Invitrogen Life Technologies). The primer sequences are as follows: NFATc coding, 5′-CCTGTCCCCTACGTCCTACA-3′, and NFATc noncoding, 5′-GACACGGTCTTCCCTGTGAT-3′; and NFATp coding, 5′-GAACGAAGAAGAGCCGAATG-3′, and NFATp noncoding, 5′-TGTTTTGAAACTGCGGACAC-3′. The PCR products were resolved in 1% agarose gel electrophoresis, and ethidium bromide-stained specific bands were visualized under UV light and photographed.

Departments of Medicine and Surgery, Medical College of Wisconsin, Milwaukee, WI 53226

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[1] Address correspondence and reprint requests to Dr. Ashwani K. Khanna, Associate Professor, Medical College of Wisconsin, Milwaukee, WI 53226. E-mail address: akkhanna@mcw.edu

[2] Abbreviations used in this paper: CsA, cyclosporine; TAC, tacrolimus; SRL, sirolimus; PTD, protein transduction domain.

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The densitometric analysis of the specific bands was done using Alpha-Imager (Alpha Innotech), and data are represented as the ratio of the specific gene to β-actin.

EMSAs

EMSAs for NF-κB, NFATp, and NFATc were performed as described by us (17) using nuclear extracts from treated or untreated activated lymphocytes. The oligonucleotides for NF-κB and NFATc and NFATp for the EMSAs were obtained from Qiagen.

Entry of exogenous p21 in to the nucleus of lymphocytes: analysis by Western blot

Lymphocytes were treated with p21 at time 0, 30 min, 1 h, and 4 h, and after each treatment, lymphocytes were washed and cytoplasmic and nuclear extracts were prepared using the NE-PER Extraction Reagent from Pierce. Western blot analysis for p21 was performed as described previously (11). In each experiment, the p21 protein added to these cells was used as control. Experiments with renal cells were performed similarly. During this experiment, cells were counted, and the viability of cells was checked at each time point using trypan blue exclusion method.

Data analysis

Differences in all quantitative data were compared across appropriate groups using ANOVA. A value of $p < 0.05$ was considered of significance.

Results

Purification and characterization of a p21 protein

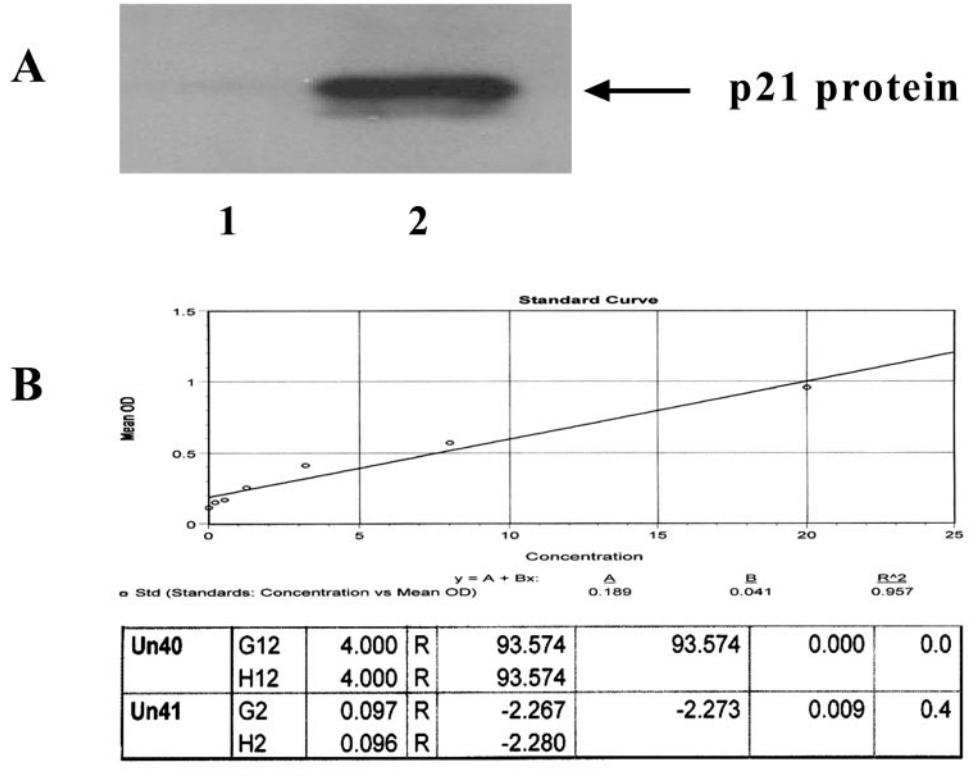
The recombinant p21 protein was characterized by silver staining of polyacrylamide gel (data not shown) and Western blot analysis. Fig. 1*A* demonstrates that the p21 immunoreactivity was observed only with the recombinant p21 protein (*lane 2*) and not with the control protein (*lane 1*). To further authenticate the purity of a p21 protein, we quantified the amount of p21 using a kit from Oncogene Research Products (WAF ELISA; catalog no. QUA18) that detects a p21 protein by the sandwich ELISA method. The standard curve obtained is shown in Fig. 1*B*, *top panel*, and the extrapolated results of the unknown values are shown in Fig. 1*B*, *bottom panel*. The amount of p21 in our preparation was estimated to be 93 U/ml, whereas the control protein did not have detectable p21 protein. Therefore, both Western blot analysis and quantification by p21-specific ELISA of the recombinant p21 protein and the control protein confirms the purity and authenticity of the recombinant p21 protein.

Effect of a p21 protein vs immunosuppressive drugs on lymphocyte proliferation

To test the efficacy of the recombinant p21 protein, we examined its effect on lymphocyte proliferation and compared it with immunosuppressive drugs. Lymphocytes were activated with and without the increasing concentrations (5–20 U/ml) of a p21 protein and the highest concentration (equivalent of 20 U/ml) of the control protein. A dose-dependent inhibition of lymphocyte proliferation with a p21 protein but not with the control protein is shown (Fig. 2*A*). Fig. 2*B* demonstrates that only the recombinant p21 protein but not the control p21 protein significantly ($p < 0.01$) inhibited proliferation of lymphocytes, which was similar to the inhibition observed with CsA ($p < 0.01$), TAC ($p < 0.01$), and SRL ($p < 0.01$). The proliferation was quantified by [^{3}H]thymidine uptake assay as described previously (11). These results indicate that the recombinant p21 protein inhibits lymphocyte proliferation as potently as observed with currently used immunosuppressive agents in organ transplantation.

Effect of the recombinant p21 protein on T cell growth factor IL-2 mRNA expression

During lymphocyte activation, T cell growth factor or IL-2 expression is increased, and its inhibition is considered to be the mechanism of action of immunosuppressive drugs. Therefore, we studied whether a p21 protein such as CsA and TAC also inhibits



Un40	G12	4.000	R	93.574	93.574	0.000	0.0
	H12	4.000	R	93.574			
Un41	G2	0.097	R	-2.267	-2.273	0.009	0.4
	H2	0.096	R	-2.280			

FIGURE 1. Characterization of the recombinant p21 protein. *A*, Western blot analysis of the recombinant p21 protein and the control p21 protein. Reactivity with anti-p21 is seen only with the recombinant p21 but not the control protein. *B*, Quantification of a p21 protein by ELISA, standard curve for a p21 protein, and calculated values for the recombinant p21 and the control protein are shown.

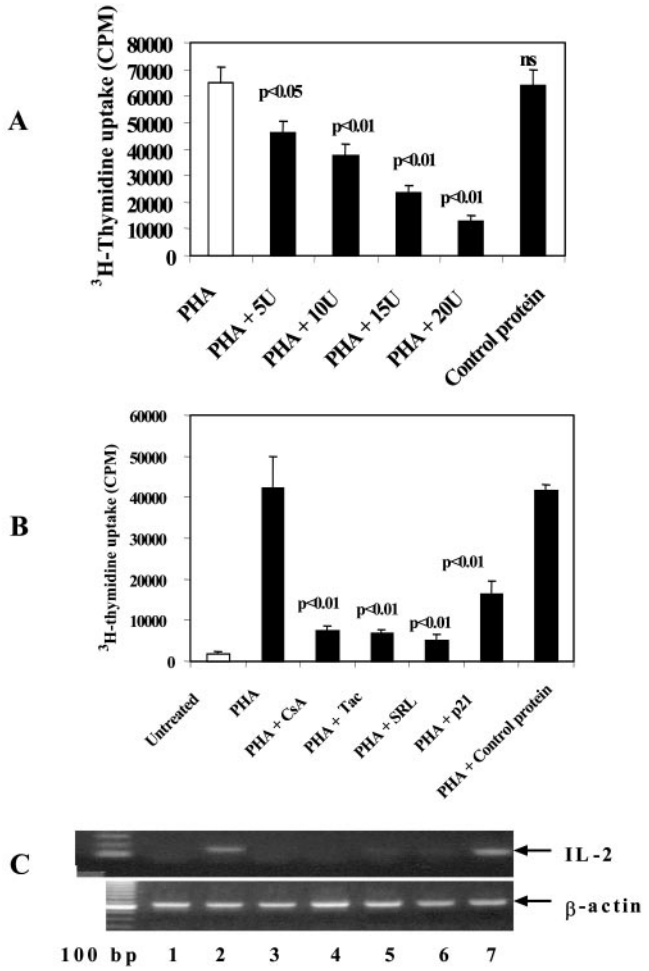


FIGURE 2. Effect of a p21 protein on lymphocyte proliferation. *A*, Determining the dilution of a p21 protein in proliferation assays: a dose-dependent inhibition of lymphocyte proliferation with a p21 protein but not with the control protein is shown. *B*, Efficacy of a p21 protein in comparison with currently used immunosuppressive drugs. Quantification of lymphocyte activation expressed as uptake of [^{3}H]thymidine (counts per minute (cpm)) indicates significant inhibition with the recombinant p21 but not with the control protein and was comparable with other immunosuppressive agents. *B*, Inhibition of IL-2 mRNA expression by p21. Inhibition of IL-2 mRNA is seen in PHA-activated lymphocytes (*lane 2*) with the recombinant p21 (*lane 6*) but not the control protein (*lane 7*). Similar inhibition can be seen with CsA (*lane 3*), TAC (*lane 4*), and SRL (*lane 5*) used as positive controls.

IL-2 mRNA expression in activated lymphocytes. Based on our previous published results (11), lymphocytes were activated with PHA (2 μg/ml) for 4 h with and without recombinant protein (20 U/ml), CsA (100 ng/ml), TAC (10 ng/ml), and SRL (10 ng/ml). Fig. 2*C* shows an example of an agarose gel electrophoresis for IL-2 and β-actin mRNA expression. Treatment with a p21 protein with PHA-activated lymphocytes (*lane 6*) but not the control protein (*lane 7*) resulted in almost complete inhibition of IL-2 mRNA expression similar to CsA (*lane 3*), TAC (*lane 4*), and SRL (*lane 5*).

Members of transcription factor NFAT family play a significant role in lymphocyte activation and inhibition. NFAT consists of a number of members with diverse functions, with NFATc and NFATp exerting opposite effects on lymphocyte functions. NFAT is considered to play a critical role in the regulation of immune activation/suppression (18). Therefore, to understand the role of NFATc and NFATp in antiproliferative effects of p21, we studied their expression by EMSA and RT-PCR analysis. We compared these results with CsA, TAC, and SRL to understand whether the effects of p21 on IL-2, NFATc, and NFATp mRNA are similar to the actions of these drugs. We studied the mRNA expression of IL-2, NFATc, and NFATp in lymphocytes from experiments described above. Fig. 3 demonstrates the mean of three experiments of the ratio of IL-2, NFATc, and NFATp with β-actin. The expression of NFATc mRNA increased upon activation and decreased with inhibition of lymphocyte proliferation with p21 but not with the control protein. Results from lymphocytes activated with CsA, TAC, or SRL were similar to those observed with the recombinant p21 protein. The pattern of NFATc mRNA upon activation and inhibition of lymphocyte proliferation was similar to

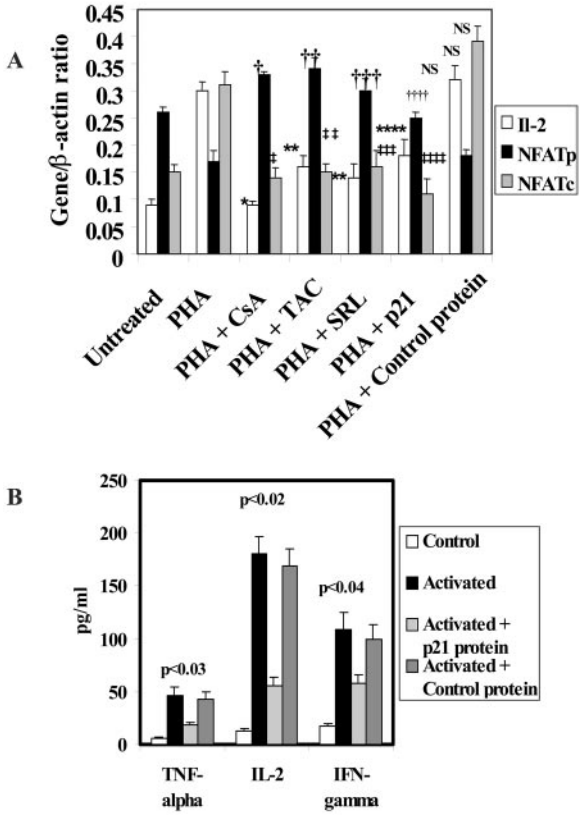


FIGURE 3. A. Differential effect of the recombinant p21 protein on NFATc and NFATp mRNA in activated lymphocytes. A significant increase in NFATc (▨) as with IL-2 (□) and decreased expression of NFATp (■) mRNA in activated lymphocytes can be seen. Inhibition of IL-2 and NFATc mRNA is seen in PHA-activated lymphocytes with the recombinant p21 but not the control protein. Similar inhibition can be seen with CsA, TAC, and SRL used as positive controls. In sharp contrast, NFATp mRNA expression was significantly higher in PHA-activated lymphocytes with the recombinant p21 but not the control protein. Similar increase can be seen with CsA, TAC, and SRL used as positive controls. (IL-2 mRNA expression; PHA vs CsA $*$, $p < 0.0003$; vs TAC $**$, $p < 0.005$; vs SRL $***$, $p < 0.006$; vs p21 protein $****$, $p < 0.02$; vs control protein NS. NFATc mRNA expression; PHA vs CsA †, $p < 0.004$ vs TAC ††, $p < 0.005$; vs SRL †††, $p < 0.01$; vs p21 protein ††††, $p < 0.006$; vs control protein NS. NFATp mRNA expression; PHA vs CsA ‡, $p < 0.01$ vs TAC ‡‡, $p < 0.001$; vs SRL ‡‡‡, $p < 0.0004$; vs p21 protein ‡‡‡‡, $p < 0.01$; vs control protein NS.) *B*, The recombinant p21 protein inhibits proinflammatory cytokines in conditioned medium from PHA-activated cells: levels of IL-2, TNF-α, AND IFN-γ were quantified in conditioned medium from PHA-activated lymphocytes with and without the recombinant p21 protein and the control protein by specific ELISA kits. A significant inhibition of these cytokines in p21 protein- but not the control protein-treated cells can be seen.

IL-2 mRNA expression. However, completely opposite results were observed with NFATp mRNA expression, which decreased upon lymphocyte activation and increased with p21-induced inhibition of lymphocyte activation. These results on IL-2, NFATc, and NFATp mRNA expression demonstrate that inhibition of lymphocyte by p21 is similar in mechanism to that observed with CsA, TAC, or SRL and suggests that p21 has the potential to be used as an immunosuppressive agent. These results demonstrate a reciprocal relationship between NFATc and NFATp mRNA in activated lymphocytes. Similar results supportive of our findings have been reported with protein expression of NFATc and NFATp in lymphocytes with and without activation (19).

Effect of a p21 protein on cytokines expression in activated lymphocytes

Because our results demonstrated inhibition of IL-2 mRNA expression by a p21 protein in activated lymphocytes, we tested whether a p21 protein also inhibited the secretion of proinflammatory cytokines by activated lymphocytes.

We performed experiments to study the effect of p21 on the levels of TNF-α, IL-2, and IFN-γ in the conditioned medium of lymphocytes activated with and without either a p21 protein (20 U/ml) or the control protein for 24 h. Cytokines were quantified by specific ELISA kits. The results (Fig. 3*B*) demonstrate that the inhibition of lymphocyte proliferation by p21 also results in decreased protein levels of IL-2, IFN-γ, and TNF-α. These experiments show that a p21 protein but not the control protein inhibited lymphocyte protein expression of proinflammatory cytokines, suggesting the potential of p21 as an anti-inflammatory and immunosuppressive agent.

How does exogenous p21 make its way to the nucleus of T cell?

Cyclin inhibitor p21 plays a unique role in cell cycle regulation. The localization of p21 in the cytoplasmic or nuclear compartments of cells determines the effect of p21 on cellular proliferation, apoptosis, and oncogenic events (20). Studies (21, 22) have demonstrated that in contrast to cytoplasmic p21, nuclear p21 inhibits cellular proliferation. Furthermore, these studies also demonstrated that although cytoplasmic p21 could interact with proliferating cell nuclear Ags, cyclin-dependent kinases, calmodulin, and cyclins, however, failed to exert an inhibitory effect on cell cycle. Because we observed that the recombinant p21 protein inhibits mRNA expression of IL-2 and NFATc and induces mRNA expression of NFATp, we were interested in testing the nuclear interaction of p21 with these transcription factors. Therefore, we designed experiments to examine the fate of a p21 protein when treated with lymphocytes. We tested whether the recombinant p21 protein enters the nucleus when mixed with lymphocytes. We performed four consecutive experiments. Lymphocytes were treated with p21 at time 0, 30 min, 1 h, and 4 h. After each treatment, lymphocytes were washed, and cytoplasmic and nuclear extracts were prepared using the NE-PER Extraction Reagent from Pierce. Results of the Western blot analysis for a p21 protein from two of these four experiments are shown in Fig. 4*A*. These results demonstrate that a p21 protein can be seen as early as 30 min in the nuclear extract and that its expression increases with time. After 4 h of treatment, cytoplasmic and nuclear expression of p21 is almost similar. These results indicate that a p21 protein localizes in the nucleus of lymphocytes. Control cells not treated with p21 did not exhibit any nuclear expression, indicating that the nuclear p21 expression seen was due to exogenous recombinant p21 added to these cells. In each experiment, the reactivity of the recombinant p21 protein added to these cells is also shown. This is the first report of nuclear translocation of a native recombinant p21 protein in lymphocytes and demonstration of its biological effect in inhibiting lymphocyte proliferation similar to that observed with immunosuppressive drugs.

To answer the question whether p21 enters the quiescent cells only, we performed similar experiments with an established renal epithelial cell line. These experiments were performed to understand the difference between the endogenous and exogenous p21 and whether continuously dividing cells behave differently compared with quiescent lymphocytes. The results shown in Fig. 4*B* demonstrate the entry of p21 into continuously growing renal epithelial cells. The time course of nuclear localization was similar to that observed with lymphocytes, with an exception of presence of endogenous p21 in renal epithelial cell line, which was similar at the time points studied. The results indicate the ability of the recombinant p21 protein to enter into cells without a carrier.

Mechanism of the cellular entry of cyclin inhibitor p21

Cellular entry of the recombinant p21 protein. The comparison with protein transduction by HIV TAT proteins is based on the studies on TAT proteins, which can enter cells either alone or can ferry smaller and larger proteins. The basis of this entry is explained through the protein transduction domains (PTDs), cell-permeable proteins, or membrane-translocating sequences that are responsible for protein transduction. These proteins have polyarginine residues. These small peptides are able to ferry much larger molecules into cells independent of classical endocytosis. We determined the percentage of arginine residues in the PTD of a TAT protein and a p21 protein. The analysis shown in Fig. 4*C* demonstrates that a p21 protein has 12% arginine residues compared with 9% in the PTD of a TAT protein. This explains the ability of a p21 protein to rapidly enter the cells. Furthermore, a p21 protein also contains the signature amino acid sequence present in the TAT protein responsible for the entry of these proteins in cells (Fig. 4*C*). This property makes PTDs ideal tools to transfer proteins and other molecules into living cells for research purposes. The mechanism by which this internalization takes place is poorly understood. By incorporating a PTD in the therapeutic gene product, the protein produced in the transfected cell might be enabled to spread to nontransfected cells, thereby creating an increased therapeutic effect. Proteins with a stretch of sequences similar to PTDs can translocate efficiently into the cytoplasm and the nucleus and exert their effects.

We also studied the consequence of nuclear localization of a p21 protein. It was hypothesized that a p21 protein after localizing to the nucleus will interact with NFATs such as NFATc, NFATp, and NF-κB. Therefore, we determined the effect of p21 on nuclear factors NFATc, NFATp, and the transcription factor NF-κB in lymphocytes activated with PHA with and without a p21 protein. We used CsA and the control protein in these experiments as positive and negative controls, respectively.

Effects of CsA and p21 on NFATc, NFATp, and NF-κB DNA-binding proteins

DNA-binding reactions were performed with nuclear extracts prepared from lymphocytes either untreated or activated with and without the recombinant p21 protein, the control protein, and CsA with ^{32}P-labeled oligonucleotide probes for NFATc and NFATp as described in *Materials and Methods*. To test sequence specificity of DNA binding, excess of cold-specific or mutant NFATc/p oligonucleotides were also added to the reaction mixture. While

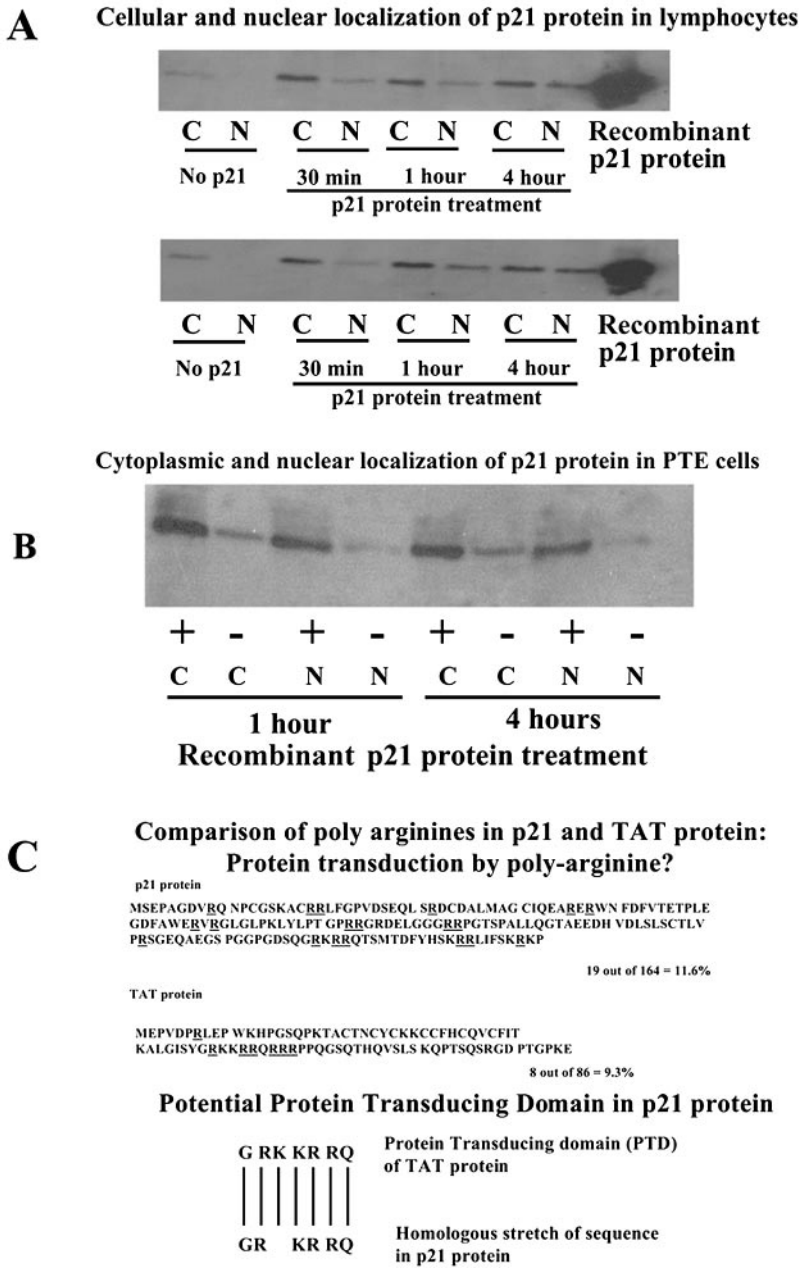


FIGURE 4. *A*, Nuclear localization of a p21 protein. Representative of three consecutive experiments of Western blot analysis of lymphocytes treated with a p21 protein at different time intervals (indicated) are shown in *A*. A gradual increase in a nuclear p21 protein can be seen. Results with untreated cells and the recombinant p21 protein as a positive control are also shown. *B*, Entry of a p21 protein into proximal tubular epithelial (PTE) cells. An increase in nuclear expression of p21 proteins after 4 h in cells treated with a p21 protein compared with untreated PTE cells is shown. At 1 h, a cytoplasmic p21 protein is more than nuclear; however, at 4 h, contents of the p21 protein are similar in both compartments, indicating the nuclear localization of a p21 protein only when exogenously p21 proteins were added. *C*, Similarities of polyarginine content and PTD sequence of a p21 protein with a HIV-TAT protein is shown.

NFATc binding increased with lymphocyte activation and was decreased upon treatment with p21or CsA, NFATp binding was opposite diametrically to that of NFATc. In the experiments described above, we also studied the transcription factors NF-κB. It is known that NF-κB activation results during lymphocyte activation and that the activation decreases during inhibition of lymphocyte activation. NF-κB activation results in the increased expression of many genes increasing, including cytokines, chemokines, growth factors, cellular ligands, and adhesion molecules relevant to alloimmune activation and inflammation in clinical transplant setting. Therefore, we also studied NF-κB nuclear-binding activity by EMSA. Fig. 5 shows the effect of a p21 protein on NFATp (*A*)- and NFATc (*B*)-binding activity. A reciprocal effect of p21 and other immunosuppressive agents, CsA, TAC, and SRL, on the binding activities of NFATc and NFATp is shown. Upon activation, NFATc binding increased and p21, but not the control protein, inhibited this activity. The opposite effects were seen with NFATp binding, which decreased after activation and increased in cells activated in the presence of p21 and other immunosuppressive agents and not with the control protein. Similarly, as shown in Fig. 5*C*, as with CsA, which is known to inhibit NF-κB (23), a p21 protein also inhibited NF-κB-binding activity, whereas the control protein had no effect on NF-κB binding in PHA-activated lymphocytes. Similar effects were seen with p60 and p50 subunits of NF-κB. The composite results are shown in Fig. 5*D*. These results demonstrate close similarities between the effects of CsA and the recombinant p21 protein with transcription factors. This suggests that CsA might be exerting these effects by inducing a p21 protein.

Effect of a p21 protein on p21 protein expression

We also prepared cell lysates to study the expression of a p21 protein in lymphocytes treated with p21 and the control protein. We have earlier reported that CsA and TAC induce p21 protein expression (11, 12), and in the present study, we report that p21 protein expression increased significantly in activated lymphocytes in the presence of a p21 protein (Fig. 5*E*). We speculate that p21

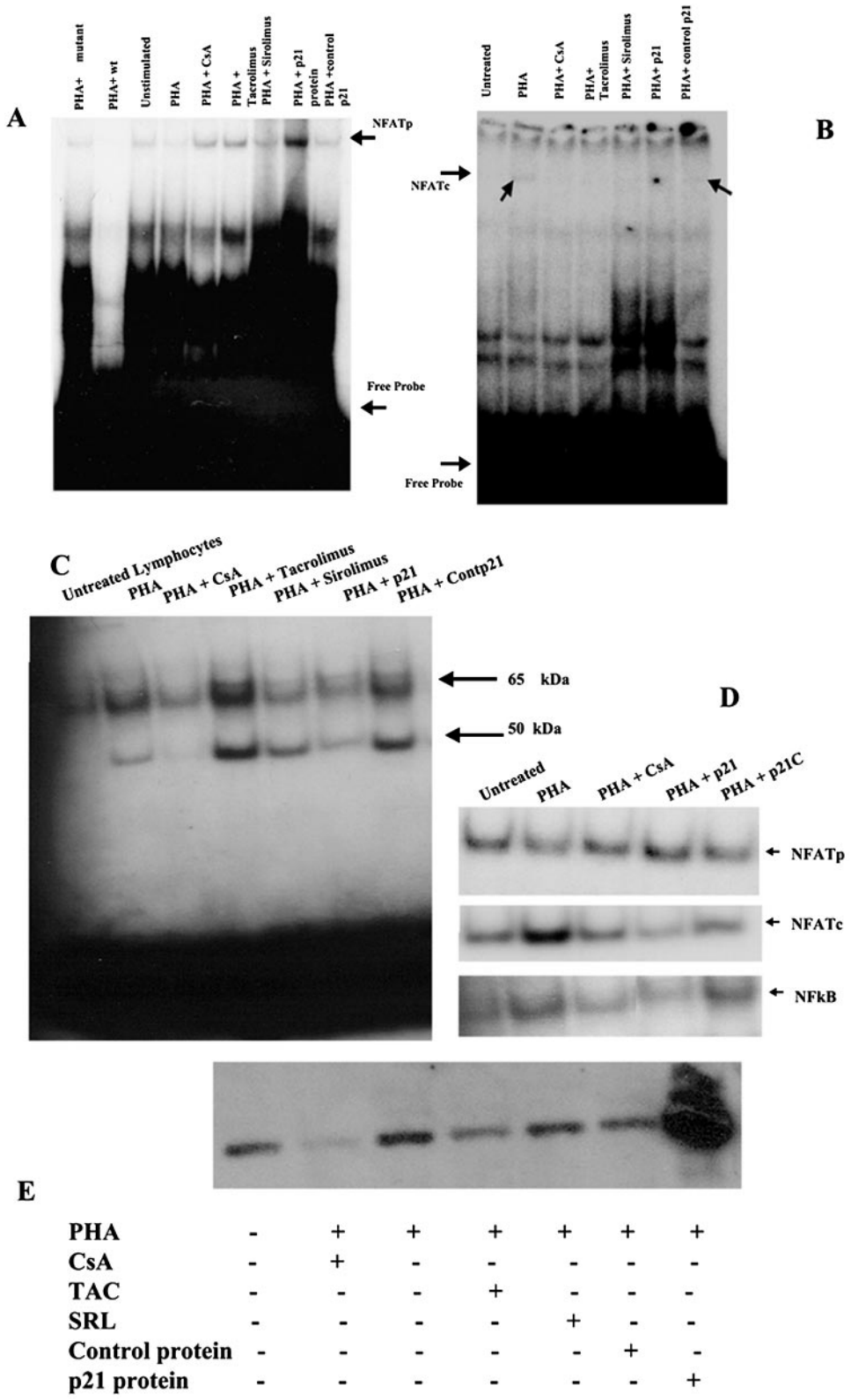


FIGURE 5. Effect of the recombinant p21 protein on NF-κB, NFATc, and NFATp transcription factors and p21 protein expression. A reciprocal effect of p21 on DNA binding of NFATp (*A*), whereas activation of NFATp can be seen in nuclear extracts from PHA-activated and p21 protein-treated cells; similar results with CsA used as a positive control can be seen. The opposite effects on NFATc binding in activated lymphocytes are shown (*B*). The arrows indicate NFATc or NFATp. An inhibition of DNA binding for NF-κB in cells treated with either p21 or immunosuppressive drugs is shown in *C*. A composite of data on the effect of the p21 protein on DNA-binding activity of NFATp, NFATc, and NF-κB in comparison with CsA is shown in *D*. Appropriate controls, including control cold probes, were used for each experiment. *E*, Effect of the recombinant p21 protein on p21 protein expression. A representative picture of Western blot analysis of cell lysates from lymphocytes activated with a p21 protein and a control p21 protein is shown. Cells treated with the p21 protein demonstrate a sharp increase in the p21 protein, and an increase in CsA-treated cells but not the control protein compared with untreated and PHA-activated cells can be seen.

may be self-regulating itself by an autoinduction mechanism or through inducing its own promoter activity. Compared with other studies (11, 24, 25), including our own, we observed p21 protein expression in nonactivated cells because in this study we used primary lymphocytes, which were not dividing, whereas in other studies, continuously dividing cell lines were used.

Discussion

The results uniquely demonstrate that the recombinant p21 protein inhibits proliferation of lymphocyte similar to that observed with currently used immunosuppressive agents CsA, TAC, and SRL. The nuclear localization of a p21 protein results in these antiproliferative effects. This observation finds support in other published

studies on the significance of nuclear localization of proteins, including p21 (26–28). These results suggested that the cytoplasmic localization of p21 is associated with increased cellular growth, whereas the nuclear localization of p21 was responsible for the inhibition of cell growth. Therefore, because the differential localization of a p21 protein exerts opposite effects on cell growth, it is conceivable that its entrance and the exit, into and out from the cell nucleus, must be regulated by some set of signaling peptides (29) that is identified by the nuclear localization signal. Similarly, studies of Dong et al. (30, 31) demonstrated the significance of the nuclear localization signal in the biological function of p21. We went steps further than these previous studies and demonstrate the postnuclear localization effects of p21 on transcription factors such as NFAT and NF-κB. Members of transcription factor NFAT family play a significant role in lymphocyte activation and inhibition. NFAT consists of a number of members with diverse functions, with NFATc and NFATp exerting opposite effects on lymphocyte functions. NFAT is considered to play a critical role in the regulation of immune activation/suppression. NFAT family consists of a number of members: NFATc1 (NFATc, NFAT2), NFATc2 (NFATp, NFAT1), NFATc3 (NFAT4, NFATx), and NFATc4 (NFAT3), each with discrete functions (32). The studies with mice deficient in either or both of these molecules have highlighted their role in immune response (33–37), demonstrating impaired lymphocyte proliferation in NFATc1-deficient lymphocytes with enhanced immune response in NFATc2-deficient animals. These studies on the role of these two members of NFATc family clearly demonstrate contrasting functions in control and regulation of immune response. We very convincingly demonstrate that at the transcription level the expression of NFATc and NFATp mRNA were increased reciprocally or decreased upon lymphocyte activation and inhibition with either p21 or one of the immunosuppressive agents. The expression of NFATc correlated with IL-2, the T cell growth factor, and increased upon lymphocyte activation, whereas NFATp mRNA increased after inhibition of lymphocyte activation. These are the first reports of reciprocal expression of mRNA for these transcription factors during lymphocyte activation and inhibition. Similar results with NFATc and NFATp protein expression in activated lymphocytes have been reported previously (19). These results indicate that a p21 protein, after localizing into the nucleus, interacts with transcription factors and renders its effects on lymphocyte activation.

The findings of this study are manyfold, especially in the context of organ transplantation. Successful graft survival depends on the control of the immune response of the recipient. Thus, by overexpressing, p21 could modify T cell activation and proliferation and inhibit the expression of cytokines, especially proinflammatory cytokines. Although in this study, we have not demonstrated inhibition of cyclins, a number of studies have shown correlation of cell cycle progression and lymphocyte activation (38, 39). In another study, we have demonstrated the role of cyclins in alloimmune activation, and the deficiency of p21 results in increased proliferation as also demonstrated earlier by other authors (40).[3] All these studies are supportive of our assumption that the expression of cyclins correlates inflammatory stress. Therefore, inhibition of cell cycle progression of lymphocyte proliferation could prevent inflammation of transplanted graft.

These results indicating that p21 localizes into the nucleus and inhibits lymphocyte proliferation are of immense significance. This is the first report on such a biological function of the native recombinant p21 protein. It is perfect to envisage the biological outcomes of a protein, which can efficiently deliver itself across the cell membrane and localizes into the nucleus. This is significant because the delivery of proteins with HIV-TAT and other carrier sequences can be very effective but requires a definitive assembly of a therapeutic protein with PTD, and the immunogenicity of the sequences ferrying these proteins can also be problematic. Therefore, the delivery of a p21 protein by itself bypasses these important problems and could serve as a potent therapeutic tool to limit cancer specifically. Our studies were performed with respect to lymphocyte proliferation; however, abnormal cell proliferation remains the key to the pathogenesis of a number of human diseases, including cancer (30, 41, 42) and atherosclerosis (43). The results from this study may suggest the use of cell growth controlling proteins, especially p21, as therapeutic measures to regulate aberrant cell growth in vascular diseases and cancer.

Even though the primary targets in drug discovery are the proteins, the inability of the proteins to cross cell membranes is the primary reason that the complete therapeutic potential of proteins cannot be realized. However, recently, newer strategies have been developed for the protein and peptide delivery into cells using specific sequences known as PTDs. These small peptides with either polyarginine residues or specific sequences can be covalently attached to small or large size proteins and then can be directly transferred to cells. The mechanism by which these sequences ferry proteins into the cells is unknown, and it is likely that the interaction of proteins with the negative cell membrane environment may allow the passage through the cell membrane. The sequence of most commonly used PTDs is consists of a 13-aa, e.g., the protein sequence of TAT consists of "**GRKKRRQRRRPQ**," which is shared by 138–145-aa "GRKRRQT" of a p21 protein. These positively charged sequences attach to the charged phospholipids in the outer side of the cellular membrane, which results in the dissociation or destabilization of the cell membrane. This is followed by structural changes in the cell membrane and by an unknown mechanism that the protein penetrates the cytoplasmic compartment. Although the participation of endocytosis-mediated entry of proteins can be ruled out. Studies have demonstrated the efficient in vitro and in vivo delivery of a large number of biologically active small and large size proteins into cells and tissues. However, the uniqueness of this study is the ability of native recombinant p21 proteins to cross cell membrane and nuclear localization without the artificial binding with these protein-transducing domains. The amino acid analysis of a p21 protein provides the explanation that such a sequence is inherently present in a p21 protein, enabling its entry into the cells. Thereby, a p21 protein may be the first protein with direct therapeutic potential to limit progression of cancers, vasculoproliferative disorders, and inflammation. This study was designed to understand the role of cyclin inhibitor p21 on cellular proliferation using lymphocyte proliferation as a model. Recombinant p21 inhibits lymphocyte proliferation, mRNA expression of cyclins, and proinflammatory cytokines. The most interesting aspect of the present study is the demonstration of nuclear localization of the recombinant p21 proteins in the nucleus of lymphocytes. Studies have demonstrated that cellular effects of p21 on proliferation can be achieved only after nuclear localization. Our results suggest that the protein enters the cells possibly using a PTD sequence, interacts with transcription factors, induces its own expression, and exerts its biological functions. We feel that p21 may provide an excellent strategy to inhibit aberrant lymphocyte proliferation and a detailed analysis of amino acid sequence of other cyclin inhibitors: p16, p27, p53, and p73; the presence of PTD is unique to p21. Because p21 will inhibit cellular proliferation, it is unlikely that its presence

[3] A. Khanna. Reciprocal role of cyclins and cyclin inhibitor $p21^{WAF1/CIP1}$ on lymphocyte proliferation, alloimmune activation and inflammation. *Submitted for publication.*

will have an effect on the quiescent cell population, avoiding unwanted complications. The benefit of this strategy will be multifold to combat diseases associated with inflammation, vasculoproliferative disorders such as atherosclerosis, cancer, and allograft rejection in organ transplantation.

Disclosures

The authors have no financial conflict of interest.

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