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The COOH-Terminal Src Kinase Csk Is a Tumor Antigen in Human Carcinoma[1]

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ABSTRACT

The cytoplasmic tyrosine kinase cSrc is involved in the regulation of many important cellular functions including cell growth and transformation, and its activity is down-regulated by phosphorylation of the Tyr530 residue by the COOH-terminal Src tyrosine kinase, Csk. Because cSrc was previously found overexpressed, activated, and in some cases mutated in carcinoma, we investigated whether it could act as a tumor antigen. We show that whereas no autoantibodies were found against cSrc or its relative Fyn, up to 20% of patients with carcinoma had high-affinity autoantibodies against Csk. Immunity mainly resulted from a secondary response, as indicated by the presence of IgG1 in the sera. Antibodies were linked to the cancer because they were not detected in healthy subjects nor in patients with unrelated diseases, and their levels decreased in the sera of patients after surgical resection. Furthermore, they behaved as early markers of epithelial transformation because they were present in sera of patients with early-stage tumors and precancerous lesions such as colorectal polyps and in sera of patients that were scored negative for other cancer serological markers (CEA, CA15–3, CA19–9, p53 antibodies). Finally the presence of these antibodies was attributed, at least in part, to a substantial elevation of Csk protein levels in the corresponding tumors. However a strong increase in Src activity was also observed in these tissues, which suggested that Csk cannot regulate Src-like activity in carcinoma. Taken together, these data demonstrate that Csk acts as an autoantigen, and the detection of anti-Csk antibodies may have potential diagnostic usefulness in the early detection and postoperative follow-up of patients with carcinoma.

INTRODUCTION

The cytoplasmic tyrosine kinases of the Src family comprise three viral oncoproteins and eight cellular members in mammals, three of which, Src, Fyn, and Yes, are widely expressed (1). A large body of evidence points to important functions for these cellular kinases in cell responses induced by a wide range of stimuli including growth factors, cytokines, and adhesion to the extracellular matrix (2). These enzymes are highly regulated *in vivo*, and deregulation of their activity can lead to oncogenic properties (1). Accordingly, Src members are found overexpressed and/or deregulated in human melanoma and carcinoma (3–8), and human oncogenic alleles of Src kinases have recently been identified (9, 10). Negative regulation of Src kinases involves phosphorylation of a tyrosine residue present in the COOH terminus sequence (Tyr530 is the human cSrc sequence; 1). Regulation of SrcTyr530 is attributable to another cytoplasmic tyrosine kinase called Csk (11). The importance of Csk in Src regulation has been illustrated by genetic analysis in mice, in which *csk* gene disruption led to embryonic lethality (12, 13). Furthermore, high Src kinase activity was detected in mouse embryo fibroblasts that do not express Csk (12, 13). Although all of these data suggest that Csk could play a negative regulatory role during carcinogenesis, its status in human cancer is ill defined.

Cancer development is frequently accompanied by immune responses against self and altered self-antigens expressed in tumor cells (14). Autoantibodies can be generated against proteins related to cell growth and found mutated in tumors. One of the best examples is the detection of autoantibodies against the tumor suppresser, p53 (15), which is frequently mutated and/or inactivated in transformed cells. Immunoreactivity has also been reported against overexpressed, nonmutated, proteins in tumors such as cErb2, a member of the epidermal growth factor receptor family (16). From these observations, several applications have emerged including potential diagnosis and new therapy (17). Because autoantibodies have been detected for several products of proto-oncogenes, we investigated whether cSrc behaves as a tumor antigen. Surprisingly, although no immunoreactivity was found for Src, we report in this study the existence of autoantibodies to its negative regulator Csk. Immunoreactivity was linked to carcinoma and was accompanied with a strong increase in Csk protein level in the corresponding tumors. Finally, our data strongly suggest that Csk-autoantibodies are early markers of carcinogenesis, which may have potential diagnostic usefulness in the early detection and postoperative, follow-up of patients with carcinoma.

MATERIALS AND METHODS

Cell Culture and Transfection

Insect cells were cultured in SF900 medium (Life Technologies, Inc.) and infected with the different recombinant baculoviruses expressing the human cytoplasmic tyrosine kinases cSrc, Fyn, or Csk. After 2 days of infection, cells were lysed in buffer A [0.1% (v/v) Nonidet P40 (NP40), 20 mM HEPES, pH 7.5, 2 mM dithiotheitol (DTT), 20 mM NaF, 100 μM sodium orthovanadate, 20 μM leupeptin, 1% (v/v) aprotinin, and 100 μM phenylmethane sulphonyl fluoride (PMSF)] as described previously (2). Simian Cos7 cells were cultured in DMEM containing 10% FCS, glutamine, and antibiotics (penicillin and streptomycin) at 37°C in a humidified 5% CO_2 atmosphere. The human colorectal cell lines Caco2, LS174T, Colo 205, and WiDr were cultured in RPMI medium (Life Technologies, Inc.) containing 10% FCS, glutamine, and antibiotics (Penicillin and Streptomycin) at 37°C in a humidified 5% CO_2 atmosphere. Transient transfection in Cos7 was performed by lipofectamine reagent (Life Technologies, Inc.) as described by manufacturer's instructions for 2 days using the pSV-Csk encoding human Csk (2).

Biochemistry

ELISA. The human cytoplasmic tyrosine kinases cSrc, Fyn, and Csk were purified from the Sf9 cell lysate by phosphotyrosine affinity column chromatography as described previously (18). Purified proteins (up to 95%, as assessed by Coomassie Blue staining after separation on SDS gels) and BSA (fraction V from Eurogentec) were adsorbed onto 96-well ELISA plates (γ-irradiated, Costar Corp.) by adding protein solution [10 μg/ml (500 ng/wells) or as indicated otherwise] in PBS overnight at 4°C. Unbound proteins were removed by extensive washing with PBS-0.1% Tween 20. Diluted sera in PBS, 0.5% BSA, and 0.1% Tween 20 (1:20 optimal dilution, as determined by serum dilution studies) were added into wells at room temperature, incubated for 3 h, washed six times with PBS-0.1%Tween and bound antibody revealed by adding antihuman IgGFc or class-specific IgG coupled-peroxidase as indicated (Sigma and Zymed Laboratories; 1:500–1:1000 dilution in PBS) for 3 h. This was followed by six washes with PBS-Tween buffer and one with distilled water, incubation for 30 min with the peroxidase substrate ABTS

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(Boehringer); and the absorbance was measured at 405 nm. Serum from patient 32, described in Fig. 1, was systematically included in the ELISA as a positive control.

Dot Blots. Purified Csk or Fyn (100 ng) was spotted onto nitrocellulose membranes, followed by blocking for 1 h at room temperature with PBS, 3% BSA, and 0.1% Tween 20. Membranes were next incubated with rabbit polyclonal anti-Csk (Transduction Laboratories), anti-cst1 (2), or human sera (dilution 1:1000 in PBS-Tween) for 1 h at room temperature, and were washed extensively with PBS-0.1% Tween 20, followed by incubation with protein A-linked peroxidase (dilution 1:5000 in PBS-Tween). Bound antibodies were revealed by the addition of chemiluminescent substrate for the peroxidase (enhanced chemiluminescence, Amersham), followed by autoradiography.

Western Blots. Purified Csk or Fyn (500 ng) were run on 9% acrylamide SDS-PAGE gels and transferred onto nitrocellulose membranes. After blocking for 1 h at room temperature with PBS, 3% BSA, and 0.1% Tween 20, membranes were incubated for 2–3 h with patients' or control sera (dilution 1:100 in with PBS, 3% BSA, and 0.1% Tween 20). After washing with PBS-0.1% Tween 20, they were incubated for 1 h at room temperature with protein A-linked peroxidase (dilution 1:5000 in PBS-Tween). After extensive washing with PBS-0.1% Tween 20, bound antibodies were revealed as described for dot blot experiments. For Cos7 cell experiments, 20 μg of proteins from total cell lysates were used and 100 μg for Csk affinity purification. For colorectal cancer cell experiments, 200 μg of protein cell lysate were used. Csk Purification was performed as follows: cell lysates was incubated for 4 h at 4°C with 20 μl of resin (Affigel 10; Bio-Rad) coupled with the phospho-peptide SALYpQVDQ, which has high affinity for Csk SH2 domain (19). After extensive washing in 20 mM HEPES, 0.1% NP40, bound material was denatured in Laemli buffer, subjected to electrophoresis on a 9% SDS-PAGE gel, and transferred onto nitrocellulose membrane. Endogenous Csk levels in control and tumor biopsies were determined using 15 μg of proteins from radioimmunoprecipitation assay tissue lysates and blotting with a commercial anti-Csk antibody (Transduction Laboratories). All of the membranes were immunoblotted as described for dot blot experiments.

***In Vitro* Kinase Assay.** Biopsies were lysed in ice-cold RIPA buffer, and proteins were immunoprecipitated (25 μg of proteins by assay) with the cst1 antibody, followed by an *in vitro* kinase assay as described previously (3). Briefly, immunoprecipitates were resuspended in kinase buffer [20 mM HEPES (pH 7.5), 1 mM DTT, and 10 mM $MnCl_2$] and incubated in the presence of 1 μCi of [γ-P^{32}]ATP and acid-denatured enolase as an exogenous substrate for 10 min at 30°C. Labeled products were separated in an SDS-PAGE gel followed by autoradiography. Radioactivity incorporated into enolase was also measured by Cherenkov counting of excised bands.

Immunohistology. Four μm of formalin-fixed paraffin-embedded human tissue sections were immunolabeled with the commercial anti-Csk antibody. Bound antibodies were visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as a chromogen and catalyzed signal amplification system (Dako Corporation).

Population Studied. Control panel included 40 healthy blood donors and 20 patients (10 females, 10 males) suffering noncancerous diseases (10 suffering from autoimmune thyroiditis and 10 from gastrointestinal inflammatory diseases). The panel of patients with cancer included 54 females and 50 males affected by adenocarcinoma (30 colorectal adenocarcinoma, 16 transitional cell carcinoma of the bladder, 21 breast adenocarcinoma, 10 ovarian adenocarcinoma, and 6 lung adenocarcinoma) and colorectal polyps (21 patients). Sera from healthy blood donors (ages <55 years according to the French Legislation) were from the Etablissement de Transfusion Sanguine de l'Hérault, Montpellier, France. Sera from patients with autoimmune tyroiditis were from the Service d'Endrocrinologie du Center Hospitalier Universitaire Lapeyronie (Montpellier, France). Levels of autoantibodies for thyroid peroxidase ranged from 94.5 to 211 units/liter (normal values, <15 units/liter) and for thyroid-stimulating hormone from 1,186 to 11,726 units/liter (normal values, <140 units/ml). Sera from patients with breast, ovary. and lung cancers were from the Center Regional de Lutte contre le Cancer (Montpellier, France). Sera from patients with digestive noncancerous diseases, colorectal adenocarcinoma (ages, 45–89 years), and transitional cell carcinoma of the bladder (ages, 49–82 years) were collected before surgery and, in some cases, 1 or 6 months after surgery. Tumor marker levels (CEA, CA19–9, and p53 autoantibodies) were determined as described previously (20). All of the sera were stored at −20°C before analysis.

RESULTS

Presence of Csk Autoantibodies in Sera of Patients with Carcinoma. To analyze whether deregulated cSrc expression in human carcinoma leads to an immune response, we performed ELISAs. Affinity-purified human cSrc was used as an antigen, plus two additional cytoplasmic tyrosine kinases as controls: purified human Fyn, a member of the Src family not found activated in carcinoma; and human Csk, a kinase distinct from the Src family. Immobilized antigens were incubated with sera of healthy controls or patients with carcinoma, and bound antibodies were revealed using an antihuman IgGFc-specific-linked peroxidase as a secondary antibody, and quantified by a colorimetric assay. An example of such experiments is shown in Fig. 1; whereas no specific antibodies were detected for the Src members, a specific immune response was obtained for Csk in the sera of two of four patients with carcinoma tested. These two patients were afflicted with early-stage tumors of two different origins, colon and bladder; they belonged to both sexes and had no other known pathologies, which suggested that the observed immune response was linked to their pathology. This observation was extended to a larger population: in a control panel comprising 40 healthy blood donors and 20 patients with inflammatory noncancerous diseases, Csk reactivity was low; mean $A_{405} \pm$ SD value was 0.262 ± 0.085, slightly exceeding that observed with BSA (0.229 ± 0.107). This indicated that Csk autoantibodies were essentially absent from these nontumor samples. In contrast, high serum reactivity was observed in 15 of 104 sera of patients with carcinoma and precancerous lesions. Deduced prevalence of patient positive for Csk immunoreactivity was in a range of 5–20%, depending on the type of carcinoma (Table 1). Binding characteristics of the immune response was next analyzed. As shown in Fig. 1*B*, immunoreactivity was antigen-dose-dependent and was specifically reversed by an excess of free Csk but not reversed by an excess of BSA (not shown) or Fyn (Fig. 1*C*). IC_{50} values deduced from binding displacement curves of two distinct sera were 0.5 and 2 nM, respectively, in agreement with the involvement of IgGs. The subclass of IgGs was next investigated using specific peroxidase-linked secondary antibodies. As shown in Fig. 1*D*, Csk autoantibodies were predominantly IgG1 in all of the sera tested; in addition, some residual IgM was also detected in two of four patients.

The presence of Csk-autoantibodies was further confirmed using two independent approaches including dot and Western blotting. In dot blot experiments, sera of three patients that were found positive in ELISA (Table 1) reacted specifically with purified human Csk but not with human Fyn (Fig. 2*A*). Reactivity of a healthy blood donor serum was low as expected, as were the sera of three patients that were found negative in ELISA. We also examined reactivity of the sera against Csk by Western blotting. As shown in Fig. 2*B*, three distinct sera that were scored positive in ELISA also recognized purified human Csk, albeit with less efficiency than in ELISA studies. Again, no specific signal was obtained from sera of healthy blood donors nor from sera of patients scored negative by ELISA (not shown). Whether positive sera also recognize Csk in mammalian cells was also investigated. As a first approach, immunoreactivity was measured by Western blotting with a total cell lysate of simian Cos7 cells overexpressing human Csk. As expected, sera immunoreacted with a large number of proteins including one migrating at M_r 50,000 that was identified as Csk by reblotting with anti-Csk commercial antibodies (data not shown). However, and to get a clearer picture, we prepared affinity purified Csk from this lysate. This was achieved by using a resin coupled with the peptide (SALYpQVDQ) with high affinity for CskSH2 domain,

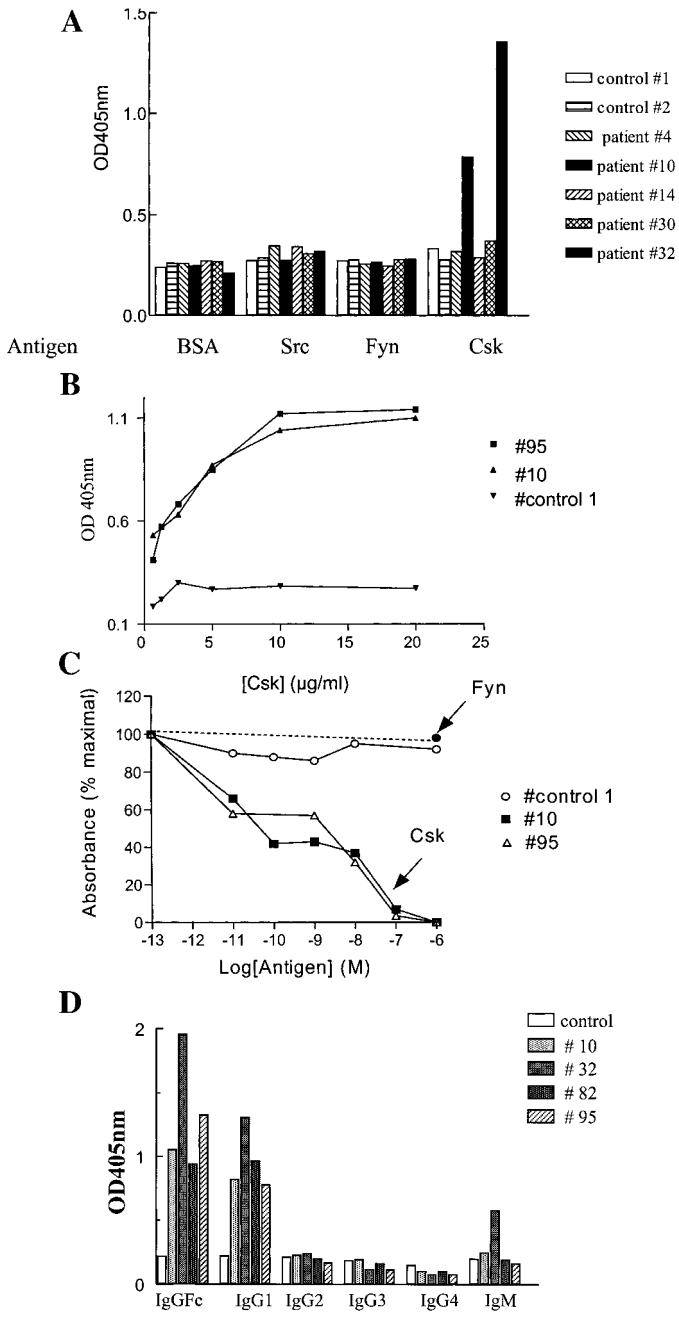

Fig. 1. Detection of anti-Csk antibodies in sera of patients with carcinoma by ELISA. In *A*, an ELISA was performed with control and carcinoma patients' sera using purified Src, Fyn, Csk, and BSA as antigens. Patients 4 and 10 had colorectal adenocarcinoma stage B, patient 14 had stage C, and patient 30 had stage D; patient 32 suffered from a transitional cell carcinoma of the bladder stage pT_a. Controls 1 and 2 are healthy blood donors. Bound antibodies were revealed using an antihuman IgGFc coupled to peroxidase, followed by the addition of the chromogenic peroxidase substrate ABTS. $A_{405\ nM}$ values are shown (*OD405nm*). In *B*, Csk immunoreactivity is antigen dose-dependent. Shown are $A_{405\ nM}$ values (*OD405nm*) obtained in an ELISA performed on sera from two positive patients, patient 10 (*described in A*) and patient 95 (who had developed colorectal polyps), and of one control (*control 1 described in A*) in the presence of increasing concentrations of bound Csk antigen. *C*, binding affinity and specificity of Csk autoantibodies. Binding of Csk autoantibodies was reversed by increasing concentrations of free Csk but not of Fyn: associated antibodies from sera of patients 10 and 95 or control 1 were incubated with increasing amounts of free Csk or of Fyn and diluted in PBS-Tween, and after extensive washing, the remaining antibodies were quantified. The percentage of maximal binding relative to increasing concentrations of free Csk is shown. In *D*, Csk autoantibodies are of IgG1 class. An ELISA was performed on one control and four carcinoma patient sera using Csk as an antigen. Bound antibodies were revealed with various antihuman immunoglobulin-coupled-to-peroxidase as indicated, and the obtained $A_{405\ nM}$ (*OD405nm*) values are shown. Patients 95 and 82 had colorectal polyps and ovary carcinoma, respectively.

Table 1 *Csk autoantibodies are linked to cancer*

	Number of positive[a]/ total number of patients	Prevalence %
Controls		
Blood donors	0/40	0
Autoimmune tyroidis	0/10	0
Noncancerous diseases of the colon	0/10	0
Patients		
Carcinoma		
Colon	6/30	20
Bladder	3/16	19
Lung	1/6	17
Ovary	1/10	10
Breast	1/21	5
Colorectal polyps	3/21	14
Total	15/104	14

[a] Percentage of sera-positive individuals in a population of 60 controls and 104 patients with cancerous or precancerous lesions. Sera with absorbance values exceeding both the mean absorbance of normal sera + 2 SD and 1.3-fold absorbance values obtained with BSA were scored as positive.

and which indeed retains the kinase with a very potent efficiency higher than that obtained using immunoprecipitation with anti-Csk COOH-terminal antibodies.[3] In these conditions Csk specifically immunoreacted with commercial anti-Csk antibody as well as with sera from patients scored positive in ELISA (Fig. 2*C*). Finally, we looked at the ability of these sera to recognize endogenous Csk from human cancer cells, and an example of such experiment is shown in Fig. 2*D*. Serum from patient 10 also immunoreacted with Csk that was purified from the human Ls174T, Colo205 and WiDr colorectal cancer cells. Specificity was confirmed by reprobing the blot with a commercial antibody specific to Csk and by the absence of specific signal obtained with a control serum (not shown). Similar results were obtained with two other positive sera (not shown).

Csk Autoantibodies Are Linked to Carcinogenesis. Whether Csk autoantibodies were linked to cancer was next confirmed by looking at Csk immunoreactivity in sera of patients after resection of the tumor. As shown in Fig. 3, Csk autoantibodies levels decreased in sera of patients postsurgery: This was observed by Western blot on affinity-purified Csk (Fig. 3*A*) and by ELISA measurements (Fig. 3*B*). One month postsurgery, the serum of patient 15 failed to immunodetect Csk by Western blot (Fig. 3*A*) and was scored negative by ELISA (Fig. 3*B*). Similar data were obtained with patient 10 (not shown and Fig. 3*B*) and with patient 32 (Fig. 3*B*). It should be noted that these three patients had well recovered and have suffered no relapse to date. Overall, these data indicate that Csk immunoreactivity is closely related to cancer.

Csk Autoantibodies Are Early Markers of Carcinogenesis. We next investigated whether detection of Csk autoantibodies could be useful for cancer diagnosis by comparing their prevalence with that of other known serological markers. We first observed that positive patients with ovary and lung cancers (Table 2) had low or median values for the classical tumor markers, CA-125 and CYFRA-21 (not shown), which suggested that Csk autoantibodies occurred at an earlier developmental stage of the cancer. This was confirmed by positive scores for patients with colorectal polyps (Table 1) and with early carcinoma, including three of three positive patients with transitional bladder cell carcinoma at stage pT_a and pT_1 (not shown) and four of six positive patients with colorectal carcinoma at stage A and B according to Dukes' classification (Table 2). The clinical history of the breast cancer patient found positive is also indicative in this respect, because we detected Csk autoantibodies 4 months before the appearance of CA15–3 and CEA markers (data not shown). Early development of a Csk immune response was confirmed when com-

[3] Unpublished data.

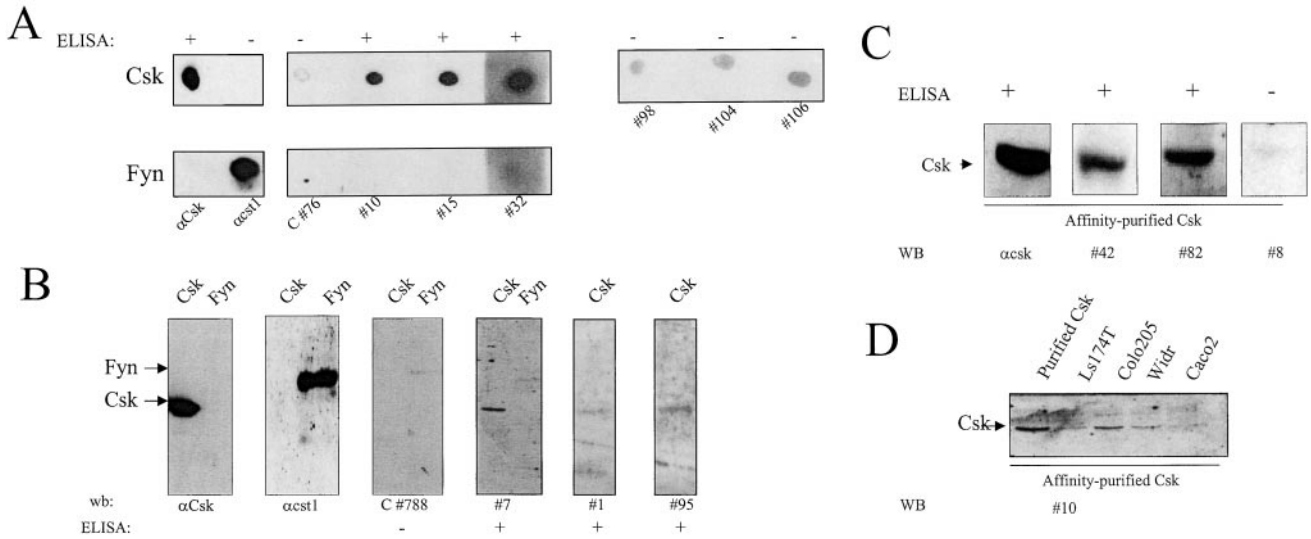

Fig. 2. Detection of anti-Csk antibodies in sera of patient with carcinoma by dot and Western blotting. *A*, detection of Csk-autoantibodies in dot blot experiments. One hundred ng of purified Csk or of Fyn were spotted on nitrocellulose membranes and incubated with αcst1 antibody, which recognizes Fyn; commercial anti-Csk antibody, or sera of a healthy blood donor (*C #76*) or of patients with carcinoma as indicated (dilution 1:1000). Shown are autoradiogramms obtained after 15-s exposure. Results obtained from these sera with ELISA are also indicated. Patients 10, 32, and 95 are described in Fig. 1. Patient 15 is described in Table 2. *B*, detection Csk autoantibodies in Western blotting experiments. Five hundred ng of purified Csk or of Fyn, separated on a SDS-PAGE gel, were transferred onto nitrocellulose membranes and immunoblotted with αcst1 antibody, which recognizes Fyn; commercial anti-Csk antibody; or sera of a healthy blood donor (*C #788*) or patients with carcinoma as indicated (dilution 1:100). Shown are autoradiogramms obtained after 15-s exposure. Results obtained from these sera with ELISA are also indicated, as well as the migration of Csk and Fyn, respectively. Patients 1 and 7 are described in Table 2. *C*, autoantibodies associate with human Csk that was expressed in simian Cos 7 cells. Csk was affinity purified from Cos7 transfected with the vector encoding human Csk as described in "Materials and Methods" and was run on SDS-PAGE gel. Proteins were next transferred onto nitrocellulose membranes and immunoblotted with commercial anti-Csk antibody or sera of 1 negative and 2 positive patients (dilution 1:100). Shown are autoradiogramms obtained after 15-s exposure. Patient 42 had a bladder carcinoma, patients 8 and 82 are described in Table 2 and Fig. 1, respectively. In *D*, autoantibodies associate with Csk expressed in human cancer cells in Western blotting. Csk was purified from Sf9 expressing the human Csk or from the indicated human carcinoma cell lines as described in "Materials and Methods," subjected to SDS-PAGE, transferred onto nitrocellulose membrane, and immunoblotted with the serum of patient 10 (dilution 1:100). Shown is the autoradiogram obtained after 30-s exposure. The presence of Csk is indicated and was confirmed by reprobing the blot with commercial anti-Csk antibody (not shown).

paring its prevalence to that of the CA19–9, ACE, and p53 autoantibodies in colorectal cancers (Table 2); three of six patients positive for Csk autoantibodies were either negative for these markers or positive for only one. CEA and CA19–9 were detected in a significant number of patient sera, one-half of them being from patients with late stages (stages C and D) colorectal cancers.

Csk Is Overexpressed in Human Carcinoma. Because the appearance of autoantibodies can be attributable to a change in the status of the protein during carcinogenesis (21), we looked at whether Csk expression was altered in human carcinoma. Csk protein level was determined in tumors relative to histologically normal epithelia obtained from the same patient. This was performed by immunoblotting the whole tissue lysate with a Csk-specific antibody, and an example is shown in Fig. 4*B*; a 10-fold increase in Csk level was found in the bladder tumor of patient 32, scored positive for Csk autoantibodies (see Fig. 1). An increase in Csk protein expression was also confirmed by an immunohistological approach and is shown in Fig. 4*A*; immunolabeling of the tumor biopsy section with specific antibodies showed a strong Csk signal in tumor cells, in agreement with our biochemical analyses. Similar data were obtained with other bladder and colorectal carcinoma from patients scored positive (see patients 22 and 37 in Fig. 4*B* as examples), suggesting a close relationship between protein overexpression and Csk-immunoreactivity (Fig. 4*B*). However, increase in Csk level was also observed in tumors of some patients that did not develop autoantibodies (see, for example, patient 31 in Fig. 4*B*), indicating that additional mechanisms may be required to trigger an autoimmune response.

Csk Overexpression Does Not Correlate with Src Activity in Human Carcinoma. Finally, the incidence of Csk overexpression was investigated on Src activity. *In vitro* kinase activity was determined from lysates of tumors relative to histologically normal epithelia from the same patients, as described in Fig. 4*B*. As shown in Fig. 4*C*, a 3- to 10-fold increase in Src activity was observed despite the observed 10-fold increase in Csk level (Fig. 4*B*). Therefore, Csk overexpression may not affect Src activation in human carcinoma in contrast to what has been observed in nontransformed fibroblasts (1). This apparent lack of regulation of Src kinase activity by Csk was observed with positive (patients 22, 32, and 37) as well as with negative patients (see patient 31, as an example), which suggests that autoimmunity against Csk may depend not solely on Csk activity.

DISCUSSION

Autoantibodies against various products of proto-oncogenes and proteins involved in the control of cytoskeleton have been previously reported for patients with cancers. These include the small GTP-binding protein p21Ras (22), the transcription factor L-myc (23), the growth factor receptor Erb2 (16), the eukaryotic translation initiation factor/4γ (eIf-4γ), and the Rho-associated $p160^{rock}$ protein (21). Humoral immune responses to tumor suppressers such as p53 have also been reported (15). Moreover, other tumor antigens of as-yet-unknown functions have also been described (21, 24), such as the recently discovered p62RNA-binding protein (25). Here we show the first example of a humoral response against a cytoplasmic tyrosine kinase. Whereas members of the Src family of tyrosine kinases have been implicated in the development of various carcinomas (3–8), no autoantibodies have been detected against these oncoproteins to date. Rather, we have shown that patients developed antibodies against their negative regulator, Csk, which raises questions about the involvement of Csk during carcinogenesis. In agreement with previous reports, Src was found deregulated in all of the tumors tested, and we found that Csk was overexpressed in most of these transformed tissues. This suggests that Csk cannot phosphorylate Src in transformed cells or that Src is activated despite its phosphorylation by Csk. The recent identification of oncogenic *Src* in advanced carcinoma (10), that were mutated at the COOH-terminal sequence and

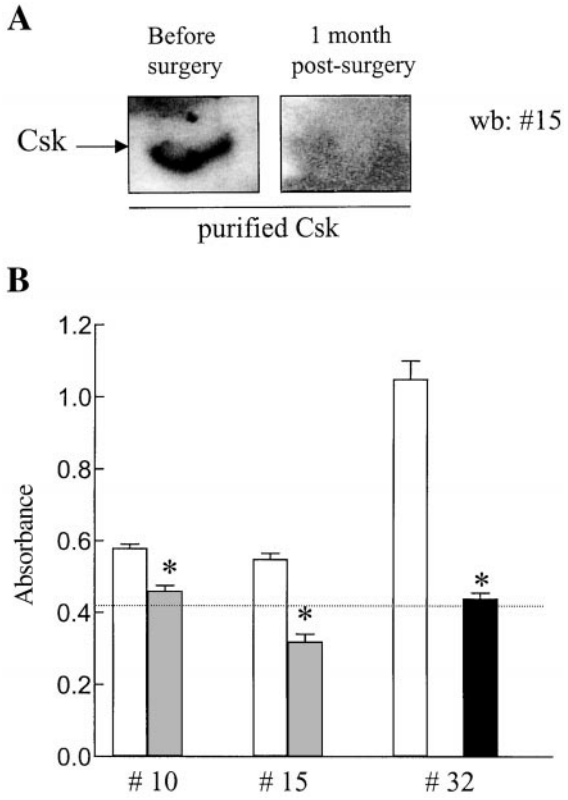

Fig. 3. Csk autoantibodies are reduced postsurgery. *A*, Csk imunoreactivity measured by Western blotting of sera of patient 15 before and 1 month postsurgery as indicated. Western blotting was performed with affinity-purified human Csk that was overexpressed in Cos7 cells as described in Fig. 2*C*, and sera were diluted 1:100 before incubation with the nitrocellulose membrane. Shown are autoradiogramms obtained after 15-s exposure. *B*, levels of Csk autoantibodies measured with an ELISA in sera form three positive patients before surgery and 1–6 months postsurgery as indicated. Patients 10 and 32 are described in Fig. 1, and patient 15 in Table 2. Shown are the $A_{450\ nm}$ values analyzed in ELISA. □, before surgery; ▨, 1 month postsurgery; ■, 6 months postsurgery; ∗, significant at $P < 0.01$, *t* test; *dotted line*, positive threshold value.

Table 2 *Comparison of Csk-autoantibodies with other serological markers in patients with colorectal carcinoma*[a]

Patient no.	Stage[b]	CEA	CA19-9	p53	Csk
1	**A**	**−**	**−**	**+**	**+**
2	A	−	−	−	−
3	B	−	−	−	−
4	B	nd[c]	nd	−	−
5	B	nd	nd	−	−
6	B	−	−	−	−
7	**B**	**−**	**−**	**−**	**+**
8	B	−	−	−	−
9	B	−	−	−	−
10	**B**	**−**	**+**	**−**	**+**
11	B	+	+	−	−
12	B	−	−	−	−
13	B	+	+	−	−
14	B	+	+	−	−
15	**B**	**−**	**−**	**+**	**+**
16	B	−	−	+	−
17	C	−	−	−	−
18	C	−	+	−	−
19	C	+	−	−	−
20	C	nd	nd	−	−
21	C	nd	nd	−	−
22	**C**	**−**	**−**	**−**	**+**
23	C	−	−	−	−
24	**C**	**−**	**−**	**−**	**+**
25	C	+	+	−	−
26	C	+	+	−	−
27	D	+	+	−	−
28	D	−	+	−	−
29	D	−	−	+	−
30	D	+	+	+	−
Total		8	10	5	6

[a] Values relative to markers CEA, CA19-9 and p53 autoantibodies have been previously described (20).
[b] Cancer stages were classified according to Dukes' classification.
[c] nd, not determined.

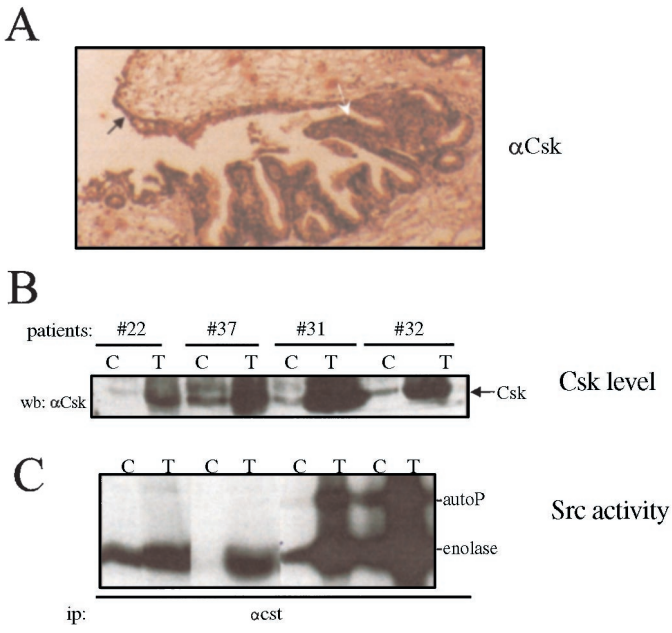

Fig. 4. Overexpression of Csk in carcinoma that exhibit a high Src-like kinase activity. *A*, immunostaining of the biopsy of patient 32 using specific anti-Csk antibody. *White arrow*, the high Csk immunolabeling in the papillary tumor; *black arrow*, the Csk immunolabeling from normal epithelium. *B*, Western blotting (*wb*) of Csk in colon (*#22*) and bladder (*#37*, *#31*, *#32*) tumor (*T*) and the corresponding nontransformed epithelium of the same patient (*C*) from tissue lysates using commercial anti-Csk. Patients 22, 37, and 32 were scored positive and patient 31 was scored negative for Csk autoantibodies. *C*, *in vitro* activity of the Src kinases from the same tissue lysates described in *B*. Src-like kinases were immunoprecipitated (*ip*) with the cst1 antibody, followed by an *in vitro* kinase assay using denatured enolase as an exogenous substrate. The phosphorylated bands corresponding to enolase and the autophosphorylation of the kinase are shown.

still phosphorylated by Csk would favor the later hypothesis; however, such an oncogenic *Src* allele was not detected in the tumor samples that we analyzed (not shown), which suggests the existence of an additional mechanism for Src deregulation in carcinoma. The mechanism by which Csk does not regulate Src in cancer is not known; however, two recent reports identified Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains, a transmembranal Csk-binding protein required for Csk membrane localization and efficient Src down-regulation (26, 27). Therefore, an attractive hypothesis would be that the absence of Src regulation is attributable to an inhibition of Csk-binding protein function in carcinoma.

Another important issue raised by these data are the mechanism by which humoral immunity against Csk is triggered in cancer. One hypothesis would be that an altered and/or overexpressed protein in tumors might provoke the immune system (21). Accordingly, autoantibodies against Csk may arise from the substantial increase in Csk protein levels observed in the tumors, perhaps leading to T-cell activation as observed previously for Erb2 (16) and suggested by the presence of the IgG1 subclass of antibodies. However the fact that Csk was also found overexpressed in tumors of some patients scored negative for Csk immunoreactivity suggests the existence of additional mechanisms. Another explanation involves the presence of mutations within the Csk sequence leading to an alteration of Csk activity and/or function *in vivo*. In agreement with this hypothesis, mutations within the peptidic sequence have been previously observed

for several tumor antigens presenting "altered-self" epitopes (15, 21). We cannot, however, exclude other possibilities such as naturally occurring autoantibodies or peptide mimicry immune responses. The former has been described for several differentiation-specific proteins linking cancer and autoimmune diseases (17). However, this seems unlikely in our case because the levels of autoantibodies against Csk were barely detectable in the normal samples. Peptide mimicry is more conceivable because numerous tumors are known to be caused by viral or bacterial infections. The cytoplasmic tyrosine kinases have oncogenic viral counterparts, and the presence of conserved, normal cellular peptide motifs in the proteins of several viral strains can subvert the host immune responses (28).

Finally, our data suggests that Csk acts as an early tumor antigen because autoantibodies were detected for carcinoma at early stages and for precancerous lesions like colorectal polyps. In contrast, most available markers including specific antigens (CEA, Cyfra 21, CA19–9, CA15–3) or p53 antibodies are detected for carcinoma of late stage. Therefore, detection of Csk autoantibodies may be useful for early noninvasive diagnosis and postoperative follow-up of patients with carcinoma.

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