Molecular Cloning of a T Cell-specific Adapter Protein (TSAd) Containing an Src Homology (SH) 2 Domain and Putative SH3 and Phosphotyrosine Binding Sites*

(Received for publication, October 9, 1997, and in revised form, December 16, 1997)

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Adapter proteins link catalytic signaling proteins to cell surface receptors or downstream effector proteins. In this paper, we present the cDNA sequence F2771, isolated from an activated CD8+ T cell cDNA library. The F2771 cDNA encodes a novel putative adapter protein. The predicted amino acid sequence includes an SH2 domain as well as putative SH3 and phosphotyrosine binding interaction motifs, but lacks any known catalytic domains. The expression of the gene is limited to tissues of the immune system and, in particular, activated T cells. The protein expressed by F2771 cDNA in transfected COS cells is localized in the cytoplasm. A polyclonal antiserum raised against an F2771-encoded peptide reacts with a tyrosine-phosphorylated 52-kDa protein expressed in phytohemagglutinin-stimulated peripheral blood mononuclear cells. The gene is localized to chromosome 1q21, a region often found to be aberrant in lymphomas. The T cell-specific expression and the rapid induction of mRNA expression upon receptor binding, as well as the lack of catalytic domains in the presence of protein interaction domains, indicate that the F2771 gene encodes a novel T cell-specific adapter protein (TSAd) involved in the control of T cell activation.

Activation of T cells involves interaction of the T cell antigen receptor (TCR)1 with complexes of peptides and major histocompatibility complex class I or II molecules. Depending on the peptide and the antigen-presenting cell, the initial contact of the TCR with the complex of peptide bound to major histocompatibility complex molecules may lead to activation, anergy, or T cell apoptosis (1). The transduction of signals from the cell membrane to the nucleus is mediated by transient interactions between various signaling proteins, among which are tyrosine kinases, phosphatases, and adapter proteins without catalytic function. The signaling process is complex and still not well understood (2).

Phosphorylation and dephosphorylation events act as triggers for transient interaction between the various signal transducing proteins. Recognition of specific protein sequences containing phosphorylated tyrosines is mediated by particular conserved modular binding domains, such as the Src homology 2 (SH2) domain (3, 4) and the phosphotyrosine binding (PTB) domain (5). Signal transducing proteins may also have binding domains, which recognize structures that do not undergo enzymatic changes during signal transduction, such as the Src homology 3 (SH3) domain. SH3 domains bind to proline-rich structures like the cytoskeleton. Additionally, SH3 domains of cytoplasmic enzymes may aid in the recruitment of specific substrates for the enzyme (3). Not all signal transducing proteins have catalytic functions. Some proteins, like Slap (8), Crk (9), Sch (10), Nck (11), Lnk (12), and Grb2 (13), function as adapters, linking catalytic signaling proteins to receptors at the cell membrane or to downstream effectors like Ras (14).

We initiated experiments to identify genes that are specifically expressed in activated CD8+ T cells using a subtractive strategy. By using this approach, we have isolated and characterized a novel gene that is strongly expressed in T cells shortly after activation. The gene continues to be expressed in long term cultures of activated T cells. We propose that this protein, due to the presence of an SH2 domain and putative SH3 and PTB binding motifs, is an adapter molecule involved in protein-protein interactions during T cell activation. This novel protein may be termed TSAd, for T cell SH2 domain-containing adapter protein.

EXPERIMENTAL PROCEDURES

Antisera—A polyclonal rabbit antiserum was raised against a synthetic peptide (AEEVPEGSLFLQAETRAWF) derived from amino acid (aa) position 59–78 of the deduced F2771 polypeptide by immunizing rabbits with peptide conjugated to keyhole limpet hemocyanin. Anti-TCR mAb T10B9 was obtained as a gift from J. S. Thomson, University of Kentucky Medical Center, Lexington, KY. Biotinylated goat anti-rabbit IgG and biotinylated goat anti-mouse IgG were obtained from Southern Biotechnology Associates (Birmingham, AL), and QIAexpress MROHisIgG was obtained from Qiagen (Chatsworth, CA). Anti-p56c-ko antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For detection of phosphotyrosine, the monoclonal antibody 4G10 (Upstate Biotechnology, Inc.) was used.

Preparation and Activation of Lymphocytes—Peripheral blood mononuclear cells (PBMC) were obtained from citrate-anticoagulated blood oruffy coats of healthy blood donors by Isopaque-Ficoll (Nycomed AS, Oslo, Norway) gradient centrifugation. Positive selection of cells fromuffy coats or PBMC using superparamagnetic Dynabeads (Dynal, Oslo, Norway) coated with mAb specific for CD4, CD8, or CD19 has been
described previously (15–17). The cells were lysed directly after isolation, or the beads were detached from the cells by incubation for 1 h at 37°C with goat anti-Fab antiserum (Detachabead, Dynal) (18). Cell subsets thus isolated contained <2% contaminating cells as assessed by flow cytometry and showed >95% viability as determined by acridine orange/nitroblue tetrazolium staining.

CD8+ cells were left overnight in RPMI supplemented with 10% pooled human serum at 37°C, 95% relative humidity and 5% CO2 before being activated by incubation with anti-TCR mAb TIB09 coated onto Dynabeads and interleukin-2 (10 units/ml). The cells were harvested after 72 h, and 30–100 × 10^6 cells were frozen as cell pellets at −70°C.

For mRNA expression studies, CD8+ and CD8+ T cells were activated with anti-TCR, tetradeacetyl phorbol acetate, or phytomyagglutinin (PhA) (19). B cells were activated with tetradeacetyl phorbol acetate. Cells were harvested at 3 days, or at different time points as shown under “Results.”

Cell Lines—Cos-1 cells (CRL-1650) (American Type Culture Collection [ATCC], Rockville, MD) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.). Jurkat (TIB-152), MOLT-3 (CRL-1552), KT-1, RAMOS (CRL-1596), THP-1 (TIB-202), and K-562 (CCL-243) cell lines (ATCC) were cultured in RPMI with 10% fetal calf serum. EBV-transformed B cell lines from healthy donors were established in our laboratory. The presence of mycoplasma was ascertained by the use of an ATCC mycoplasma PCR detection kit (ATCC).

cDNA Library—A cDNA library in the pcDNA1 plasmid vector was custom made by Invitrogen (San Diego, CA) from 100 × 10^6 CD8+ T cells activated 3 days previously with anti-TCR coated Dynabeads as described above. cDNA synthesis was performed with a mixture of oligo(dt) primer and random priming. The cDNAs smaller than 800 bp was removed. The resulting cDNA library contained 2.23 × 10^6 primary recombinants.

RNA Preparation—Poly(A)+ RNA was isolated directly from 50–70 × 10^6 lysed cells using 5 mg of Dynabeads oligo(dt) (Dynal) as described previously (20). Seven to 10 µg of mRNA were obtained from 50–70 × 10^6 cells (lymphoid cell lines).

Total RNA was extracted from fresh or frozen pellets of 50 × 10^6 cells by the guanidine thiocyanate method (21).

Subtraction Probe—Preparation of a subtraction probe was performed as described previously in detail (22). Briefly, 10 µg of mRNA was isolated from Jurkat cells (subtractor) using oligo(dt)20, as described above and first strand cDNA was synthesized while mRNA was radiolabeled and used as a probe for library screening. This PCR product was purified using a DNA blotting kit (Amersham, UK). Alternatively, immunoprecipitation of proteins from the cell lysates was performed using the indicated antibodies and Sepharose A 4B beads. The presence of the correct sequence was verified by sequencing.

Sequence Analysis—Data base searches were performed using the BLAST (National Center for Biotechnology Information) and Fasta and pfscan programs from the GCG program package (27).

2 Protein sequence analysis programs are also available via the World Wide Web (http://expasy.hgcuge.org/www/tools.html).

The pfscan program is available via the World Wide Web (http://ulrec3.unil.ch/software/profilescan.html).
tein motifs was also performed using the compilation of protein sequence analysis programs at the UK HGMP Resource Center, Cambridge, UK. The alignment of multiple SH2 domains was performed using the pfscan program.

RESULTS

Isolation of F2771, a Novel Human cDNA Clone Encoding a Polypeptide Containing an SH2 Domain—Using a subtractive approach.

**FIG. 1.** **A** F2771 nucleotide and deduced amino acid sequences. A, the translation of the F2771 sequence is shown below the nucleotide sequence. Nucleotide and amino acid positions are indicated on the left of each lane. The ATG171 representing an alternate start site and the putative poly(A) addition signal AATAAA at position 1554 are underlined. The SH2 domain is underlined, and proline-rich sequences are stippled. An arrow (↓) indicates the position of a 10-aa insertion (RVRPPLSVTH) found in one cDNA clone (F2771–45). Prolines and arginines separated by two amino acids are marked in bold. Tyrosine residues residing within an NPXY motif is double underlined. Filled circles (●) indicate six putative Ser/Thr phosphorylation sites. An N-glycosylation site is indicated with open diamonds (◊). The cDNA sequence is available through GenBank (accession no. AJ000553). B, alignment of the SH2 domain of F2771 (aa 94–186) with the SH2 domains of other proteins. The sequences of the SH2 domain proteins are taken from the SwissProt database. Boxes are drawn by the PrettyPlot program in the extended GCG program package (27). C, schematic presentation of the F2771 protein.
strategy, we isolated a novel cDNA clone, F2771, derived from anti-TCR activated human CD8+ T cells. Initial Northern blot analysis showed that the F2771 cDNA hybridized to a single band of approximately 1700 bp, expressed in activated CD8+ cells and not in the Jurkat cell line used as subtractor, thus confirming the efficiency of the subtraction (data not shown).

The entire F2771 cDNA sequence was found to consist of 1592 bp, excluding the nucleotide polyadenylate stretch (Fig. 1A). A common AAUAAA polyadenylation signal is localized at position 1556. The F2771 sequence has an open reading frame.

FIG. 2. Chromosomal localization of the F2771 gene; chromosomal localization performed by FISH. The idiogram and the chromosome 1 counterstained with 4,5-diamino-2-phenyleindole (a) and propidium iodide (b), respectively, illustrate the chromosomal localization at 1q21. Arrowheads indicate chromosomes 1 with a fluorescent signal at 1q21 band on a partial normal metaphase counterstained with propidium iodide (c).

Fig. 3. Expression of F2771 in different human tissues. Panel A represents commercial tissue blots (multiple human tissue blot 1 and 2, CLONTECH). Each lane shows hybridization to 2 μg of mRNA of the indicated tissue. Panel B represents a Northern blot of 1 μg of mRNA/lane from positively selected B cells, CD4+ cells, and CD8+ cells using Dynabeads oligo(dT)25.

chromosome 1

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from position 2 to position 1254. Attempts to identify a transcription start site upstream of the obtained F2771 sequence, either by primer extension analysis of RNA or PCR amplification of the 5’ F2771 ligated to the pcDNA1 vector, failed to reveal a more 5’ start site (data not shown). Thus, the first putative initiation codon is at nucleotide 87, within a sequence (GCTCTCATGG) that shows moderately good similarity to the Kozak translation initiation consensus sequence (XCGCCATGG; Ref. 28). This putative initiation codon yields a predicted polypeptide chain of 389 aa with a predicted molecular mass of 43 kDa. Two other F2771 hybridizing cDNAs representing two different alternate splice variants of the F2771 gene were isolated. One of these cDNAs may employ a different start site at nucleotide 171 (ACAGACATGA) yielding a polypeptide of 361 aa. The other cDNA includes a 30-bp insertion at position 399, yielding a predicted polypeptide of 399 aa. The F2771 sequence showed only low overall homology to any genes or gene families. However, several expressed sequence tags (29) with high degree of similarity to F2771 were identified in the EST (expressed sequence tag) Data Bank.

The predicted polypeptide of F2771 contains a putative SH2 domain, which is located at aa positions 95–186 (Fig. 1, A and B) (30, 31). In the C-terminal part of the F2771 sequence (aa 239–389), the motif RPKPXP is repeated twice, and there are also several other repeats of ((R/P)XX)X. These motifs may represent binding sites for SH3 domains (32). Two of the 10 tyrosines of the F2771 sequence are found within an NPXY motif, i.e. Tyr-216 and Tyr-280 (Fig. 1A). When phosphorylated on tyrosine, NPXY motifs may represent binding sites for PTB domains (33). A schematic representation of the F2771 cDNA is given in Fig. 1C.

The F2771 Gene Is Localized to Chromosome 1q21—By using a monochromosomal cell hybrid DNA panel and F2771-specific primers, we localized the F2771 gene to chromosome 1 (data not shown). These data were further confirmed by FISH analysis. Fluorescent signals were observed in the 1q21 position (Fig. 2) on 20 human metaphases from two unrelated healthy donors (one male and one female), respectively.

The F2771 Gene Is Abundantly Expressed in Lymphoid Tissue—The initial Northern blot analysis demonstrated that the F2771 cDNA hybridized to a single band of approximately 1700 bp, expressed in activated CD8+ cells but not in the T leukemic cell lines Jurkat. When examining the tissue distribution, the F2771 gene was found to be expressed in peripheral blood leukocytes (PBL), thymus, and spleen (Fig. 3A). Furthermore, we found that the F2771 gene was expressed in tetradeanoyl phorbol acetate-activated CD4+ and CD8+ cells, but not in resting CD4+ and CD8+ T cells, nor in B cells (Fig. 3B). The resting cells were obtained by positive selection from buffy coats using anti-CD4, anti-CD8, or anti-CD19 Dynabeads, respectively, and directly lysed after a 30-min incubation at 4 °C with the beads. Several lymphoid cell lines (the KT-1 and MOLT-3, the B lymphoma cell line Ramos, the erythroleukemia cell line K 562, and the myelocyte cell line THP-1), as well as the monkey fibroblast cell line COS-1, did not express F2771 mRNA as demonstrated by Northern blot analysis (data not shown).

When probing 105 cDNA clones from the cDNA library of activated CD8+ T cells using the initially isolated F2771 cDNA as a probe, we isolated 16 different F2771 clones. Thus, we were able to estimate the frequency of F2771 expression to be higher than 1/10,000 transcripts.

The Expression of the F2771 Gene Is Rapidly Induced after Activation of T Cells—Exposure of freshly isolated PBM to the plastic of culture bottles activate monocytes and thereby possibly T cells (34). We observed that after a 2-h incubation of PBM on plastic at 37 °C to remove adherent cells, the F2771 gene was already induced to maximal mRNA expression in the nonadherent fraction of PBM incubated in unstimulated culture in a plastic bottle for 2 h at 37 °C (labeled 2 h PBM). Freshly isolated PBM (0 h PBM) and PBM stimulated with PHA and interleukin-2 for 6 days are included as controls. Panel B represents CD4+ T cells positively selected with immunomagnetic beads covered with anti-CD4. Positively selected cells were incubated with beads present at 37 °C for the indicated time points, whereupon RNA was extracted. Panel C represents F2771 expression in positively selected CD4+ or CD8+ cells, detached from the beads and incubated for 24 h at 37 °C (lanes labeled 0 h CD4-cells or 0 h CD8-cells). These cells were then activated with anti-TCR-coated immunomagnetic beads, and RNA was extracted at the indicated time points. Right part of panel C shows expression of F2771 gene in long term cultures of PBM or CD8+ T cells activated with PHA or anti-CD3 cross-linking. Number of days in culture is indicated above each lane.

By the use of “analysis and prediction of protein sorting signals” (PSORT; Ref. 35), the F2771 protein was found to lack a hydrophobic
signal sequence, transmembrane regions and nuclear localization signals. This indicates that the F2771 protein is an intracellular protein.

To verify this prediction, three cDNA clones (F2771–19, F2771–21, and F2771–45) encoding F2771 polypeptides of 361, 389, and 399 aa, respectively, were modified to encode a C-terminal histidine tag (MRGS-His6). Microscopy of COS-1 cells transiently transfected with these constructs, visualized using an anti-His-specific antibody and the alkaline phosphatase anti-alkaline phosphatase technique, showed staining of the cytoplasm and not the nuclei of positive cells (Fig. 5). This observation supports the prediction that the F2771 protein is an intracytosolic protein.

An F2771-specific Antiserum Identifies a Tyrosine-phosphorylated 52-kDa Protein in Cell Lysates from PHA-stimulated PBM—A polyclonal rabbit antiserum was raised against a peptide derived from aa 56 to aa 78 in the predicted F2771 polypeptide sequence. In Western blots of cell lysates, this antiserum detected one protein band at 52 kDa expressed in PHA blasts and freshly isolated PBM, but not in Jurkat, KT-1, MOLT-3, or K562 leukemic cell lines, or in EBV-transformed B cells (Fig. 6A). The 52-kDa protein could be detected by Western blot in freshly isolated CD4+ and CD8+ T cells (Fig. 6B). Protein expression was increased in cells left overnight in unstimulated culture (cells labeled 0 h CD4-cells and 0 h-CD8 cells). A further increase in protein expression was observed in these T cells after 1 day of PHA stimulation (Fig. 6B). This corresponds to the observed changes in F2771 mRNA expression in positively selected CD4+ and CD8+ T cells treated in a similar way (Fig. 4C).

The F2771 antiserum could immunoprecipitate a 52-kDa protein from lysates of PHA blasts (Fig. 7A). F2771 immunoprecipitates from lysates of PBM stimulated with PHA for 1 and 2 days reacted with an anti-phosphotyrosine antibody. However, phosphorylation of the immunoprecipitated protein was also observed in lysates from freshly isolated PBM (Fig. 7B).

Attempts to coprecipitate the p56lck protein from lysates of PHA blasts, using the anti-F2771 antiserum or vice versa using an antiserum against the p56lck protein, failed to reveal an association between the two proteins (Fig. 7A and data not shown).

DISCUSSION

We have identified and characterized a novel human cDNA (F2771) encoding a putative adapter protein with T cell-specific expression. The F2771 cDNA encodes a protein with predicted molecular mass of 43 kDa, including an SH2 domain, as well as putative SH3 and PTB interaction motifs. The polypeptide sequence contains no homology to known catalytic domains.

The presence of an SH2 domain in the F2771 sequence indicates that the F2771 protein is involved in phosphotyrosine-dependent protein interactions. SH2 domains are conserved regions of approximately 100 aa, found in many cytoplasmic signaling molecules (3, 31). Tyrosine phosphorylation acts as a switch to induce binding of SH2 domains, thereby mediating
arginine that is crucial for phosphotyrosine binding (37). Thus, SH2 domains described so far, contains at position 120 an arginine that is crucial for phosphotyrosine binding (37). Thus, SH2 domains described so far, contains at position 120 an arginine that is crucial for phosphotyrosine binding (37). Thus, SH2 domains described so far, contains at position 120 an arginine that is crucial for phosphotyrosine binding (37).

Thus, the putative SH2 domain region in the F2771 sequence probably forms a functional phosphotyrosine binding domain. However, we have isolated one variant cDNA, where a 10-aa insertion is included in the SH2 domain at aa position 102 (Fig. 1A). This insertion may distort the structure of the SH2 domain, thus rendering the domain dysfunctional.

The deduced F2771 protein displays other sequences that may mediate protein-protein interactions. Many signaling proteins, with or without catalytic function, contain SH3 domains (2), which are conserved regions of 60–85 aa residues involved in cell polarization and in subcellular localization of proteins (38). The consensus SH3 binding motif is PXXP, with different SH3 domains having different preferences for particular sequence motifs (3, 32). Arginine at position +3 or –3 from the PXXP motif also seems to be part of the SH3 binding motif (32). The C-terminal proline-rich F2771 sequence contains, in addition to eight PXXP motifs, seven RXXP or PXXR motifs (Fig. 1A). Furthermore, the motif RPKPXXP is found twice in the F2771 sequence. Thus, it is highly probable the F2771 protein contains binding sites for SH3 domains.

Two NPYX motifs are found in the F2771 sequence. Phosphorylated NPYX have been found to be bound by the PTB domains of Shc and insulin receptor substrate-1 (33). We observed that the F2771 protein is tyrosine-phosphorylated in vivo. If the tyrosines of the NPYX motifs of the F2771 sequence are phosphorylated, these motifs could interact with PTB domain-containing proteins.

The expression of F2771 at the mRNA level was extensively studied. The gene is not expressed in normal resting or activated B cells, nor in several transformed cell lines and various tissues tested. However, mRNA expression was observed in cells derived from thymus or spleen. While the F2771 gene was expressed at low levels in unstimulated T cells, it was rapidly induced in T cells after triggering through the CD4, CD8, or the CD3 molecules, and also shortly after exposure to adhesion activated monocytes. This rapid induction of mRNA expression could indicate that the gene is involved in the early stages of T cell activation. However, the gene continued to be expressed in long term cultures of activated T cells, indicating a functional role also in the later stages of T cell activation.

Proteins involved in signal transmission in T cells may be expressed mainly or exclusively in T cells, but the majority are also expressed in other tissues. The tyrosine kinase ZAP-70 are mainly expressed in T cells (39), whereas the p56\textsuperscript{Lck} and the p59\textsuperscript{Lyn} kinases are also found in B cells (40, 41) and the central nervous system (42, 43). Several adapter proteins have been reported to have lymphocyte-specific expression. Lnk, containing an SH2 domain and a tyrosine phosphorylation site, is

**Fig. 6.** Expression of the F2771 protein in lymphoid cells. A, Western blot of cell lysates derived from PHA blasts, the T leukemic cell lines Jurkat, K1, MOLT-3, EBV-transformed B cells, the erythroleukemic cell line K562, and freshly isolated PBM. The blot was stained with a polyclonal rabbit antiserum specific for a F2771 peptide. Position of 52 kDa band is indicated with an arrow. B, Western blot of lysates from positively selected CD4+ and CD8+ T cells lysed directly after isolation, lysed after 1 day in unstimulated culture (labeled 0 h CD4 or CD8), and after 1 day of PHA stimulation. The blot was stained with a polyclonal rabbit antiserum specific for a F2771 peptide.
expressed in murine T cells (44). SKAP55 (45) and FYB (46) are adapter proteins that associate with p56lck and that are mainly expressed in human T cells. Based on the structural features of the F2771 sequence and the pattern of expression, we suggest that the F2771 protein is an adapter protein involved in T cell signaling.

F2771 mRNA expression is rapidly induced upon triggering of the CD4 and CD8 receptors. Thus, a possible candidate with which the F2771 protein might interact is the p56lck tyrosine kinase, since p56lck is associated with these coreceptors (47). However, we were not able by immunoprecipitation studies to demonstrate an association between the p56lck protein and the F2771 protein in PHA blasts. This does not exclude the possibility that the F2771 protein could play a role in the CD4-p56lck-dependent signal transduction pathway.

We found by FISH analysis that the F2771 gene is located on chromosome 1q21. Aberrations of chromosome 1 with breakpoint at 1q21-q22 have repeatedly been found in Hodgkin’s lymphomas (48). Amplification of 1q21-q22 has been found in lymphomas (48). Amplification of 1q21-q22 has been found in Hodgkin’s lymphoma (49). Points at 1q21-q22 have repeatedly been found in Hodgkin’s lymphoma. Amplification of chromosome 1 with breakpoints at 1q21 has been observed in CD4-p56lck.

We failed to demonstrate expression of the F2771 gene, either at the mRNA or at the protein level, in several lymphoid cell lines derived from different lineages. The absence of expression, the sequence structure and the probable cytoplasmic localization suggest that F2771 encode a T cell-specific adapter protein. Thus we propose that the F2771 protein be termed T cell SH2 domain-containing adapter protein (TSAd).

Acknowledgments—Arne Deggerdal, Finn Eirik Johansen, Guttorm Haraldsen, and Ludvig M. Sollid have contributed to this work with A. Forus, personal communication.

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Anne Spurkland, Jan E. Brinchmann, Gunnar Markussen, Florence Pedeutour, Else Munthe, Tor Lea, Frode Vartdal and Hans-Christian Aasheim

J. Biol. Chem. 1998, 273:4539-4546.
doi: 10.1074/jbc.273.8.4539

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