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25. The following oligonucleotides (5′ to 3′) were used for first strand synthesis (i), PCR (ii), and primer extension (iii), with the calculated melting temperature shown in parentheses. E_β coding: (i) AGGGAACTCTGGGGAGCCACACCAT (64°C); (ii) GGAGAGGAGACACAGGAGTCAGAGG (64°C); (iii) GGAGACACAGGAGTCAGAGGGGAAGGC (68°C). E_β noncoding: (i) TCTCACAAATATCCAGCTGCCTCTG (59°C); (ii) TGAGTGCTGGGATATGAGGCATGGG (62°C); (iii) GCTGGGATATGAGGCATGGGCCAGCAG (67°C). E_α coding: (i) GAACAGCAATGAAGAAAAATCTTAA (51°C); (ii) CTCCAATTGTGGCCATTTTCTTCTT (56°C); (iii) GCCATTTTCTTCTTGGGTGTTTGGTGGG (62°C). E_α noncoding: (i) ATTGGGACAGAAGATGTGTATTTTA (52°C); (ii) GAAATTTTTGTCCTGTTTGTCTACAGCC (56°C); (iii) primer (ii) was also used for primer extension. A_β coding: (i) GATCTGCAGAGCCATCTCTAAGGCA (61°C); (ii) TAATGCCAGTCACCAGGACTCACAC (61°C); (iii) AGGACTCACACCTGCTGTGCCCCCTTC (67°C). A_β noncoding: (i) TACTTCACGCACTTTTCTCTTTAAA (52°C); (ii) CATCCAGTGGGGCTCATGAAGAAAC (61°C); (iii) CCAGTGGGGCTCATGAAGAAACAGACC (64°C). A_α coding: (i) CGAGGACCCCCAGAATCAGAGCTCT (64°C); (ii) GGGAGGTCTCTGCAGCTGCTCTCCT (66°C); (iii) GGGAGGTCTCTGCAGCTGCTCTCCTGAG (68°C). A_α noncoding: (i) CAGAAGCACAAGAGAAAGGGTCTGA (59°C); (ii) CAGAGCCTGCAGCAGGTCCAGGGAG (67°C); (iii) GCCTGCAGCAGGTCCAGGGAGTTCCCC (70°C).
26. No band corresponding to the guanine at position −89 of the noncoding strand of A_β was observed in LMPCR reactions with naked DNA as was expected from the published sequence (*23*). Subsequent sequencing of this region of genomic DNA revealed an adenine at this position, and thus the A_β sequence in Fig. 2 reflects this finding. The guanine at position −70 of the coding strand of A_α was also not detected in LMPCR reactions with naked DNA. This was not due to an error in the published sequence, but rather may reflect some characteristic of the sequence that prevents completion of one of the steps in LMPCR before amplification of the fragments, and thus this fragment is "lost" from the final amplification products. Therefore, we cannot determine whether this residue is protected in in vivo methylated DNA.
27. The following oligonucleotides (5′ to 3′) were used for first strand synthesis, PCR, and primer extension, respectively, for the DR_α coding strand (melting temperature shown in parentheses): TCCACTTATGGCCATTTTCTTCTTG (56°C), TTGGGAGTCAGTAGAGCTCGGGAGT (62°C), and AGAGCTCGGGAGTGAGGCAGAACAGAC (65°C).
28. We thank C. Griscelli, B. Lisowska-Grospierre, M. Eibl, D. Pious, R. Accolla, C. Stiles, and S. Burakoff for cell lines; P. Mueller for advice on in vivo footprinting; M. Boothby and M. Grusby for helpful discussions; and P. Finn and M. Grusby for careful review of the manuscript. Supported by NIH grants GM36864 and A121163 and by the Leukemia Society of America.

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Presence of an SH2 Domain in the Actin-Binding Protein Tensin

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The molecular cloning of the complementary DNA coding for a 90-kilodalton fragment of tensin, an actin-binding component of focal contacts and other submembraneous cytoskeletal structures, is reported. The derived amino acid sequence revealed the presence of a Src homology 2 (SH2) domain. This domain is shared by a number of signal transduction proteins including nonreceptor tyrosine kinases such as Abl, Fps, Src, and Src family members, the transforming protein Crk, phospholipase C–γ1, PI-3 (phosphatidylinositol) kinase, and guanosine triphosphatase–activating protein (GAP). Like the SH2 domain found in Src, Crk, and Abl, the SH2 domain of tensin bound specifically to a number of phosphotyrosine-containing proteins from v-src–transformed cells. Tensin was also found to be phosphorylated on tyrosine residues. These findings suggest that by possessing both actin-binding and phosphotyrosine-binding activities and being itself a target for tyrosine kinases, tensin may link signal transduction pathways with the cytoskeleton.

FOCAL CONTACTS (ALSO CALLED ADhesion plaques) are local, electron-dense regions of plasma membrane that contain vinculin, talin, integrin, fibronectin, actin, and other cytoskeletal components that mediate the attachment of many cultured cells to their substrate (*1*). Stress fibers composed of bundles of actin filaments are often anchored to the membrane in these regions. Growth factors, transforming oncogenes, and tumor promoters affect focal contacts and the organization of actin filaments (*2*). The composition, structure, and dynamics of focal contacts may therefore be influenced by the interaction between signal transduction pathways and the actin cytoskeleton.

Vinculin was initially thought to be the crucial linking factor that anchors actin filaments to the focal contacts. This assumption was based on evidence that certain vinculin preparations possessed actin filament capping and bundling activities (*3*). However, these activities were absent from highly purified vinculin preparations (*4*). Instead, they were found in a contaminating fraction of the cruder vinculin preparations (*5*). Antibodies to these contaminants recognized focal contacts in chicken embryo fibroblasts (CEF), Z-lines of skeletal muscle fibrils, and cultured embryonic heart cells (*5*). On protein immunoblots, the antibodies recognized two immunologically related species with apparent molecular sizes of 150,000 and 200,000, which are distinct from that of talin (*5*). Analysis of peptide maps showed that the 150-kD polypeptide is closely related to the 200-kD polypeptide (*5a*). The 150-kD polypeptide isolated from chicken gizzard, which was shown to interact with actin in vitro (*6*), was used for immunization of rabbits. The resulting polyclonal antibodies recognized focal contacts (*7, 8*) as well as both the 150-kD and 200-kD proteins. These two polypeptides are called tensin for their putative function in maintaining tension in the microfilaments at their point of anchorage.

The polyclonal antibodies against 150-kD tensin were used to screen a λgt11 cDNA library from CEF that contained short in-

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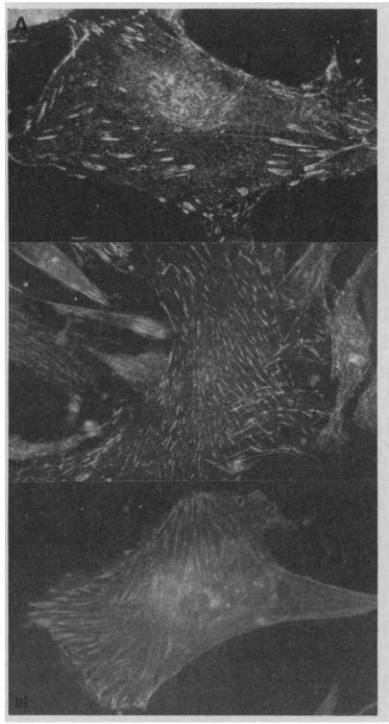
serts (*9*). The insert (200 bp) from a positive clone was then used to probe a CEF cDNA library with larger inserts (*9*), which yielded a clone that contained a 3.5-kb insert. Three lines of evidence indicated the authenticity of this clone: (i) proteins produced from this insert in bacteria were recognized on protein immunoblots by the polyclonal antibodies specific for tensin. By constructing and expressing nonoverlapping fragments of the clone, we demonstrated that the antibodies recognized at least two independent epitopes on the bacterially expressed protein; (ii) in immunofluorescence experiments, focal contacts were recognized by antibodies raised in rabbits to both the bacterially expressed protein and a synthetic peptide that corresponded to a segment of the deduced amino acid sequence (Fig. 1A); and (iii) on protein immunoblots, both of these antibodies recog-



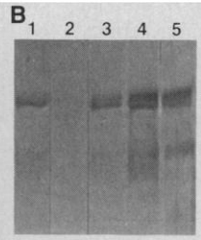


Fig. 1. Verification of authenticity of tensin cDNA using polyclonal antibodies from rabbit. The cDNA insert was cloned into the expression vector pRX (*28*). Fusion proteins were produced in protease deficient bacteria (*28*) and inclusion bodies were purified (*29*). (**A**) Immunofluorescence staining of CEF with antibodies to 150-kD tensin (I), antibodies to bacterially produced cloned protein (II), antipeptide antibodies (III). Fixations were for 2 min at −20°C in methanol (I, II), or acetic acid (5%) in ethanol (III). Cells were incubated at 37°C with antibodies before incubation with rhodamine-conjugated goat antibody to rabbit immunoglobulin G. (**B**) Protein immunoblot analysis of CEF proteins with antibodies to tensin. Proteins extracted from CEF cells were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with affinity-purified antibodies to the peptide alone (lane 1), the same antibodies with peptide (1 μg/ml) (lane 2), the same antibodies with an unrelated peptide (CTHTVDFTFNKFNKSYNGTVD) (1 μg/ml) (lane 3), affinity-purified antibodies against inclusion bodies (lane 4), or affinity-purified antibodies against the 150-kD species of tensin (lane 5). Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

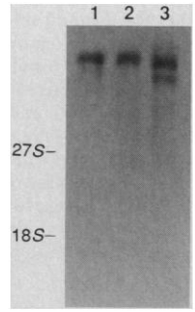


Fig. 2. Analysis of RNA from various chicken tissues. Total cellular RNA was isolated from CEF or from chicken tissues by the guanidinium isothiocyanate method. Samples (10 μg) of RNA were subjected to electrophoresis on agarose gels (1%) that contained formaldehyde, transferred to GeneScreen Plus filters (New England Nuclear), and probed with ^{32}P-labeled tensin cDNA. Molecular sizes were estimated by comparison with the positions of ribosomal RNA and commercial RNA size markers.

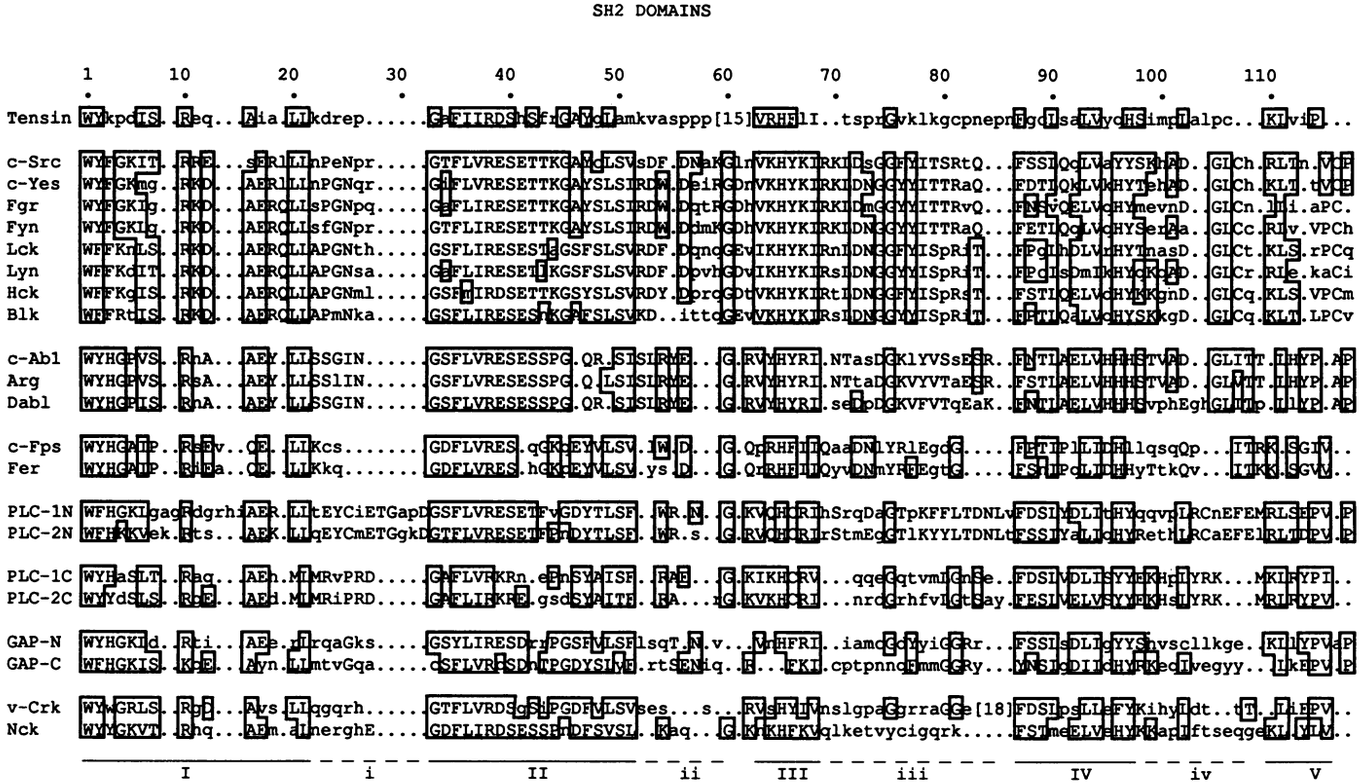


Fig. 3. Alignment of the SH2 domain of tensin sequences with other SH2 sequences. Gaps introduced to improve the alignment of the sequences are indicated by dots. Positions with highly conserved residues are boxed. Sequences were taken from the PIR database.

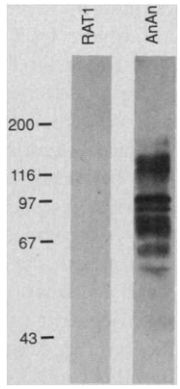


Fig. 4. Binding of phosphotyrosine-containing proteins to the SH2 domain of tensin. A recombinant fusion protein that contained the SH2 domain of tensin and glutathione transferase sequences was synthesized and purified (*30*). Supernatant (1 ml) from untransformed rat fibroblasts (Rat-1) or RSV-transformed rat fibroblasts (AnAn) was incubated with 30 μg of SH2-glutathione transferase fusion protein and 50 μl of beaded agarose conjugated with glutathione (50% slurry in phosphate-buffered saline) for 2 hours at 4°C. The SH2-binding proteins captured on the beads were washed three times with RIPA buffer (*30*), dissociated in SDS-polyacrylamide gel sample buffer (25 mM tris, pH 6.8, 1% SDS, 1 mM EDTA, 0.5% β-mercaptoethanol, and 5% glycerol) and analyzed by protein immunoblotting with a monoclonal antibody specific for phosphotyrosine (*25*). No phosphotyrosine-containing protein was detected when SH2-containing fusion protein was substituted with glutathione transferase in the above experiment.

nized protein bands at 150 and 200 kD, identical in size to the bands recognized by antibodies to tensin itself. Incubation of the antibodies to the peptide with excess peptide specifically inhibited recognition of the blotted protein bands, whereas incubation with an unrelated peptide did not (Fig. 1B).

Northern (RNA) blot analysis from various tissues with the cDNA insert as probe revealed unusually long messages. We estimated that the messages are about 11 kbin CEF and chicken cardiac muscle, and about 10 kb and 8 kb in chicken gizzard (Fig. 2).

The nucleotide sequence of the 3.5-kb insert was determined (GenBank accession number M63606). There was an open reading frame that extended through the first 2.5 kb of the insert, with a coding capacity of ~90 kD. The remaining 1000 bp were noncoding. A computer search of the Protein Identification Resource (PIR) data bank (Dana-Farber Cancer Institute) revealed similarity between residues 573 to 681 of the amino acid sequence and a region called the Src homology domain 2 (SH2) shared by several proteins that function in signal transduction (*10*) (Fig. 3). These include the family of nonreceptor tyrosine kinases (such as Src, Abl, Fgr, Fps, Yes, Fyn, Lck, Lyn, Hck, Blk, Fer) (*11*), the transforming protein Crk (which is similar to the tyrosine kinases but does not contain a catalytic domain for tyrosine phosphorylation) (*12*), phospholipase C–γ1 (PLC-γ1) (*13*), PI-3 kinase, and GAP (*14*). The tensin sequences thus far available showed no similarity with a domain, SH3, which is found in many SH2-containing proteins and in some cytoskeletal proteins.

The SH2 domain is not required for the activity of tyrosine kinases (*10*). However, insertions and deletions in the SH2 domain can reduce or abolish transforming activity of oncogenes (*15*), and other mutations in the domain can activate c-Src (*16*). The observations that PLC-γ1 and GAP (*17*) are substrates for some tyrosine kinase growth factor receptors (*18*), and Crk activates an endogenous cellular tyrosine kinase (*12*) suggested that this region might direct intermolecular interactions with tyrosine kinases. SH2 domains of cytoplasmic tyrosine kinases interact with the adjacent kinase domain to regulate kinase activity (*19, 20*) and are required for the binding of specific substrates (*19, 21, 22*). SH2 domains, but not SH3 domains, have been implicated as binding sites for phosphotyrosine residues. SH2 domains bind tyrosine phosphorylated proteins and these interactions are induced by tyrosine phosphorylation of the ligand (*20, 22, 23*). In addition, SH2-containing cytoplasmic signaling proteins such as Src, GAP, PLC-γ1, and PI-3 kinase have been shown to form stable complexes with autophosphorylated growth factor receptors; the SH2 domains are both necessary and sufficient for these associations (*24*). We therefore explored whether the SH2 domain from tensin would also bind phosphotyrosine-containing proteins.

An expression vector system producing a glutathione transferase fusion protein was used to overexpress a fragment of tensin that contained the SH2 domain in bacteria. The fusion protein was isolated by binding to glutathione-conjugated beads. The SH2-containing fusion protein was then incubated with lysates of either untransformed Rat-1 (fibroblasts) or Rat-1 cells transformed with Rous sarcoma virus (RSV) and glutathione-conjugated beads. The bound proteins were analyzed by protein immunoblotting with a monoclonal antibody specific for phosphotyrosine (*25*). Phosphotyrosine-containing proteins (160 kD, 120 to 130 kD, 95 kD, 90 kD, 70 to 80 kD, 66 kD, and 60 kD) specifically bound to the SH2 domain derived from tensin and their amount was increased in RSV-transformed cells as compared to untransformed cells (Fig. 4). The molecular sizes of these phosphotyrosine-containing proteins are similar to those reported for the tyrosine phosphorylated proteins that bind to the SH2 domains in Src, Crk, and Abl (*20, 22–23*).

Most of the SH2-containing cytoplasmic signaling proteins are also substrates for tyrosine kinases. In tensin, the SH2 region is preceded at its NH_2- terminus by a potential tyrosine phosphorylation site. We therefore examined whether tensin could also be phosphorylated on tyrosine. Tensin from CEF and RSV-transformed CEF was immunoprecipitated with polyclonal antibodies to recombinant tensin and analyzed by protein immunoblotting with a monoclonal antibody specific for phosphotyrosine. In another experiment, phosphotyrosine-containing proteins from CEF and RSV-transformed CEF were immunoprecipitated with the monoclonal antibody to phosphotyrosine and analyzed by protein immunoblotting with antibodies to recombinant tensin. These results (Fig. 5) indicate that tyrosine phosphorylation of tensin occurs and is increased in RSV-transformed cells. Although the consequence of this phosphorylation is unknown, it might effect tensin's cellular localization, binding to actin and other cytoskeletal components, interactions with integrin, growth factor receptors, and other signal transduction proteins, or its sensitivity to proteolysis.

Although other structural proteins such as myosin I (*26*) and the α-subunit of spectrin (*27*) contain SH3-like domains, tensin is the first cytoskeleton-associated protein found to contain an SH2 domain. The SH2 domain may allow tensin to translate tyrosine kinase or other regulatory signals into a direct effect on the structure of the cytoskeleton. By binding to actin filaments and tyrosine phosphorylated proteins, tensin may serve as a nucleus around which signaling complexes can be assembled, and hence,

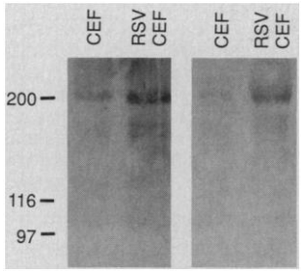


Fig. 5. Tyrosine phosphorylation of tensin. CEF or RSV-transformed CEF were extracted with RIPA buffer as described above. (**A**) Cell lysates were immunoprecipitated with rabbit antibodies to recombinant tensin covalently conjugated to Affi-Gel HX beads (Bio-Rad, Richmond, California). The immunoprecipitates were washed once with RIPA buffer, twice with 10 mM tris buffer (pH 8.0) that contained $LiCl_2$ (0.5 M) and NP-40 (0.5%), once with 50 mM tris buffer (pH 8.0), and analyzed by protein immunoblotting with mouse monoclonal antibody specific for phosphotyrosine. (**B**) Cell lysates were immunoprecipitated with mouse monoclonal antibody to phosphotyrosine covalently conjugated to protein A–Sepharose beads. The immunoprecipitates were washed as above and analyzed by protein immunoblotting with antibodies to recombinant tensin.

link the cytoskeleton with the signal transduction pathways.

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30. A Hinc II restriction fragment of tensin (530 bp from 1547 to 2076) was subcloned into pGEX-3X (*31*). The transformed cells (DH5α) were grown overnight, induced with isopropyl β-D-thiogalactopyranoside (0.1 mM) for 2 hours, washed in PBS, and sonicated. After centrifugation (15,000*g* for 10 min) the supernatant was incubated with beaded agarose (4%) conjugated with glutathione (Sigma) for purification of the fusion protein between glutathione transferase and SH2 domain of tensin. Confluent untransformed or RSV-transformed rat fibroblasts grown in Dulbecco's modified Eagle's medium supplemented with calf serum (10%) (iron-supplemented, HyClone) were lysed in RIPA buffer (2 ml of 1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 25 mM tris, pH 8.0) supplemented with 2 mM diisopropylfluorophosphate, 2 mM phenylmethylsulfonyl fluoride, pepstatin (5 μg/ml), leupeptin (10 μg/ml), 2.5 mM EDTA, and 100 μM sodium vanadate on ice for 20 min and clarified by centrifugation at 16,000*g*.
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Protection Against Malaria by Vaccination with Sporozoite Surface Protein 2 Plus CS Protein

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The circumsporozoite (CS) protein has been the target for development of malaria sporozoite vaccines for a decade. However, immunization with subunit vaccines based on the CS protein has never given the complete protection found after immunization with irradiated sporozoites. BALB/c mice immunized with irradiated *Plasmodium yoelii* sporozoites produced antibodies and cytotoxic T cells against a 140-kilodalton protein, sporozoite surface protein 2 (SSP2). Mice immunized with P815 cells that had been transfected with either SSP2 or CS genes were partially protected, and those immunized with a mixture of SSP2 and CS transfectants were completely protected against malaria. These studies emphasize the importance of vaccine delivery systems in achieving protection and define a multi-antigen sporozoite vaccine.

IMMUNIZATION WITH RADIATION-ATTENUATED sporozoites protects animals and humans against malaria (*1, 2*). Antibodies and cytotoxic T lymphocytes (CTLs) against the CS protein are thought to be primary in mediating this immunity, and immunization of mice with peptides from the *P. berghei* CS protein (*3*) or with *Salmonella typhimurium* transformed with the *P. berghei* CS protein gene (*4*) partially protects against moderate sporozoite challenge. However, no CS subunit vaccine has produced protection against *P. berghei* comparable to that induced by irradiated sporozoites or provided any protection against highly infectious 17XNL *P. yoelii* sporozoites (*5, 6*). This observation suggested that other sporozoite or liver stage antigens contribute to this $CD8^+$ T cell–dependent immunity (7).

A monoclonal antibody (MAb), Navy Yoelii Sporozoite 4 (NYS4), produced by immunization of BALB/c mice with irradiated *P. yoelii* sporozoites, recognizes the surface of *P. yoelii* sporozoites and a 140-kD protein in extracts of sporozoites (*8*); this 140-kD protein is designated SSP2 (*9*). The *P. yoelii* SSP2 gene encodes a protein of 826 amino acids. It has a sequence distinct from that of the CS protein, but, like the CS protein, it has a sequence of six amino acids tandemly repeated multiple times [QGPGAP for the CS protein; NPNEPS for SSP2 (*10*)] and an area with significant similarity to the conserved region II of CS proteins (*9*).

To determine if mice immunized with *P. yoelii* sporozoites produced CTLs against SSP2, we transfected a 1.5-kb fragment of the SSP2 gene into P815 mastocytoma cells ($H\text{-}2^d$) (*11*). This fragment encodes 477 amino acids, including two regions of short, tandemly repeated peptide sequences, PNN and NPNEPS, and the region of similarity to CS region II (*9*). The fragment does not include any sequence similar to the only known CTL epitope on the *P. yoelii* CS protein (*12*). We derived nine different

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