In human T-lymphocytes the Src family protein tyrosine kinase p59(fyn) associates with three phosphoproteins of 43, 55, and 85 kDa (pp43, pp55, and pp85). Employing a GST-Fyn-Src homology 2 (SH2) domain fusion protein pp55 was purified from lysates of Jurkat T-cells. Molecular cloning of the pp55 cDNA reveals that the pp55 gene codes for a so far nondescribed polypeptide of 359 amino acids that comprises a pleckstrin homology domain, a C-terminal SH3 domain, as well as several potential tyrosine phosphorylation sites, among which one fulfills the criteria to bind Src-like SH2 domains with high affinity. Consistent with this observation, pp55 selectively binds to isolated SH2 domains of Lck, Lyn, Src, and Fyn but not to the SH2 domains of ZAP70, Syk, Shc, SLP-76, Grb2, phosphatidylinositol 3-kinase, and e-abl in vitro. Based on these properties the protein was termed SKAP55 (src kinase-associated phosphoprotein of 55 kDa). Northern blot analysis shows that SKAP55 mRNA is preferentially expressed in lymphatic tissues. SKAP55 is detected in resting human T-lymphocytes as a constitutively tyrosine phosphorylated protein that selectively interacts with p59(fyn). These data suggest that SKAP55 represents a novel adaptor protein likely involved in Fyn-mediated signaling in human T-lymphocytes.
MATERIALS AND METHODS

Cells and Antibodies—Jurkat cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (Sigma), 1% penicillin-streptomycin, and 2% gluatin (Life Technologies, Inc.), in 7% CO₂, humidified atmosphere at 37 °C. Resting human T-lymphocytes were prepared from heparinized blood of healthy donors as described previously (23). The anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY) and used at 1:100 (v/v) dilution in immunoprecipitation experiments.

Cloning of SKAP55, a p59 fyn-associated Protein—Large scale purification of proteins interacting with the SH2 domain of Fyn was performed using a GST-Fyn-SH2 fusion protein as described previously (20). Pre-cipitated proteins were separated by two-dimensional gel electrophoresis and subjected to amino acid microsequencing.

Degenerate oligonucleotides corresponding to the sequenced peptides (namely, 5′-AARGGNGNCARGARCTNGA-3′ and 5′-TTTYGCCAYT-CRCTNCRAA-3′) were synthesized and used in polymerase chain reactions to amplify Jurkat cells cDNA. The resulting fragment was sequenced and used further as a probe to screen a λ ZAP cDNA library derived from human blood (Stratagene). The DNA sequence of the positive clones was determined by standard DNA sequencing of both strands.

RESULTS AND DISCUSSION

In an attempt to identify proteins that interact with p59<sup>fn</sup>, Nonidet P-40 lysates of freshly prepared resting human T-lymphocytes were subjected to immunoprecipitation using specific antisera directed against p59<sup>fn</sup> and p56<sup>fyn</sup>. The proteins that co-precipitate with each kinase were subsequently resolved by two-dimensional gel electrophoresis and subjected to radiolabeling in vitro kinase assays. A polyclonal antiserum directed against SKAP55 was used in immunoblot analysis. The sequence of the peptides obtained by microsequencing is shown in bold. The deduced amino acid sequence of pp55 contains a SH3 domain (boxed), a SH2 domain (double-lined), and several phosphorylation sites (circled). Amino acid sequences homologous to Src-like SH2 domains binding sites are marked with a dashed line.

Northern Blot Analysis—Two Northern blot filters containing approximately 2 μg of poly(A)<sup>+</sup> RNA/lane from various human tissues (CLONTECH) were hybridized with a radiolabeled probe corresponding to nucleotides 298–325 of clone 5.2 according to the manufacturer’s procedures.

Immunoprecipitation, in Vitro Kinase Assay, and Immunoblot Analysis—Freshly prepared human resting T-lymphocytes (40 × 10<sup>6</sup>) were lysed in Nonidet P-40 lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 10 mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) at 4 °C for 1 h. Post-nuclear lysates were subjected to anti-p56<sup>fyn</sup> or anti-p59<sup>fn</sup> immunoprecipitation and when necessary subjected to in vitro kinase assay as described previously (20). Alternatively, SH2 domains from various signaling molecules expressed as GST fusion protein were used (20). Precipitated proteins were released using 1% Triton X-100 lysis buffer supplemented with 8 μM urea and separated by means of two-dimensional gel electrophoresis or by conventional SDS-10% PAGE. Western blot analysis was performed using standard procedures that have been described elsewhere (20).

RESULTS AND DISCUSSION

In an attempt to identify proteins that interact with p59<sup>fn</sup>, Nonidet P-40 lysates of freshly prepared resting human T-lymphocytes were subjected to immunoprecipitation using specific antisera directed against p59<sup>fn</sup> and p56<sup>fyn</sup>. The proteins that co-precipitate with each kinase were subsequently resolved by two-dimensional gel electrophoresis and subjected to
Cloning of SKAP55, a p59<sup>brn</sup>-associated Protein

**Fig. 3. Constitutive tyrosine phosphorylated pp55 is an integral part of the fyn complex.** Immunoprecipitation was performed on lysates of resting T-lymphocytes using anti-pp55 (A and B) or anti-pp56<sup>brn</sup> (C and D) antibodies. Proteins were detected by two-dimensional Western blotting with anti-phosphotyrosine (A and C) or anti-pp55 (B and D) antibodies. Blots shown in B and D were obtained after stripping of those presented in A and C, respectively. IEF, isoelectric focusing.

Fig. 1A demonstrates that under these experimental conditions three tyrosine-phosphorylated proteins with apparent molecular masses of 43, 55, and 85 kDa are detectable in the Fyn immunoprecipitate. The same proteins are phosphorylated in an *in vitro* kinase assay of a Fyn immunoprecipitate prepared from resting T-cells (Fig. 1B). In addition, the 55- and 85-kDa proteins are also precipitated by a recombinant Fyn-SH2 domain from Nonidet P-40 lysates of Jurkat cells (Fig. 1C), suggesting that their interaction with Fyn could be mediated via the SH2 domain of the kinase. In contrast to Fyn, the anti-Lck precipitate consists of a unique protein spot that corresponds to p56<sup>brn</sup> (Fig. 1D). Furthermore, none of the Fyn-associated phosphoproteins was found to associate with the third Src PTK expressed in T-lymphocytes, p62<sup>brn</sup> (not shown). These data suggest a selective association between Fyn and tyrosine phosphorylated pp43, pp55, and pp85 and further corroborate the idea of differential compositions of the Fyn and Lck complexes in human T-lymphocytes as suggested by others and us (20, 21, 26, 27).

Because none of the Fyn-associated proteins described above was identified by antibodies or antisera directed against known signaling molecules expressed in human T-lymphocytes, we attempted their purification and molecular characterization. To this end a GST-Fyn-SH2 fusion protein coupled to glutathione-Sepharose beads was employed to precipitate tyrosine phosphorylated polypeptides from lysates of pervanadate-treated Jurkat T-cells. Whereas the amounts of pp43 and pp85 were below the level of detection under these conditions, high amounts of the 55-kDa Fyn-associated protein (pp55) were obtained. The Coomassie spots corresponding to pp55 were excised from the gels, digested with endoproteinase-Lys-C, purified by HPLC, and subjected to microsequencing. Amino acid sequence was obtained from two peptides (Fig. 2).

Combinations of degenerate oligonucleotides deduced from these two peptides were used in polymerase chain reactions on Jurkat cDNA and led to the isolation of a 100-base pair fragment. This fragment was used to screen a λ ZAP cDNA library from human blood, which resulted in the identification of several cDNA clones. Fig. 2 shows the DNA sequence, determined from both strands, of the longest clone (clone 5.2). DNA sequencing of the remaining clones indicated that they were derived from the same gene as clone 5.2. The cDNA sequence was further confirmed by sequencing the products resulting from 5′ and 3′ rapid amplification of cDNA ends experiments performed on human leukocyte cDNA (not shown).

As shown in Fig. 2, clone 5.2 contains a 1542-base pair cDNA insert with an open reading frame that starts at nucleotide 71 and terminates at nucleotide 1150. The cDNA sequence codes for a 359-amino acid polypeptide with a calculated molecular mass of 41.3 kDa, which also contains the two peptides that were identified by microsequencing of purified pp55. Transfection of Jurkat cells with pp55 cDNA resulted in the overexpression of a 55-kDa protein that co-migrates with the endogenously expressed pp55 (not shown), suggesting that conformational effects and/or post-translational modifications could influence the mobility of pp55 in SDS-PAGE. Using a polyclonal pp55 antiserum, a constitutively tyrosine phosphorylated protein of 55 kDa is precipitated from lysates of resting human T-lymphocytes (Fig. 3, A and B). Perhaps more importantly, analysis of a Fyn immunoprecipitate by means of anti-pp55 Western blotting revealed that the 55-kDa phosphoprotein that co-precipitates with pp55<sup>brn</sup> in resting T-lymphocytes exactly co-migrates with pp55 (Fig. 3, compare C and D). Collectively these data demonstrate that clone 5.2 represents a full-length cDNA clone coding for the Fyn-associated pp55 molecule.

Comparison of the predicted amino acid sequence of pp55 with all available data bases showed that the pp55 cDNA encodes a so far not described protein that contains a PH domain (residues 106–205), a C-terminal SH3 domain (residues 300–356), and several putative phosphorylation sites (Fig. 2). Importantly, one potential tyrosine phosphorylation site is part of the peptide sequence <sup>EDIYEVLPY</sup><sup>274</sup>, which would be predicted to bind to isolated SH2 domains of Src PTKs with high affinity based on its homology with the EXXYXXL motif (28).

To further assess this possibility we investigated the capacity of SH2 domains derived from p56<sup>brn</sup>, p59<sup>brn</sup>, p60<sup>brn</sup>, PI 3-kinase, ZAP70, Syk, c-abl, Shc, SLP-76, and Grb2 to bind to endogenously expressed pp55. To this end the individual SH2 domains were expressed as GST fusion proteins, coupled to glutathione-Sepharose beads, and incubated with a Nonidet P-40 lysate of Jurkat T-cells. Bound proteins were resolved by SDS-PAGE, transferred onto nitrocellulose, and probed with anti-pp55 antiserum. Fig. 4 demonstrates that pp55 preferentially binds to the isolated SH2 domains of Fyn, Lyn, Src and Lck, whereas no signal is obtained when the SH2 domains of PI 3-kinase, ZAP70, Syk, Shc, SLP-76, Grb2, or c-abl are used for precipitation. Due to the highly selective interaction with Src-like SH2 domains, we termed the protein SKAP55. The finding that SKAP55 equally binds to the isolated Fyn and Lck SH2 domains whereas it is not detectable as a tyrosine phosphorylated protein in Lck immunoprecipitates (see Fig. 1) obtained from resting T-lymphocytes resembles the situation we have previously reported for α-tubulin (20).
SKAP55 mRNA expression was determined by Northern blot analysis employing poly(A)" RNA obtained from a variety of primary human tissues. Fig. 5A shows that the 1.5-kilobase SKAP55 transcript is highly expressed in thymocytes and peripheral blood lymphocytes and to a lesser extent in spleen cells. A positive signal was also detected in testis at a slightly higher molecular mass. This could indicate the existence of an alternatively spliced form of pp55 or of a highly related gene expressed in this organ. The latter possibility is supported by our finding that hybridization of human genomic DNA with a full-length SKAP55 cDNA probe results in appearance of several distinct bands (not shown). No SKAP55-specific transcript was observed in any of the other tissues that were analyzed (Fig. 5A). These data show that pp55 is preferentially expressed in lymphatic organs. Among transformed cells of hematopoietic origin SKAP55 was exclusively detected in Fyn-SH2 precipitates prepared from lysates of Jurkat cells but not in precipitates obtained from the erythroleukemic cell line K562, the Epstein-Barr virus-transformed B-cell line LAZ-509 and the myeloid cell lines HL60 and U937 (Fig. 5B). Identical results as shown here for Fyn-SH2 precipitates were obtained when whole cell lysates were probed with SKAP55 antibodies (not shown), supporting the idea that SKAP55 is preferentially expressed in T-lymphocytes.

In summary we have identified and cloned a novel cytoplasmic protein, SKAP55, which is preferentially expressed in human T-lymphocytes where it specifically interacts with the protein-tyrosine kinase p59<sup>56k</sup>. The presence of a PH domain and a SH3 domain suggests that SKAP55 is capable of interacting with several other intracellular proteins and thus could represent a novel adaptor protein involved in Fyn-mediated signaling. The constitutive tyrosine phosphorylation and Fyn-association of SKAP55 in nonstimulated T-lymphocytes further could suggest that SKAP55 exerts its signaling function even in the absence of external stimuli. Consistent with this assumption is that we have so far observed no alterations in the level of tyrosine phosphorylation of SKAP55 or in its association with Fyn following short time stimulation of T-lymphocytes through the TCR-CD3 complex. Vanadate treatment of T-lymphocytes, which is believed to concomitantly activate PTKs and inhibit protein-tyrosine phosphatases, also not alters the tyrosine phosphorylation status of SKAP55 or its association with Fyn (not shown). However, the quality of the signal that is mediated via SKAP55 as well as potential modifications of this signal during T-cell activation have to be determined in further studies. In addition it will be of importance to identify the 43- and the 85-kDa phosphoproteins, which also constitutively associate with Fyn in T-lymphocytes.

Acknowledgments—We thank Drs. S. Ratnofsky, S. Fischer, and J. Wienands for kindly providing the GST-SH2 domain constructs and Dr. T. Gibson for help in the identification of the PH domain. C. Schwarz is thanked for technical assistance.

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