Identification of a Mouse Protein Homologous to the Human CD6 T Cell Surface Protein and Sequence of the Corresponding cDNA[1]

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CD6 is a 105/130 kDa monomeric T cell surface glycoprotein that has been shown to play a role in human T cell activation. Recently a partial mouse CD6 cDNA sequence was described. We have isolated full-length cDNA clones including the initiation codon and sequence encoding the full signal peptide, as well as an additional 39 amino acids within the cytoplasmic domain as compared to the previously reported clone. The predicted full-length mouse CD6 protein contains 665 amino acids and has the features of a type I integral membrane protein. The extracellular domain of mouse CD6 is composed of three repeated cysteine-rich domains similar to those in human CD6, mouse and human CD5, and other members of a family of proteins whose prototype is the type I macrophage scavenger receptor. In marked contrast to the previously published human CD6 sequence, the mouse sequence predicts a long cytoplasmic tail that is not closely related to other proteins and possesses two proline-rich motifs containing the SH3-domain binding consensus sequence, three protein kinase C phosphorylation site motifs, nine casein kinase-2 phosphorylation site motifs, and a serine-threonine-rich motif repeated three times. Northern blot analysis revealed that mouse CD6 mRNA is expressed predominantly in thymus, lymph node, and spleen. A polyclonal antiserum was raised against mouse CD6 by gene gun plasmid DNA immunization of rabbits with the mouse CD6 cDNA in an expression vector. In immunofluorescence analysis this polyclonal antiserum positively stained the surface of cells transfected with the mouse CD6 cDNA in an expression vector, as well as most normal mouse thymocytes and peripheral T cells. CD6 protein is expressed on most $CD4^+CD8^+$ double-positive and $CD4^+$ or $CD8^+$ single-positive thymocytes, and is expressed at highest levels on mature $CD3^{high}$ thymocytes. The expression of mouse CD6 in thymocytes and peripheral T cells correlates closely with the expression of the related CD5 molecule. The polyclonal rabbit anti-mouse CD6 Abs immunoprecipitated a major polypeptide of 128 kDa from resting and 130 kDa from PMA- and FCS-activated mouse thymocytes and lymph node cells; it is likely that this increase in size upon activation is due to phosphorylation of mouse CD6 as has been described for human CD6. These data demonstrate that mouse thymocytes and T cells express a 130-kDa cell surface protein homologous to human CD6. ***The Journal of Immunology,*** **1995, 155: 4739–4748.**

Human CD6 is a T cell surface glycoprotein that is expressed at high levels by peripheral blood T cells and medullary thymocytes, and is also expressed by cortical thymocytes, a small fraction of peripheral blood B cells, some B cell chronic lymphocytic leukemias, and some cells of the brain (1–7). Most human CD6-specific mAbs can enhance T cell proliferation induced by suboptimal doses of CD3-specific mAb, but with other methods of T cell stimulation, mAb specific for distinct CD6 epitopes have been shown to have different activation properties (5, 8–12). For example, the human CD6-specific mAb T12 stimulates T cell proliferation in a macrophage-dependent (and IL-2-dependent) fashion and is minimally co-mitogenic with PMA (10); mAb UMCD6 is also macrophage dependent but is synergistic with PMA (11); mAb 2H1 can stimulate T cell proliferation in conjunction with a CD2-specific mAb (anti-$T11_3$) or PMA and is macrophage independent (5, 9). Serine and threonine residues in the cytoplasmic domain of human CD6 are constitutively phosphorylated in resting T cells (9, 13–15), and activation by anti-CD3 mAb cross-linking induces phosphorylation of tyrosine and hyperphosphorylation of serine residues (15). The CD6-specific mAb T12 has been used successfully as an immunosuppressive agent for patients undergoing kidney or bone marrow allograft rejection and to purge donor bone marrow T cells prior to allogeneic bone marrow transplantation (2, 16, 17). CD6-specific mAbs have been shown to partially inhibit interaction of thymocytes with thymic epithelial cells (18), and human CD6 was recently shown to bind to the 100-kDa activated leukocyte cell adhesion molecule (ALCAM)[3] expressed on the surface of thymic epithelial cells (19, 20).

As a type I integral membrane glycoprotein, human CD6 exists on the cell surface as a 105- or 130-kDa monomer with intrachain disulfide bonds (9, 13, 14, 21, 22). The size difference between

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Received for publication May 4, 1995. Accepted for publication August 29, 1995.

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[1] This work was supported by National Institutes of Health Grants CA46507 and CA68675 (formerly AI30155) to J.R.P. W.H.R. and S.S.P. received support from Training Grant AI07290 from the National Institutes of Health.

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[3] Abbreviations used in this paper. ALCAM, activated leukocyte cell adhesion molecule; SRCR, scavenger receptor cysteine-rich domains; PKC, protein kinase C.

0022-1767/95/$02.00

these two forms is due to differences in phosphorylation (14). Activation of human PBLs with PMA and FCS results in the hyperphosphorylation of CD6 on serine residues and is accompanied by an increase of the 130-kDa form and a relative decrease of the 105-kDa form (14). Aruffo et al. (22) isolated a human CD6 cDNA (CD6-15) encoding a mature polypeptide of 444 amino acids from an HPB-ALL cDNA library. Transient transfection of this clone into Cos cells yielded surface expression of a 90-kDa polypeptide (22); the size of this encoded protein was notably smaller than the 105- and 130-kDa wild-type CD6 polypeptide immunoprecipitated from human PBLs (9, 13, 14, 21) or from HPB-ALL (22). Human CD6 is heavily glycosylated (primarily *N*-linked sugars) and contains a polysulfated O-linked oligosacharide (13), and Aruffo et al. (22) attributed the difference in molecular mass between their transfected human CD6 cDNA product and wild-type CD6 to incomplete glycosylation (23). Human CD6 is homologous to CD5 and to the family of proteins containing cysteine-rich domains defined by that of the type 1 macrophage scavenger receptor (22).

Recently Whitney et al. reported a partial mouse CD6 cDNA sequence (24) missing the sequence encoding the amino-terminal portion of the signal peptide as well as part of the cytoplasmic domain, but to date mouse CD6 has not been serologically, biochemically, or functionally characterized. We have used the human CD6-15 cDNA probe to isolate homologous full-length mouse CD6 cDNA clones. The predicted full-length mouse CD6 protein sequence from these clones is similar to that of human CD6-15 (22) in its signal sequence, extracellular domain, and transmembrane domain. However, the predicted cytoplasmic domain possesses 243 amino acids as compared with the 44 amino acid cytoplasmic domain reported by Aruffo et al. (22) for human CD6-15. This long cytoplasmic domain of mouse CD6 bears no striking homology to other described proteins. We show that CD6 mRNA is expressed by mouse lymphoid tissues. We used gene gun DNA immunization to raise a rabbit polyclonal antiserum highly specific for mouse CD6. We have used this antiserum to immunoprecipitate the mouse CD6 protein from the surface of transfectants expressing the mouse CD6 cDNA as well as from the surface of mouse thymocytes and lymph node cells. We have also used this antiserum to demonstrate expression of CD6 protein on the cell surface of mouse thymocytes and peripheral T cells.

Methods and Materials

Isolation of mouse CD6 cDNA clones by hybridization

The 1.2 kb *Nsp*I-*Eco*RI fragment from the human CD6 cDNA clone CD6-15 (22), representing nucleotides 121 to 1349, was used to screen a cDNA library generated in the Lambda-ZAP II vector (Stratagene, La Jolla, CA) from thymocyte mRNA of BALB/c and C57BL/6 mice by J. Jorgensen and M. Davis (Stanford University, Stanford, CA). The hybridization probe was labeled with ^{32}P using the random hexamer priming method (25), and plaque hybridization was performed at 42°C in 40% deionized formamide, 4X SSC, 20 mM Tris-HC1 (pH 7.5), 1X Denhard's solution, 0.1% SDS, 10% dextran sulfate, and 20 μg/ml herring sperm DNA. The filters were washed at room temperature in 2X SSC/0.05% SDS followed by 0.1 × SSC/0.05% SDS at 50°C. After three rounds of screening, six positively hybridizing plaques were excised in the Bluescript phagemid using the helper phage ExAssist (Stratagene) as recommended by the manufacturer. The cDNA inserts of these phagemid clones range in size from 1.4 to 3.0 kb, and all represent overlapping clones of mouse CD6 based on nucleotide sequence analysis. The two largest clones, mouse CD6-4 and CD6-6, were sequenced fully in both directions using the dideoxy chain-termination method (26).

Northern blotting

The 1.4-kb *Xho*I-*Eco*RI fragment representing nucleotides 1 to 1380 of mouse CD6-4 was labeled with ^{32}P by random hexamer priming (21) and used as a hybridization probe. The multi-tissue Northern blot was obtained from CloneTech (South San Francisco, CA). Using RNAzol (Cinna/Biotecx, Friendswood, TX), total RNA was isolated from single-cell suspensions generated from mouse spleen, thymus, lymph node, and bone marrow as well as from the following cell lines: the CD4$^+$ T cell lymphoma line C6VL obtained from J. Allison (University of California at Berkeley, Berkeley, CA) (27), Gross leukemia virus transformed early T cell lines KKA (Thy-1$^+$, CD4$^-$, CD8$^-$, CD3$^-$) and KgV (Thy-1$^+$, CD4$^-$, CD8$^-$, CD3$^+$) obtained from K. Blank (University of Pennsylvania, Philadelphia, PA) (28), the CD8$^+$ T cell lymphoma line Eb288 (29), as well as the thymoma cell line EL4, the mature B cell lymphoma line WEHI-231, and the differentiation-inducible mature B cell leukemia lymphoma line BCL$_1$ obtained from the American Type Culture Collection (Rockville, MD). Northern blotting and hybridization were performed as previously described (30).

Transfection of NB2 cells

NB2-6TG (NB2) is a rat T cell line obtained from H. Waldmann (University of Cambridge, Cambridge, GB). The 2979-bp mouse CD6-4 cDNA was subcloned into the *Xho*I restriction enzyme site of the pBJ-neo expression vector (31). Transfection of NB2 cells with pBJ-neo-mouse CD6-4 cDNA expression constructs was performed by electroporation using a Bio-Rad (Richmond, CA) gene pulser at 675 V/cm and 960 μF according to the manufacturer's instructions. CD6 cDNA transfectants were selected by growth in the presence of 1.2 mg/ml G418 (Life Technologies, Inc., Grand Island, NY).

Preparation of polyclonal rabbit antiserum

Polyclonal antiserum to mouse CD6 was raised using a gene gun to inoculate rabbits with a CD6-expressing plasmid DNA (32–34). The CD6-expressing plasmid DNA was constructed by inserting a PCR-amplified fragment representing the coding region of mouse CD6-4 into the *Hin*dIII and *Bam*HI sites of the pJW4303 expression vector (5′ PCR primer: 5′-TATATAAGCTTGGCAGGCGTGAGTAGCAGTGG-3′, derived from the 5′ untranslated region and containing an added *Hin*dIII restriction enzyme site, and 3′ PCR primer: 5′-GGCGGATCCGAGTGAGATAAGGC CTCAGGG-3′, derived from the 3′ untranslated region and containing an added *Bam*HI restriction enzyme site). The pJW4303 expression vector was the generous gift of J. Arthos (Department of Microbiology and Immunology, Stanford University, Stanford, CA) and contains the cytomegalovirus immediate early promoter and the bovine growth hormone gene polyadenylation sequence (35). The gene gun inoculations consisted of 40 shots of 0.5 μg of 0.95-mm gold beads coated with 1.25 μg of pJW4303-CD6-4 DNA. DNA was precipitated onto the gold beads using spermidine and calcium as described by Fynan et al. (32). Shots were delivered to abdominal skin at 450 psi using the helium pulse Accell gene delivery system (Agracetus, Middleton, WI). The abdominal epidermis was prepared by shaving. Sera were collected prior to immunization (preimmune serum) and 4 wk following a 40-shot vaccination (polyclonal CD6-specific antiserum).

Immunofluorescence analysis

One million transfected or untransfected NB2 cells were stained with a 1/100 dilution of preimmune rabbit serum or the rabbit anti-mouse CD6 antiserum, followed by FITC-conjugated goat anti-rabbit IgG-specific Ab (Tago, Burlingame, CA). One million thymocytes or lymph node cells from a C57BL/6 mouse (The Jackson Laboratory, Bar Harbor, ME) were stained with 0.5 μg of protein A-Sepharose-purified preimmune rabbit serum Ab or protein A-Sepharose-purified rabbit anti-mouse CD6 Ab followed by phycoerythrin-conjugated CD3-, CD5-, CD4- or CD8-specific mAb (PharMingen, San Diego, CA). Immunofluorescence was determined by analysis on a modified FACScan (Becton Dickinson, Mountain View, CA) FACS.

Cell-surface iodination and immunoprecipitation

Thymocytes and lymph node cells were activated with 20 ng/ml PMA and 10% FCS for 2 h at 37°C, and resting cells were kept in 5% FCS for 2 h on ice. Twenty million untransfected and mouse CD6-4 transfected NB2 cells as well as 3×10^7 thymocytes and lymph node cells were surface labeled with 0.5 mCi ^{125}I (1 mCi = 37 GBq, Amersham, Arlington Heights, IL) using the lactoperoxidase method as previously described (30). Cells were washed in PBS and lysed in 0.5 ml lysis buffer (1% Nonidet P-40, 10 mM Tris-HC1 (pH 7.5), 1 mM EDTA, 0.15 M NaCl, 1 mM PMSF, and 1 mM iodoacetamide). NB2 cell lysates were precleared twice with 12 μl preimmune rabbit serum followed by protein A-Sepharose. Mouse CD6 was then precipitated by adding 12 μl rabbit anti-mouse CD6 antiserum followed by 150 μl 10% protein A-Sepharose for 1 h at 4°C. Thymocyte and lymph node cell lysates were precleared with 15 μg

protein A-Sepharose-purified preimmune rabbit serum Ab and precipitated with rabbit anti-mouse CD6 Ab. The precipitates were washed five times as previously described (30). Half of each sample was resuspended in 1X loading buffer containing 1% 2-ME (reducing) and the other half in 1X loading buffer without 2-ME (nonreducing), and the samples were electrophoresed on 8% SDS-polyacrylamide gels. The gels were dried onto Whatman chromatography paper (Whatman International Ltd., Maidstone, England) and the protein bands were detected by autoradiography.

Results

Isolation and sequence of cDNA clones encoding mouse CD6

To isolate the mouse homologue of human CD6, we screened a cDNA library constructed from RNA isolated from mouse thymus. We used a 5′ coding sequence fragment of the human CD6-15 cDNA clone (22) as a hybridization probe and low stringency wash conditions. After three successive rounds of screening, six positively hybridizing plaques were chosen for restriction enzyme analysis and nucleotide sequencing and were excised into the Bluescript vector using the helper phage ExAssist. Nucleotide sequence analysis revealed that all six clones were overlapping, and the two longest clones, mouse CD6-4 and mouse CD6-6, were sequenced fully in both directions. Mouse CD6-4, the longest clone, contains 2979 bp, which corresponds well with the 3-kb mRNA we have identified in mouse tissues (see below). This clone contains an open reading frame coding for a 665 amino acid polypeptide chain containing the typical features of a type I membrane protein (Fig. 1). A 24-amino acid NH_2-terminal signal sequence is predicted by the method of Von Heijne (36). Removal of this NH_2-terminal signal sequence results in a mature protein of 641 amino acids with a predicted m.w. of 70 kDa. The predicted 372-amino acid extracellular domain possesses eight possible *N*-linked glycosylation sites (Asn-X-Ser/Thr with X representing a nonconserved residue). Like mouse and human CD5 and like human CD6, mouse CD6 possesses three repeated extracellular domains of about 100 amino acids (SRCR-1, SRCR-2, and SRCR-3) (Fig. 1), each of which is homologous to the cysteine-rich domain of the macrophage scavenger receptor type 1 (SRCR) family of proteins (22, 37, 38).

Mouse CD6-4 also encodes a 26-amino acid hydrophobic membrane-spanning domain and a 243-amino acid cytoplasmic domain. We used the Quest program to search the cytoplasmic domain of mouse CD6 for sequence motifs present in the Prosite 12 database (IntelliGenetics, Mountain View, CA). We found three motifs containing the PKC phosphorylation site consensus sequence (39) and nine motifs containing the casein kinase-2 phosphorylation site consensus sequence (40) (Figs. 1 and 2). There are 10 tyrosines, 9 threonines, and 32 serines in the cytoplasmic domain of mouse CD6.

Visual inspection of the predicted cytoplasmic tail of mouse CD6-4 revealed a serine-threonine-rich amino acid motif repeated three times with the consensus sequence Asp-Ser-Ser-Ser/Thr-Y-Ser-X-X-Glu-X-Tyr (X represents a nonconserved residue, and Y represents a nonconserved residue or a gap in the alignment) at amino acid positions 475, 559, and 614 (Figs. 1 and 2). The cytoplasmic domain contains the proline rich sequences Cys-Pro-Gly-Pro-Pro-Gly-Pro-Gln at position 641 and Gln-Pro-Pro-Pro-Gln-His-Pro at position 628, which contain the class I SH3 domain-binding consensus motif X-Pro-X-Pro-Y-X-Pro (X represents a nonconserved residue, and Y represents proline or a nonconserved residue) (Figs. 1 and 2) (41). We also identified the Tyr-Asp-Asp-Ile motif that could represent a target for binding of the SH2 domain of c-*fgr* or a different Src kinase family member (42).

Mouse CD6-4 possesses 144 nucleotides of protein-coding sequence not present in the previously reported partial mouse CD6 cDNA sequence (24) (Fig. 1). This coding sequence includes 27 nucleotides containing the initiation codon and encoding the first 9 amino acids of the protein (Figs. 1 and 2); two of these amino acids are not conserved in human CD6 (Fig. 2). This additional coding sequence also includes 117 nucleotides encoding 39 amino acids starting at amino acid position 461 in the cytoplasmic domain. These 117 nucleotides correspond to an exon in the mouse CD6 gene, and the mouse CD6 cDNA clone of Whitney et al. (24) most likely resulted from splicing out of that exon (24). Notably, sequence corresponding to this exon is present in full length human CD6 cDNA (42a). Mouse CD6-4 also possesses 158 nucleotides of 5′ untranslated region and 124 nucleotides of 3′ untranslated region not present in the reported (24) partial mouse CD6 cDNA sequence (Fig. 1). The additional 3′ untranslated region sequence contains a potential, although not perfect, polyadenylation signal sequence ATTAAA near the 3′ terminus at position 2963.

Of the shorter clones, mouse CD6-6 lacks 462 nucleotides that encode part of the cytoplasmic domain and the 3′ untranslated region of mouse CD6-4, from position 1828 to 2289 of mouse CD6-4, and is otherwise identical in nucleotide sequence to mouse CD6-4 (Fig. 1). Mouse CD6-6 contains an open reading frame coding for a polypeptide of 565 amino acids (Fig. 1). In constrast to the predicted 243-amino acid cytoplasmic domain encoded by mouse CD6-4, mouse CD6-6 encodes a 143-amino acid residue cytoplasmic domain, the last nine residues of which distinguish it from that encoded by mouse CD6-4 (Fig. 1).

Comparison of mouse and human CD6 protein sequences

We compared the amino acid sequence predicted from the mouse CD6-4 cDNA clone with that of human CD6-15 (Fig. 2). The sequences are highly conserved with 68% identical amino acid residues overall (316 identical residues/468 total residues including gaps in the alignment and including only the first 44 amino acids of the cytoplasmic domain). In the extracellular domain, the SRCR-2 and SRCR-3 are the most highly conserved domains with 85% (88/104) and 79% (80/101) identity, respectively, while the membrane distal SRCR-1 domain has only 56% (64/115) identity between mouse and human. In each of the SRCR domains of mouse CD6 the six cysteine residues shared by the other members of the SRCR family are all conserved, as well as the two additional cysteine residues unique to CD5 and human CD6 (22, 37, 38). Two single amino acid gaps in the alignment are present in the extracellular domain, located in the SRCR-1 domain and in the region between SRCR-3 and the transmembrane domain. Both of these regions with gaps in the alignment are not as highly conserved as the SRCR-2 and SRCR-3 domains (Fig. 2). Six of the eight potential sites for *N*-linked glycosylation present in the extracellular domains of both mouse and human CD6 are conserved (Fig. 2). The transmembrane domains of mouse and human CD6 exhibit 69% (18/26) identity and the first 44 amino acid residues of the cytoplasmic domains 59% (26/44) identity.

Expression of mouse CD6 mRNA in lymphoid cells

On Northern blot analysis the 1.4 Kb 5′ *Xho*I-*Eco*RI fragment from mouse CD6-4 hybridized to a 3.0-kb mRNA species expressed in thymus, lymph node, spleen, and the $CD4^+$ T cell lymphoma line C6VL (Fig. 3). Substantially lesser amounts of this same mRNA species was present in RNA isolated from lung and skeletal muscle (Fig. 3*A*) (note, Fig. 3*A* was highly overexposed to highlight the fainter bands in tissues other than spleen). CD6 protein is reported to be expressed in human brain (7), and we found a faint band representing a 2.4-kb species in RNA isolated from mouse brain (Fig. 3*A*). No hybridizing mRNA was detected in liver, kidney, testis, bone marrow, the Abelson-transformed pre-B

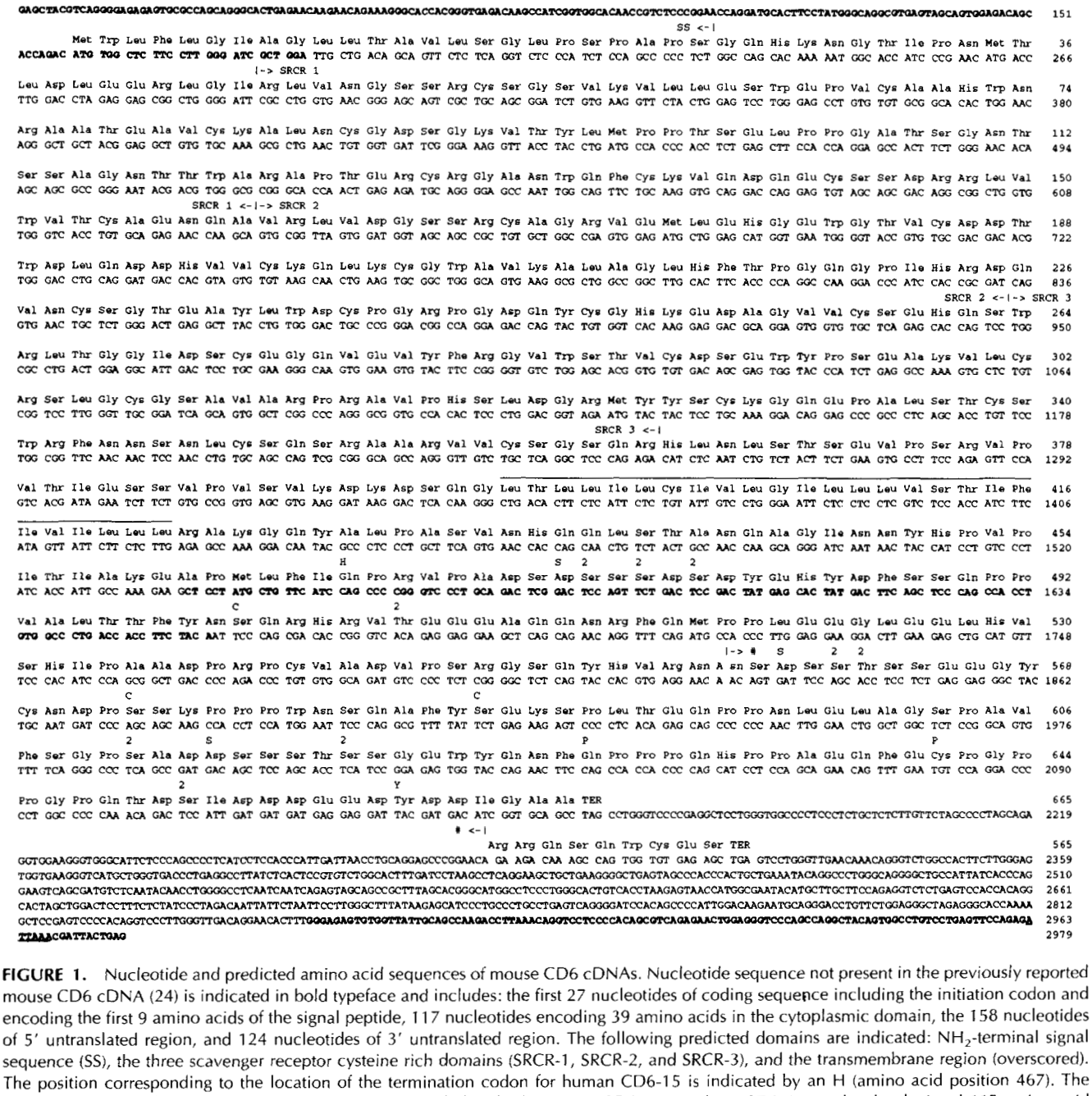

FIGURE 1. Nucleotide and predicted amino acid sequences of mouse CD6 cDNAs. Nucleotide sequence not present in the previously reported mouse CD6 cDNA (24) is indicated in bold typeface and includes: the first 27 nucleotides of coding sequence including the initiation codon and encoding the first 9 amino acids of the signal peptide, 117 nucleotides encoding 39 amino acids in the cytoplasmic domain, the 158 nucleotides of 5′ untranslated region, and 124 nucleotides of 3′ untranslated region. The following predicted domains are indicated: NH_2-terminal signal sequence (SS), the three scavenger receptor cysteine rich domains (SRCR-1, SRCR-2, and SRCR-3), and the transmembrane region (overscored). The position corresponding to the location of the termination codon for human CD6-15 is indicated by an H (amino acid position 467). The potential polyadenylation signal sequence ATTAAA is underlined. The mouse CD6 cDNA clone CD6-4 encodes the depicted 665 amino acid protein. The mouse CD6 cDNA clone CD6-6 lacks 462 nucleotides between positions 1828 and 2289 as indicated by a # and encodes a 565 amino acid protein with the indicated distinguishing nine carboxyl-terminal amino acids. Two proline-rich sequences that contain the SH3-domain-binding consensus motif X-Pro-X-Pro-Y-X-Pro are indicated by Ps. The three Asp-Ser-Ser-Ser/Thr-Y-Ser-X-X-Glu-X-Tyr motifs are indicated by Ss. The Tyr-Asn-Asn-Ile motif is indicated by a Y. The nine motifs containing the casein kinase-2 phosphorylation site consensus sequence Ser/Thr-X-X-Asp/Glu are indicated by 2's. The three motifs containing the PKC phosphorylation site consensus sequence Ser/Thr-X-Arg/Lys are indicated by Cs. The accession number of our mouse CD6-4 cDNA sequence is U37543 and of our mouse CD6-6 cDNA sequence is U37544 at the sequence data base at the National Center for Genome Resources.

cell line L1.2, the mature B cell line WEHI-231, the differentiation-inducible B cell line BCL_1, the Gross leukemia virus transformed immature thymocyte lines KKA and KgV, the thymoma cell line EL4, and the mature $CD8^+$ T cell line Eb288 (Fig. 3). This pattern of mRNA expression is consistent with the observed pattern of expression for human CD6, which is predominantly expressed in peripheral T cells and mature medullary thymocytes. Based on flow cytometry analysis, all of these cell lines except KKA and KgV express CD5 (data not shown), and thus the expression of CD6 does not correlate precisely with expression of the homologous CD5 protein.

Cell-surface staining of NB2 cells transfected with the mouse CD6-4 cDNA with rabbit anti-mouse CD6 antiserum

The rat T cell line NB2 was transfected with the mouse CD6-4 cDNA in the pBJ-neo expression vector. Polyclonal antiserum specific for mouse CD6 was raised by gene gun inoculation of rabbits

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                               SS |                    | SR-1   *               +            +    *
mCD6-4     MWLFLGIAGLLTAVLSGLPSPAPSGQHKNGTIPNMTLDLEERLGIRLVNGSSRCSGSVKVLLE.SWEPVCAAHWNRAATEAVCKALNCGD 89
hCD6-15    ----F--T-----A---H-----PD-LNTSSAESELWEPG---PV--T----S---T-E-R--A----A-G-L-DSR-A----R--G--G 90
                                                 +       +      +          +     | SR-2   *
mCD6-4     SGKVTYLMPPTSELPPGATSGNTSSAGNTTWARAPTERCRGANWQFCKVQDQECSSDRRLVWVTCAENQAVRLVDGSSRCAGRVEMLEHG 179
hCD6-15    AEAASQ-A---P----PPAA----V-A-A-L-G--ALL-S--E-RL-E-VEHA-R--G-RAR------R-L-----GGA---------- 180
                +            +    *                         +         +        +         +     | SR-3
mCD6-4     EWGTVCDDTWDLQDDHVVCKQLKCGWAVKALAGLHFTPGQGPIHRDQVNCSGTEAYLWDCPGRPGDQYCGHKEDAGVVCSEHQSWRLTGG 269
hCD6-15    ---S--------E-A----R--G-----Q--P-------R-----------A---------L--QH--------------------- 270
              *               +            +    *                     +         +         +         +
mCD6-4     IDSCEGQVEVYFRGVWSTVCDSEWYPSEAKVLCRSLGCGSAVARPRAVPHSLDGRMYYSCKGQEPALSTCSWRFNNSNLCSQSRAARVVC 359
hCD6-15    A-R-------H-----N----------------Q-----T--E--KGL----S-------N-E-LT--N-------------L----L- 360
          SR-3 |                                 ___________________________
mCD6-4     SGSQRHLNLSTSEVPSRVP.VTIESSVPVSVKDKDSQGLTLLILCIVLGILLLVSTIFIVILLLRAKGQYALPASVNHQQLSTANQAGIN 448
hCD6-15    -A-RSLH----P---AS-QT-------T-KIEN-E-RE-M---PS--------G-L---AFI---I--K----VM----H-P-TIP--S- 450
                                     S2 2 2                    C     2
mCD6-4     NYHPVPITIAKEAPMLFIQPRVPADSDSSSDSDYEHYDFSSQPPVALTTFYNSQRHRVTEEEAQQNRFQMPPLEEGLEELHVSHIPAADP 538
hCD6-15    S-Q------P--G-GPAP                                                                        466
                               @  22         C            C                        2  @    2        P
mCD6-4     RPCVADVPSRGSQYHVRNNSDSSTSSEEGYCNDPSSKPPPWNSQAFYSEKSPLTEQPPNLELAGSPAVFSGPSADDSSSTSSGEWYQNFQ 628
                       P         2       Y
mCD6-4     PPPQHPPAEQFECPGPPGPQTDSIDDDEEDYDDIGAA                                                     665
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FIGURE 2. Comparison of the predicted amino acid sequence of mouse CD6-4 with human CD6-15. Identical residues are denoted by dashes, gaps in the alignment are denoted by dots, the transmembrane region is overscored, the boundaries of the three SRCR domains (SR-1, SR-2, and SR-3) are indicated by vertical bars. Cysteine residues shared by all members of the SRCR family are denoted by pluses, and the two cysteine residues shared only by CD5 and CD6 are denoted by asterisks. Other motifs are indicated according to the legend to Figure 1.

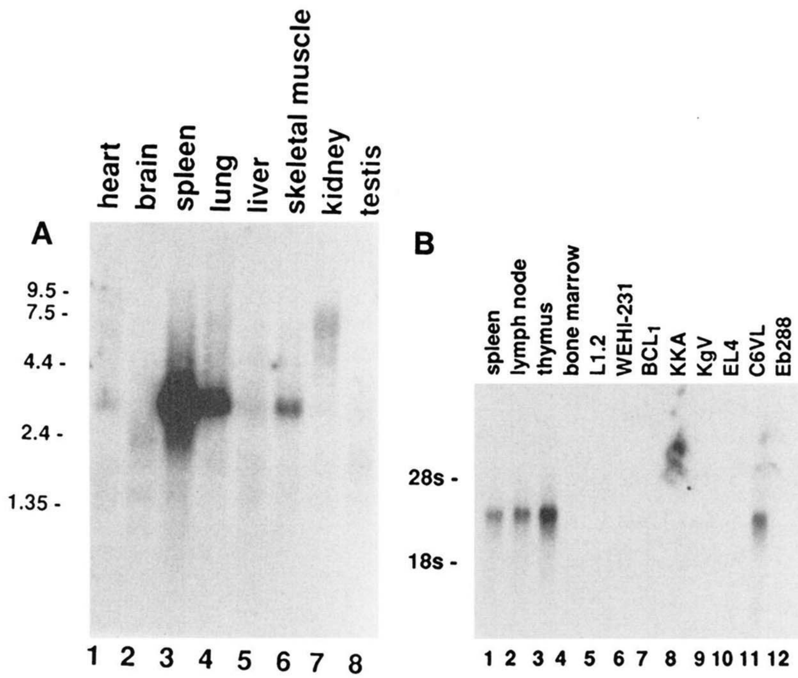

FIGURE 3. Northern blot analysis demonstrating expression of mouse CD6 mRNA in lymphoid cells. Northern blots were hybridized with a ^{32}P-labeled mouse CD6-4 cDNA probe. The blots contain total RNA isolated from the following sources: *A*) heart (*lane 1*), brain (*lane 2*), spleen (*lane 3*), lung (*lane 4*), liver (*lane 5*), skeletal muscle (*lane 6*), kidney (*lane 7*), and testis (*lane 8*); *B*) spleen (*lane 1*), lymph node (*lane 2*), thymus (*lane 3*), bone marrow (*lane 4*), L1.2 (*lane 5*), WEHI-231 (*lane 6*), BCL_1 (*lane 7*), KKA (*lane 8*), KgV (*lane 9*), EL4 (*lane 10*), C6VL (*lane 11*), and Eb288 (*lane 12*). The migration positions of *A*) the 28S and 18S rRNA or *B*) ssRNA size markers (Kb) are indicated in the left margins.

with the mouse CD6-4 cDNA in the pJW4303 expression vector. As compared with immunofluorescence staining with preimmune serum, the rabbit anti-mouse CD6 antiserum did not stain untransfected NB2 cells (Fig. 4*A*) and positively stained mouse CD6-4 cDNA-transfected NB2 cells (Fig. 4*B*).

Cell-surface staining of normal mouse T lineage cells with rabbit anti-mouse CD6 Ab

Purified Ab from the polyclonal rabbit anti-mouse CD6 antiserum was used to stain bulk thymocytes and lymph node cells isolated from a C57BL/6 mouse (Figs. 5 and 6). As compared with immunofluorescence staining with preimmune Ab, the rabbit anti-mouse CD6 Ab positively stained the cell surface of most $CD4^+CD8^+$ double-positive thymocytes and most $CD4^+$ or $CD8^+$ single-positive thymocytes (Fig. 5). Staining with the rabbit anti-mouse CD6 Ab was highest on the mature $CD3^{high}$ thymocytes (Fig. 5). The rabbit anti-mouse CD6 Ab also positively stained most or all $CD3^+$, $CD5^+$, $CD4^+$, and $CD8^+$ peripheral lymph node and splenic T cells, and few or no B cells (Fig. 6) (data not shown). The level of CD6 expression on most mouse thymocytes, splenocytes,

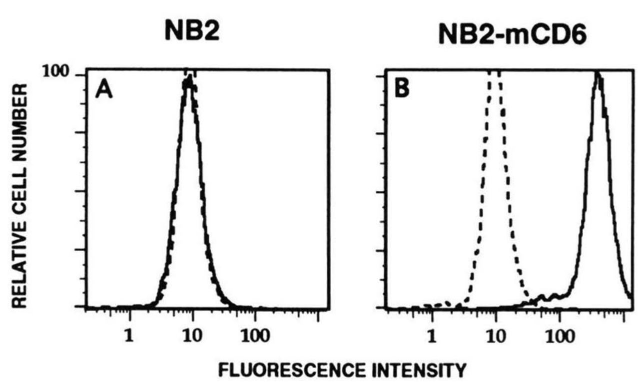

FIGURE 4. Flow cytometric analysis of NB2 cells transfected with the mouse CD6-4 cDNA and cell-surface stained with polyclonal rabbit anti-mouse CD6 antiserum. Untransfected (*A*) and mouse CD6-4 transfected (*B*) NB2 cells were stained with preimmune rabbit serum (dashed lines) or polyclonal mouse CD6-specific rabbit antiserum (solid lines), followed by FITC-conjugated goat anti-rabbit Ig-specific secondary Ab.

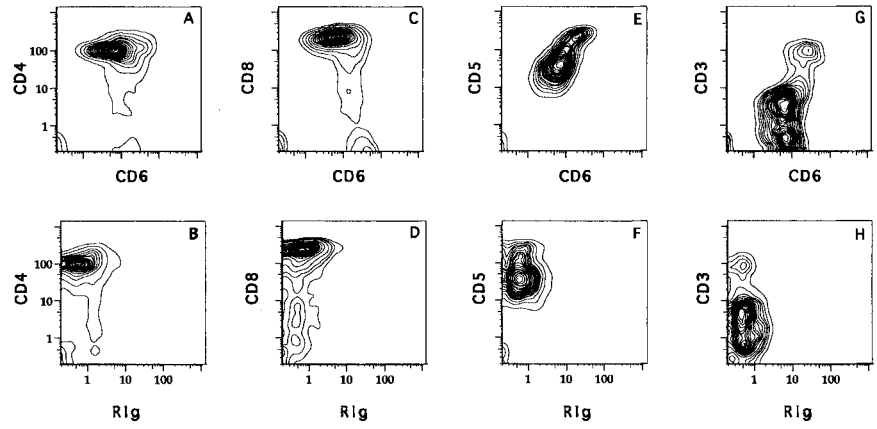

FIGURE 5. Flow cytometric analysis of mouse thymocyte cell surface stained with rabbit anti-mouse CD6 Ab. A single-cell suspension of mouse thymocytes was stained with protein A-Sepharose-purified rabbit anti-mouse CD6 Ab (*A, C, E,* and *G*) or preimmune rabbit Ab (Rlg) (*B, D, F,* and *H*) followed by FITC-conjugated goat anti-rabbit Ig-specific secondary Ab. The thymocytes were then washed and stained with phycoerythrin-conjugated CD4- (*A* and *B*), CD8- (*C* and *D*), CD5- (*E* and *F*), or CD3- (*G* and *H*) specific mAb.

and lymph node T cells was proportional to the level of CD5 expression (Figs. 5 and 6) (data not shown).

Immunoprecipitation of CD6 from NB2 cell transfectants expressing the mouse CD6-4 cDNA with rabbit anti-mouse CD6 antiserum

We performed immunoprecipitations from lysates of radioiodinated untransfected or mouse CD6-4 cDNA-transfected NB2 cells using the polyclonal rabbit anti-mouse CD6 antiserum (Fig. 7). When analyzed under reducing conditions, the polyclonal rabbit antiserum precipitated a major polypeptide band of 125 kDa and a minor polypeptide band of 100 kDa. When analyzed under nonreducing conditions, the polyclonal antiserum precipitated a major polypeptide band of 120 kDa and a minor polypeptide band of 95 kDa. We believe these polypeptide bands represent mouse CD6 based on the similarity of their m.w. to the observed m.w. of human CD6 and the predicted m.w. of the polypeptide encoded by the mouse CD6-4 cDNA. In addition, the precipitated polypeptides have similar properties to the human CD6 polypeptide in that the nonreduced polypeptide migrates faster than the reduced polypeptide in SDS-polyacrylamide gel analysis, suggesting the presence of intrachain disulfide bonds (9, 13, 22). The polyclonal rabbit anti-mouse CD6 antiserum did not precipitate any specific bands from untransfected NB2 cells under reducing or nonreducing conditions.

Immunoprecipitation of CD6 from normal mouse T lineage cells with rabbit anti-mouse CD6 antiserum

We performed immunoprecipitations from lysates of radioiodinated resting or PMA- and FCS-activated thymocytes and lymph node T cells (Fig. 8). When analyzed under reducing conditions, the rabbit anti-mouse CD6 Ab precipitated a major polypeptide band of 128 kDa from resting and 130 kDa from activated thymocytes and lymph node cells. These reduced immunoprecipitates from resting and activated cells also contain minor polypeptide bands of 103 and 105 kDa, respectively. The CD6 polypeptide bands precipitated from mouse thymocytes and lymph node cells are slightly larger than the 125-kDa polypeptide immunoprecipitated from the NB2 cell transfectants expressing the CD6-4 cDNA. This is most likely due to differences in glycosylation and/or phosphorylation.

Discussion

We report isolation of cDNA clones encoding full-length mouse CD6 from a mouse thymic cDNA library by cross-hybridization with the cDNA encoding human CD6. The sequence of our clones differs from a recently reported mouse CD6 cDNA sequence (24) in that it contains the complete leader sequence (encoding the signal peptide) as well as part of the cytoplasmic domain that was apparently spliced out in the mRNA that gave rise to the reported cDNA sequence. As compared with the reported sequence, our cDNA clones also possess 5′ untranslated region sequence as well as additional 3′ untranslated region sequence containing a potential polyadenylation signal sequence. Northern blot analysis demonstrated expression of a 3.0-kb CD6 mRNA species in lymphoid tissues and thus our 2979-bp CD6-4 cDNA clone is likely to be representative of the full-length mouse CD6 mRNA expressed in these tissues.

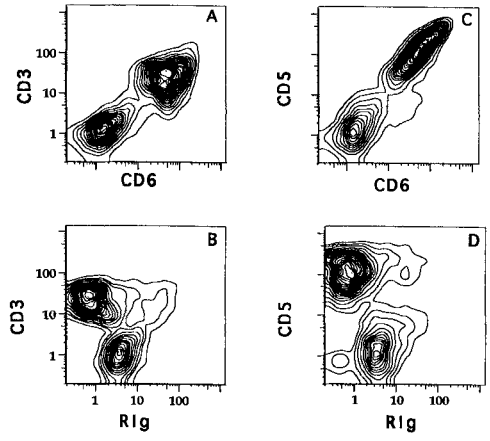

FIGURE 6. Flow cytometric analysis of mouse lymph node cells surface stained with rabbit anti-mouse CD6 Ab. A single-cell suspension of mouse lymph node cells was stained with protein A-Sepharose-purified preimmune rabbit Ab alone (*B* and *D*) or rabbit anti-mouse CD6 Ab (*A* and *C*), followed by FITC-conjugated goat anti-rabbit Ig-specific secondary Ab. The lymph node cells were then washed and stained with phycoerythrin-conjugated CD3- (*A* and *B*) or CD5- (*C* and *D*) specific mAb.

We found that mouse CD6 is expressed at the RNA level in thymus, lymph node, spleen, and the $CD4^+$ T cell lymphoma line C6VL. Relative to the levels in lymphoid tissues, a very small amount of CD6 mRNA was detected in skeletal muscle and a small amount was detected in lung. To confirm that the CD6 mRNA detected in lung was not due to spillage of the splenic mRNA sample (in the adjacent lane) at the time of loading the gel, we probed an independent Northern blot with the CD6 cDNA and obtained the same result. No expression of human CD6 has been reported in these tissues. Although it is possible that the cells composing these tissues express low levels of mouse CD6, we believe

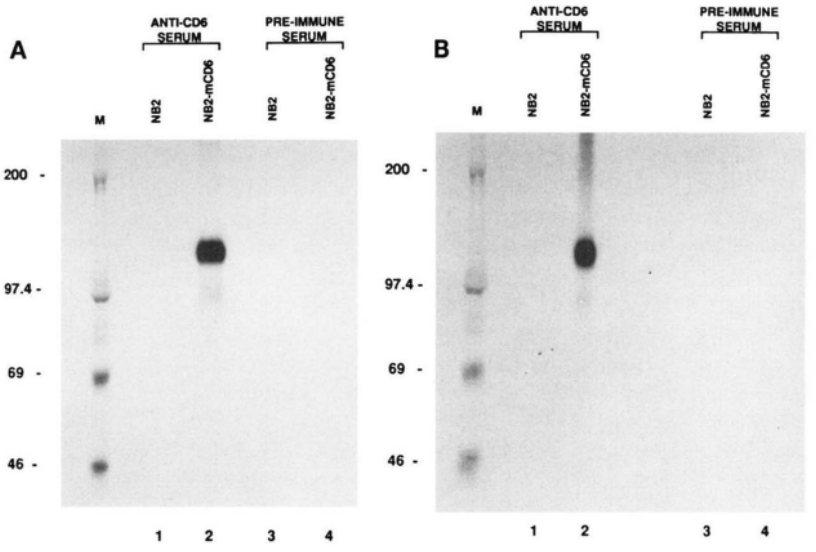

FIGURE 7. Immunoprecipitation of mouse CD6 from cells lysates of NB2 cell transfectants expressing the mouse CD6-4 cDNA. Untransfected NB2 cells (*A* and *B*, *lanes 1* and *3*) and NB2 cells transfected with the mouse CD6-4 cDNA in the pBJ-neo expression vector (*A* and *B*, *lanes 2* and *4*) were surface labeled with ^{125}I, lysed, and the lysates precleared twice with preimmune rabbit serum (the second preclearing is represented in *A* and *B*, *lanes 3* and *4*) before immunoprecipitation with gene gun-generated polyclonal rabbit anti-mouse CD6 antiserum (*A* and *B*, *lanes 1* and *2*). Samples were electrophoresed on 8% SDS-polyacrylamide gels under reducing (*A*) or nonreducing (*B*) conditions. The migration positions of co-migrated ^{14}C-labeled M_r standards ($\times 10^{-3}$) are shown in the left margin.

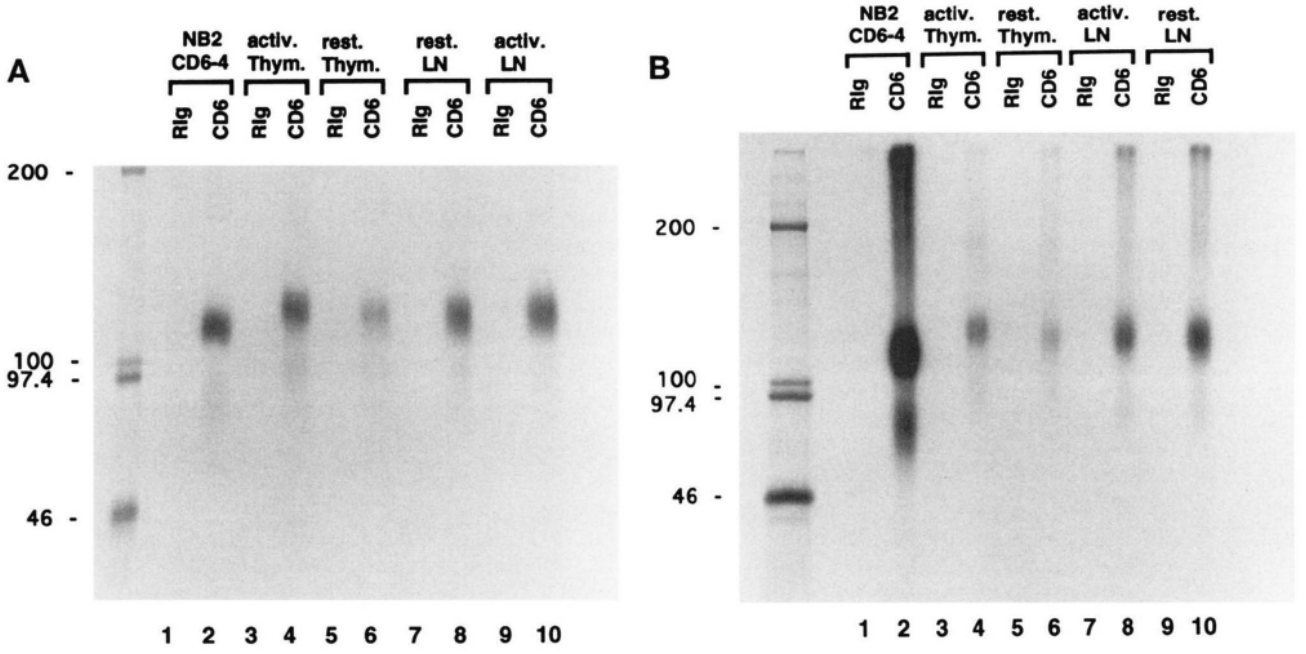

FIGURE 8. Immunoprecipitation of mouse CD6 from cells lysates of mouse thymocytes and lymph node cells. NB2 transfectants expressing the mouse CD6-4 cDNA (*lanes 1* and *2*), thymocytes activated with PMA and FCS (*lanes 3* and *4*), resting thymocytes (*lanes 5* and *6*), resting lymph node cells (*lanes 7* and *8* in *A*, and lanes *9* and *10* in *B*), and lymph node cells activated with PMA and FCS (*lanes 9* and *10* in *A*, and *lanes 7* and *8* in *B*) were surface labeled with ^{125}I, lysed, and the lysates precleared twice with protein A-Sepharose-purified preimmune rabbit Ab (the second preclearing is represented in *A* and *B*, *lanes 1*, *3*, *5*, *7*, and *9*) before immunoprecipitation with protein A-Sepharose-purified rabbit anti-mouse CD6 Ab (*A* and *B*, *lanes 2*, *4*, *6*, *8*, and *10*). Samples were electrophoresed on 8% SDS-polyacrylamide gels under reducing (*A*) or nonreducing (*B*) conditions. The migration positions of co-migrated ^{14}C-labeled M_r standards ($\times 10^{-3}$) are shown in the left margin.

it most likely that the CD6 mRNA detected in lung and skeletal muscle are derived from lymphoid cells associated with or present in the blood within these tissues, as no specific attempt was made to free these organs of blood prior to RNA isolation. In addition, a small amount of a shorter 2.2-kb CD6 mRNA is present in brain. Human CD6 protein is expressed in brain (7), and it is possible that an alternatively spliced form of CD6 mRNA (and possibly the encoded protein) is expressed in mouse brain. The faint band representing a 7-kb RNA species in kidney most likely does not represent expression of an alternative CD6 mRNA due to the large disparity in the size of this band as compared with that of the CD6 mRNA expressed in lymphoid tissues; this band could represent artifactual cross-hybridization of the CD6 cDNA probe to a larger, different mRNA species expressed in kidney, or perhaps an only partially spliced CD6 transcript. It is therefore unlikely that this mRNA species would encode CD6 protein.

Using gene gun plasmid DNA immunization of rabbits with the mouse CD6-4 cDNA in an expression vector, we generated a polyclonal antiserum specific for mouse CD6. This antiserum positively stained NB2 cells transfected with the mouse CD6-4 cDNA in an expression vector and did not stain untransfected NB2 cells. Using this antiserum we demonstrated that CD6 protein is expressed on the cell surface of most mouse thymocytes and is expressed at the highest levels on the mature $CD3^{high}$ population. In

addition, most or all peripheral lymph node and splenic T cells express CD6. Few or no lymph node or splenic B cells express mouse CD6. This pattern of expression is consistent with that described for human CD6, which is expressed at highest levels on mature medullary thymocytes and peripheral T cells (1–5). We also found that the level of CD6 expression on most mouse thymocytes and peripheral T cells is proportional to the level of expression of the homologous CD5 protein. Nevertheless, Northern blot analysis of various mouse T and B cell lines demonstrated that expression of CD6 does not necessarily correlate precisely with expression of CD5.

The rabbit anti-mouse CD6 Ab immunoprecipitated under reducing conditions a major polypeptide band of 128 kDa from resting thymocytes and lymph node T cells and 125 kDa from the NB2 cell transfectants expressing the CD6-4 cDNA. This small difference in size is most likely due to differences in glycosylation and/or phosphorylation. The mouse CD6 immunoprecipitates each contain a minor polypeptide band of 95 to 105 kDa under reducing conditions, which could represent the product of an alternatively spliced form of mouse CD6 mRNA (e.g., corresponding to the CD6-6 or another cDNA) or a form of CD6 that differs in its degree of phosphorylation, glycosylation, and/or degradation. Two forms of human CD6, 130, and 105/110 kDa, have been described and their difference in size can be accounted for by phosphorylation of the larger relative to the smaller form (14). Activation of human PBL with PMA and FCS results in phosphorylation of CD6 on serine and tyrosine and a concomitant 20- to 25-kDa increase in the apparent molecular mass (9, 13–15). Activation of mouse thymocytes or lymph node cells with PMA and FCS resulted in only a 2-kDa increase in size of the CD6 polypeptide, which is most likely due to phosphorylation of the larger relative to the smaller form. It is important to note that FCS induces phosphorylation of human CD6 in PBL (14) and that the "resting" mouse thymocytes and lymph node cells were kept in 5% FCS on ice for 2 h before radioiodination and immunoprecipitation. FCS-induced phosphorylation of human CD6 required incubation at 37°C for 2 h with 10% FCS to transform 50% of the CD6 polypeptide to the larger 130-kDa species (14). Nevertheless, it is possible that treatment of mouse cells with 5% FCS on ice for 2 h could induce phosphorylation of the majority of CD6, and that true resting mouse cells actually possess a less-phosphorylated major CD6 polypeptide of 100 to 105 kDa. It is also possible that mouse CD6 possesses a constitutive level of phosphorylation much higher than that of human CD6, which would be represented by the observed 128-kDa CD6 polypeptide band immunoprecipitated from thymocytes and lymph node cells kept in 5% FCS on ice for 2 h. Regardless, our immunoprecipitations demonstrate that activation of mouse thymocytes and lymph node cells with PMA and 10% FCS for 2 h at 37°C induces in a 2-kDa increase in size of the major CD6 polypeptide band as compared with incubation in 5% FCS on ice; it is likely that this difference in size is due to phosphorylation.

There is a 199 amino acid difference in the size of the predicted cytoplasmic domain encoded by the mouse CD6-4 cDNA clone as compared with the reported human CD6-15 cDNA clone (22). In nucleotide sequence comparisons we found a high degree of nucleotide identity in the mouse CD6-4 cDNA region encoding the cytoplasmic domain as compared with the 3′ untranslated region of the reported human CD6-15 cDNA, suggesting that the 3′ untranslated region of human CD6-15 might, in fact, represent coding sequence. The predicted m.w. of the unmodified human CD6-15 polypeptide chain of 48 kDa is 40 kDa smaller than the observed m.w. of 88 kDa for nascent CD6 polypeptide in both thymocytes and peripheral blood T cells (13, 22). In addition, the observed m.w. of 90 kDa for cell-surface human CD6-15 polypeptide immunoprecipitated from transiently transfected Cos cells is 15 and 40 kDa smaller, respectively, than the 105-/110- and 130-kDa m.w. observed for cell-surface human CD6 immunoprecipitated from thymocytes, PBLs, and the HPB-ALL cell line (13, 14, 22). These large discrepancies led us to clone the 3′ region of human CD6 cDNA from RNA isolated from PBLs. We found a small insertion that results in a frame shift as well as a second larger insertion relative to the reported sequence of human CD6-15; these changes together result in a predicted cytoplasmic domain highly identical to that of mouse CD6 (42a). We anticipate that this long cytoplasmic domain in mouse and human CD6 plays an important role in signal transduction by this protein. Based on the exon/intron boundaries that they have identified in the mouse CD6 gene, Whitney et al. (24) predicted that the human CD6-15 clone might have resulted from alternative mRNA splicing, and our sequence of human CD6 from PBLs indicates that this is likely to be the case (42a).

The mouse CD6-6 cDNA lacks 462 nucleotides present in the CD6-4 cDNA and encodes a 143-amino acid cytoplasmic domain with nine distinct amino acids at its carboxyl terminus, as compared with the 243-amino acid cytoplasmic domain of CD6-4 (Figs. 1 and 3). Based on the preliminary genomic organization of the mouse CD6 gene in which "at least ten exons" were identified by Whitney et al. (24), the boundaries of the sequence missing in CD6-6 do not correspond to any of the identified exon-intron junctions. The CD6-6 cDNA may therefore result from a cloning artifact, an alternatively spliced mRNA transcript making use of exon/intron junctions not previously identified or conceivably the product of a second CD6 gene. Minor polypeptide bands potentially representing the CD6-6 species of protein are present in CD6 immunoprecipitates from thymocytes and lymph node cells, although a similar band is also present in the NB2 transfectants expressing the CD6-4 cDNA. If the minor band in the NB2 transfectants represents the same protein species as seen in lymphoid cells, it would have to represent post-translational modification of the CD6 protein (e.g., differences in phosphorylation, glycosylation, or potentially protein degradation).

We compared the predicted amino acid sequence of mouse CD6 with all the protein sequences available in the National Biomedical Research Foundation database (Georgetown University, Washington, DC) using the FASTA program (43). Mouse CD6 is homologous to human CD6, mouse and human CD5, as well as the other members of the SRCR family. We also compared the predicted amino acid sequence of the cytoplasmic domain of mouse CD6 with the National Biomedical Research Foundation database and found no significant homologies. Using the Quest program and the Prosite 12 database (IntelliGenetics, Mountain View, CA) to search for amino acid sequence motifs in the full-length cytoplasmic domain of mouse CD6 we found three motifs containing the PKC phosphorylation site consensus sequence Ser/Thr-X-Arg/Lys (where X represents a nonconserved residue, and serine or threonine is phosphorylated) (39) and nine motifs containing the casein kinase-2 phosphorylation site consensus sequence Ser/Thr-X-X-Asp/Glu (where X represents a nonconserved residue, and serine or threonine is phosphorylated) (40). These motifs represent potential sites for phosphorylation of the cytoplasmic domain of mouse CD6 by PKC, casein kinase-2, or a different, related kinase. PKC and casein kinase-2 phosphorylate several different transmembrane protein receptors (39, 40), and it is intriguing that activation of human PBL with PMA, an activator of PKC, induces hyperphosphorylation of serine residues in human CD6 (14).

The cytoplasmic tail of mouse CD6 contains the serine-threonine-rich motif Asp-Ser-Ser-Ser/Thr-Y-Ser-X-X-Glu-X-Tyr in triplicate (X represents a nonconserved residue, and Y represents a nonconserved residue or a gap in the alignment). These motifs

are potential sites for phosphorylation of the cytoplasmic tail on serine, threonine, and/or tyrosine as well as for interaction of CD6 with cytoplasmic signaling or structural proteins. The predicted cytoplasmic domain of CD6-4 also possesses the proline-rich sequences Cys-Pro-Gly-Pro-Pro-Gly-Pro at position 641 and Gln-Pro-Pro-Pro-Gln-His-Pro-Pro at position 628, which contain the class I SH3 domain-binding consensus motif X-Pro-X-Pro-Y-X-Pro (X represents a nonconserved residue, and Y represents proline or a nonconserved residue) (41). This second proline-rich sequence at position 628 is closely related to the Gln-Pro-Pro-Pro-Leu-Ala-Pro SH3-domain binding motif of chicken YAP65 (41). These proline-rich motifs are potential recognition sites for SH3-domain containing structural or signaling proteins involved in CD6 function. The mouse CD6 cytoplasmic region also possesses the proline-rich sequence Asp-Pro-Ser-Ser-Lys-Pro-Pro-Pro-Trp at position 571 (Figs. 1 and 2), although this sequence does not possess significant identity to the SH3 domain-binding consensus sequences (41).

We also observed the amino acid motif Tyr-Asp-Asp-Ile at position 659 in the cytoplasmic domain of mouse CD6, and this motif has been designated as a possible recognition specificity of the SH2 domain of c-*fgr* based on the screening of phosphopeptide libraries using affinity columns containing the c-*fgr* SH2 domain (42). c-*fgr* is expressed in normal differentiated bone marrow-derived myeloid cells including granulocytes, monocytes, and alveolar macrophages (44). Although EBV-transformed human B cells also express c-*fgr*, no c-*fgr* expression has been observed in T cells, NK cells, and untransformed B cells (44), and thus c-*fgr* is not likely to be involved in CD6-mediated signaling in T cells although a different *src* family member may be.

Our data demonstrate that mouse T cells express a previously undescribed 130-kDa protein representing the mouse homologue to human CD6. In addition to the homology of the extracellular domains of CD5 and CD6, both CD5 and human CD6 can provide signals to costimulate T cell proliferation, and both are phosphorylated on tyrosine and serine upon T cell activation (8–12, 14, 15, 45–47). CD5 binds to the B cell surface protein CD72 (48). Recently human CD6 was shown to bind ALCAM, a molecule expressed on thymic epithelial cells (20). We demonstrated that the cytoplasmic domain of mouse CD6 contains 243 amino acids, which is significantly larger than the 94-amino acid cytoplasmic domains of mouse and human CD5 (49, 50).

We also found that the pattern of expression of mouse CD6 does not correlate entirely with that of mouse CD5 on T cell lines. Together these data suggest that CD5 and CD6 may transduce different context-dependent signals to T cells. Better understanding of the pattern of expression of CD5 and CD6 relative to each other as well as delineation of the cytoplasmic and cell-surface molecules with which they interact is critical to generating further hypotheses regarding the potential different functional roles of these molecules in T cell development and activation.

Acknowledgments

We thank Drs. M. Davis and J. Jorgensen for the cDNA library, Dr. B. Seed for the human CD6-15 cDNA, and Drs. J. Arthos and J. Mullins for the JW4303 expression vector. We thank Drs. P. Lu and H. E. N. de Vegvar for valuable advice.

References

1. Reinherz, E. L., S. Meuer, K. A. Fitzgerald, R. E. Hussey, H. Levine, and S. F. Schlossman. 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. *Cell 30:735.*
2. Reinherz, E. L., R. Geha, J. M. Rappeport, M. Wilson, A. C. Penta, R. E. Hussey, K. A. Fitzgerald, J. F. Daley, H. Levine, F. S. Rosen, and S. F. Schlossman. 1982. Reconstitution after transplantation with T-lymphocyte-depleted HLA haplotype-mismatched bone marrow for severe combined immunodeficiency. *Proc. Natl. Acad. Sci. USA 79:6047.*
3. Kamoun, M., M. E. Kadin, P. J. Martin, J. Nettleton, and J. A. Hansen. 1981. A novel human T cell antigen preferentially expressed on mature T cells and shared by both well and poorly differentiated B cell leukemias and lymphomas. *J. Immunol. 127: 987.*
4. Bastin, J. M., S. Granger, N. Tidman, G. Janossy, and A. J. McMichael. 1981. Recognition of a human T-lymphocyte differentiation antigen by an IgM monoclonal antibody. *Clin. Exp. Immunol. 46:597.*
5. Morimoto, C., C. E. Rudd, N. L. Letvin, M. Hagan, and S. F. Schlossman. 1988. 2H1—A novel antigen involved in T lymphocyte triggering. *J. Immunol. 140: 2165.*
6. Endres, N., G. Riethmuller, and E. P. Rieber. 1989. Functional characterization of a novel B-cell subset defined by the CD6 antigen. White cell differentiation antigens. In *Leucocyte Typing IV.* W. Knapp, B. Dorken, W. R. Gilks, E. P. Rieber, R. E. Schmidt, H. Stein, and A. E. G. Kr. von dem Borne, eds. Oxford University Press, New York, NY, p. 340.
7. Mayer, B., I. Funke, B. Seed, G. Riethmuller, and E. Weiss. 1990. Expression of the CD6 T lymphocyte differentiation antigen in normal human brain. *J. Neuroimmunol. 29:193.*
8. Walker, C., F. Bettens, and W. J. Pichler. 1987. Activation of T cells by cross-linking an anti-CD3 antibody with a second anti-T cell antibody: mechanism and subset-specific activation. *Eur. J. Immunol. 17:873.*
9. Swack, J. A., R. M. Gangemi, C. E. Rudd, C. Morimoto, S. F. Schlossman, and P. L. Romain. 1989. Structural characterization of CD6: properties of two distinct epitopes involved in T cell activation. *Mol. Immunol. 26:1037.*
10. Gangemi, R. M., J. A. Swack, D. M. Gaviria, and P. L. Romain. 1989. Anti-T12, an anti-CD6 monoclonal antibody, can activate human T lymphocytes. *J. Immunol. 143:2439.*
11. Bott, C. M., J. B. Doshi, C. Morimoto, P. L. Romain, and D. A. Fox. 1993. Activation of human T cells through CD6: functional effects of a novel anti-CD6 monoclonal antibody and definition of four epitopes of the CD6 glycoprotein. *Int. Immunol. 5:783.*
12. Osorio, L. M., C. A. Garcia, M. Jondal, and S. C. Chow. 1994. The anti-CD6 mAb, IOR-T1, defined a new epitope on the human CD6 molecule that induces greater responsiveness in T cell receptor/CD3-mediated T cell proliferation. *Cell. Immunol. 154:123.*
13. Swack, J. A., J. W. Mier, P. L. Romain, S. R. Hull, and C. E. Rudd. 1991. Biosynthesis and post-translational modification of CD6, a T cell signal-transducing molecule. *J. Biol. Chem. 266:7137.*
14. Cardenas, L., A. C. Carrera, E. Yague, R. Pulido, F. Sanchez-Madrid, and M. O. de Landazuri. 1990. Phosphorylation-dephosphorylation of the CD6 glycoprotein renders two isoforms of 130 and 105 kilodaltons: effect of serum and protein kinase C activators. *J. Immunol. 145:1450.*
15. Wee, S. F., G. L. Schieven, J. M. Kirihara, T. T. Tsu, J. A. Ledbetter, and A. Aruffo. 1993. Tyrosine phosphorylation of CD6 by stimulation of CD3: augmentation by the CD4 and CD2 coreceptors. *J. Exp. Med. 177:219.*
16. Kirkman, R. L., J. L. Araujo, G. J. Busch, C. B. Carpenter, E. L. Milford, E. L. Reinherz, S. F. Schlossman, T. B. Strom, and N. L. Tilney. 1983. Treatment of acute renal allograft rejection with monoclonal anti-T12 antibody. *Transplantation 36:620.*
17. Soiffer, R. J., C. Murray, P. Mauch, K. C. Anderson, A. S. Freedman, S. N. Rabinowe, T. Takvorian, M. J. Robertson, N. Spector, R. Gonin, K. B. Miller, R. A. Rudders, A. Freeman, K. Blake, F. Coral, L. M. Nadler, and J. Ritz. 1992. Prevention of graft-versus-host disease by selective depletion of CD6 positive T lymphocytes from donor marrow. *J. Clin. Oncol. 10:1191.*
18. Vollger, L. W., D. T. Tuck, T. A. Springer, B. F. Haynes, and K. H. Singer. 1987. Thymocyte binding to human thymic epithelial cells is inhibited by monoclonal antibodies to CD-2 and LFA-3 antigens. *J. Immunol. 138:358.*
19. Patel, D. D., S.-F. Wee, L. P. Whichard, M. A. Bowen, J. M. Pesando, A. Aruffo, and B. F. Haynes. 1995. Identification and characterization of a 100-kD ligand for CD6 on human thymic epithelial cells. *J. Exp. Med. 181:1563.*
20. Bowen, M. A., D. D. Patel, X. Li, B. Modrell, A. R. Malacko, W.-C. Wang, H. Marquardt, M. Neubauer, J. M. Pesando, U. Franke, B. F. Haynes, and A. Aruffo. 1995. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. *J. Exp. Med. 181:2213.*
21. Yssel, H., J. E. De Vries, J. Borst, and H. Spits. 1987. Distribution and functional analysis of a 120–130 kDa T-cell surface antigen. *Cell. Immunol. 105:161.*
22. Aruffo, A., M. B. Melnick, P. S. Linsley, and B. Seed. 1991. The lymphocyte glycoprotein CD6 contains a repeated domain structure characteristic of a new family of cell surface and secreted proteins. *J. Exp. Med. 174:949.*
23. Seed, B., and A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA 84:3365.*
24. Whitney, G., M. Bowen, M. Neubauer, and A. Aruffo. 1995. Cloning and characterization of murine CD6. *Mol. Immunol. 32:89.*
25. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem. 132:6.*
26. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74:5463.*
27. Allison, J. P., and L. L. Lanier. 1985. Identification of antigen receptor-associated structures on murine T cells. *Nature 314:107.*
28. Hashimoto, Y., and K. J. Blank. 1990. T cell receptor gene and T cell development in virus transformed early T cell lines. *J. Immunol. 144:1518.*
29. Altevogt, P., J. T. Kurnick, A. K. Kimura, K. Bowsslet, and V. Schirrmacher. 1982. Different expression of Lyt differentiation antigens and cell surface glycoproteins by a murine T lymphoma line and its highly metastatic variant. *Eur. J. Immunol. 12:300.*

30. Zamoyska, R., A. C. Vollmer, K. C. Sizer, C. W. Liaw, and J. R. Parnes. 1985. Two Lyt-2 polypeptides arise from a single gene by alternative splicing patterns of mRNA. *Cell 43:153.*
31. Lin, A. Y., B. Devaux, A. Green, C. Sagerstrom, J. F. Elliott, and M. M. Davis. 1990. Expression of T cell antigen receptor heterodimers in a lipid-linked form. *Science 249:677.*
32. Fynan, E. G., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene gun inoculations. *Proc. Natl. Acad. Sci. USA 90:11478.*
33. Tang, D.-C., M. De Vit, and S. A. Johnson. 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature 336:152.*
34. Eisenbraun, M. D., D. H. Fuller, and J. R. Haynes. 1993. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. *DNA Cell Biol. 12:791.*
35. Chapman, B. S., R. M. Thayer, K. A. Vincent, and N. L. Haigwood. 1991. Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res. 19:3979.*
36. Von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res. 14:4683.*
37. Freeman, M., J. Ashkenas, D. J. G. Rees, D. M. Kingsley, N. G. Copeland, N. A. Jenkins, and M. Krieger. 1990. An ancient, highly conserved family of cysteine-rich protein domains revealed by cloning of the type I and type II murine macrophage scavenger receptors. *Proc. Natl. Acad. Sci. USA 87:8810.*
38. Bazan, J. F. 1990. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA 87:6934.*
39. Pinna, L. A. 1990. Casein kinase-2: an "eminence grise" in cellular regulation? *Biochimi Biophys Acta 1054:267.*
40. Woodgett, J. R., K. L. Gould, and T. Hunter. 1986. Substrate specificity of protein kinase C. *Eur. J. Biochem. 161:177.*
41. Feng, S., J. K. Chen, H. Yu, J. A. Simon, S. L. Schreiber. 1994. Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. *Science 266:1241.*
42. Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, J. Hanafusa, B. Schaffhausen, and L. C. Cantly. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell 72:767.*
42a. Robinson, W. H., M. E. Neuman de Vegvar, S. S. Prohaska, J. W. Rhee, and J. R. Parnes. Human CD6 possesses a large, alternatively spliced cytoplasmic domain. *Eur. J. Immunol. In press.*
43. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res. 12:387.*
44. Bolen, J. B., P. A. Thompson, E. Eiseman, and I. D. Horak. 1991. Expression and interactions of the Src family of tyrosine protein kinases in T lymphocytes. *Adv. Cancer Res. 57:103.*
45. Spertini, F., W. Stohl, N. Ramesh, C. Moody, and R. S. Geha. 1991. Induction of human T cell proliferation by a monoclonal antibody to CD5. *J. Immunol. 146: 47.*
46. Burgess, K. E., M. Yamamoto, K. V. Prasad, and C. E. Rudd. 1992. CD5 acts as a tyrosine kinase substrate within a receptor complex comprising T-cell receptor zeta chain/CD3 and protein-tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. *Proc. Natl. Acad. Sci. USA 89:9311.*
47. Davies, A. A., S. C. Ley, and M. J. Crumpton. 1992. CD5 is phosphorylated on tyrosine after stimulation of the T-cell antigen receptor complex. *Proc. Natl. Acad. Sci. USA 89:6368.*
48. Van de Velde, H., I. von Hoegen, W. Luo, J. R. Parnes, and K. Thielemans. 1991. The B-cell surface protein CD72/Lyb-2 is the ligand for CD5. *Nature 351:662.*
49. Jones, N. H., M. L. Clabby, D. P. Dialynas, H-J. S. Huang, L. A. Herzenberg, and J. L. Strominger. 1986. Isolation of complementary DNA clones encoding the human lymphocytes glycoprotein T1/Leu-1. *Nature 323:346.*
50. Huang, H-J. S., N. H. Jones, J. L. Strominger, and L. A. Herzenberg. 1987. Molecular cloning of Ly-1, a membrane glycoprotein of mouse T lymphocytes and a subset of B cells: molecular homology to its human counterpart Leu-1/T1 (CD5). *Proc. Natl. Acad. Sci. USA 84:204.*