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Immunology

Delineation of a T-cell activation motif required for binding of protein tyrosine kinases containing tandem SH2 domains

(T-cell receptor/protein–protein interaction/interleukin 2/calcium/crosslinking)

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ABSTRACT **To define the T-cell receptor signal transduction motif, we have transfected human and murine T-cell lines with a chimeric receptor consisting of the extracellular and transmembrane domains of human CD8α and the membrane-proximal portion of CD3ζ containing at its C terminus either an 18-amino acid segment (NQLYNELNLGRREEYDVL) or alanine-scanning point mutant derivatives. Crosslinking of the extracellular domain of the chimera is sufficient to initiate Ca^{2+} flux, interleukin 2 production, and tyrosine phosphorylation of cellular proteins including the chimera. Subsequently, the chimera becomes associated with several tyrosine-phosphorylated proteins, among them the 70-kDa protein tyrosine kinase ZAP70. Mutational data identify the T-cell activation motif as $Y(X)_2L(X)_7Y(X)_2L$ and show that each of the four designated residues is necessary for the above activation events. Recombinant protein containing the two tandem SH2 domains derived from ZAP70 binds to a synthetic peptide corresponding to the above 18-amino acid motif but only when both tyrosines are phosphorylated; in contrast, little or no binding is observed to monophosphorylated or nonphosphorylated analogues. These results imply that after receptor crosslinking in T cells, and by inference also in B cells and mast cells, the motif is phosphorylated on both tyrosine residues, thereafter serving as a docking site for protein tyrosine kinases containing tandem SH2 domains.**

The degenerate amino acid sequence $D/E(X)_7D/E(X)_2Y(X)_2L(X)_7Y(X)_2L/I$ found in CD3γ, δ, ε, ζ, and η subunits of the T-cell receptor (TCR), Igα and β subunits of the B-cell antigen receptor, and β and γ subunits of the high-affinity IgE receptor has been implicated in signaling through these receptors (1). Studies with chimeric receptors consisting of the cytoplasmic domain of CD3ζ and extracellular domains of distinct receptors have shown that the cytoplasmic portion of CD3ζ is sufficient for transmitting signals through the chimeric receptors upon crosslinking of the extracellular domains (2–5). To precisely define the T-cell signal-transduction motif, we have transfected human and murine T-cell lines with a chimeric receptor (CD8α–ζΔ) consisting of the extracellular and transmembrane domains of human CD8α and the membrane-proximal 35 amino acids of CD3ζ containing at its C terminus either an 18-amino acid segment (NQLYNELNLGRREEYDVL) or alanine scanning point mutant derivatives. This is the first of three such related sequences in the CD3ζ cytoplasmic tail. Studies with these chimeric receptors unequivocally identify the T-cell activation motif as $Y(X)_2L(X)_7Y(X)_2L$ and show that each of the designated residues is necessary for T-cell activation events. Furthermore, we demonstrate that both tyrosine residues in a single motif must be phosphorylated for interaction of this motif with the protein tyrosine kinase (PTK) ZAP70 containing tandem SH2 domains.

MATERIALS AND METHODS

Generation of CD8α–ζΔ Chimeras and Transfectants. The CD8α–ζΔ construct was created by PCR as described (6) except that a 3′ antisense oligonucleotide 5′-CGCG-AGATCTTTACAAAACATCGTACTCCTCTCT-3′ including nt 333–312 of ref. 7 (underlined), a termination codon (double underlined), and *Bgl* II restriction site (boldface type) was used instead of a 3′ antisense oligonucleotide derived from the 3′ end of the CD3ζ cDNA coding region. The chimeric PCR product contains a termination codon TAA at amino acid position 87 (numbered according to ref. 7). The chimeric PCR product was gel-purified, digested with *Bgl* II, and subcloned into the *Bam*HI site of the pPink-2 vector (8). Specific mutations were made by standard site-directed mutagenesis (Amersham) with oligonucleotides designed to mutate each individual amino acid to alanine or each tyrosine to phenylalanine or glutamic acid. The $CD3\zeta^-\eta^-$ mouse T-cell hybridoma MA5.8 cells (9) or the TCR^- Jurkat variant 31.13 cells (10) were transfected with the chimeric construct by electroporation, and cells expressing the chimeric receptors were screened with anti-human CD8α monoclonal antibody (mAb) 21Thy2D3 as described (6).

Interleukin 2 (IL-2) Production Assay. Approximately 2×10^5 cells were cultured in an Immulon ELISA plate coated with 145-2C11 (anti-mouse CD3ε) at 1 μg per well or 21Thy2D3 at 1 μg per well for 24 h. Culture supernatants were examined for IL-2 activity with an indicator cell line CTLL-20 as described (11, 12). Recombinant IL-2 (Biogen; 4×10^6 units/mg) was used as a standard.

Ca^{2+} Assay. Approximately 2×10^6 cells were resuspended in 200 μl of medium containing 1 mM acetoxymethyl ester of Indo-1 (Molecular Probes). The cells were incubated for 30 min at 37°C and then diluted 1:10 with medium. Analysis was performed with an Epics V cell sorter as described (10).

Analysis of Tyrosine Phosphorylation. Immunoprecipitation was performed as described (12). Approximately $0.5–1 \times 10^8$ cells were stimulated with biotinylated 21Thy2D3 at 15 μg/ml and avidin at 25 μg/ml for 5 min. Cells were then washed with ice-cold PBS and lysed in 5 ml of a lysis buffer solution containing 1% Triton X-100 as described (12). Chimeras were immunoprecipitated with 20 μl of packed CNBr-activated Sepharose CL4B beads coupled to 7Pt3F9 (5 mg/ml), which recognizes a different epitope on human CD8α than 21Thy2D3. SDS/PAGE and Western blot analysis with 4G10

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Abbreviations: TCR, T-cell receptor; PTK, protein tyrosine kinase; GST, glutathione *S*-transferase; IL-2, interleukin 2; mAb, monoclonal antibody; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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mAb against phosphotyrosine (13) were performed as described (12).

Production of Recombinant SH2 Domains Derived from ZAP70. A glutathione *S*-transferase (GST) fusion protein (GST–NC) consisting of the N-terminal tandem SH2 domains (amino acids 1–267) of ZAP70 (14) was constructed by PCR amplification using primers with *Bam*HI sites. RNA from the Jurkat T cell line J77 was used as a template for reverse transcription. The PCR product was *Bam*HI-digested and subcloned into the vector pGEX-2TK (15, 16). Purification of GST–NC and control GST proteins was as described (16, 17). For production and purification of the isolated tandem SH2 domains, a PCR product containing amino acids 1–256 was made using a 5′ primer that included an *Nde* I restriction site and a 3′ primer that included three termination codons and a *Hin*dIII site and was subcloned into the *Nde* I–*Hin*dIII sites of pRSETA (Invitrogen). Subsequently, the NC protein was purified as described (18). The nucleotide sequence of each construct was confirmed prior to expression.

Biospecific Interaction Analysis. All the peptides were made on an ABI 431A peptide synthesizer by FastMOC HBTU/NMP chemistry with on-line real time UV-monitoring for the fluoren-9-ylmethoxycarbonyl (Fmoc) deprotection at each cycle using an LKB UVicord. All the reagents were obtained from Applied Biosystems, except Fmoc-phosphotyrosine and MBHA amide resin, which were obtained from Nova Biochem. Both diphosphopeptide (Y4 + Y15) and monophosphopeptide (Y15) were made during the same synthesis and split into two just before the variation occurred. The other monophosphopeptide (Y4) and nonphosphopeptide were synthesized in a second synthesis. The peptides were dissolved in 1 M NaCl/10 mM Hepes, pH 7.4, at 1 mg/ml (lyophilized powder weight). Individual peptides (0.2–0.5 mg/ml) were coupled to the sensor chip with twice the standard concentration of *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Solutions (7–20 μg/ml) of GST, GST–NC, NC, and anti-phosphotyrosine mAb 4G10, each diluted in 0.2 M NaCl/10 mM Mes, pH 6.0, were passed across the immobilized peptide surface. The bound protein was removed by pulses of 0.2 M NaCl/0.1 M $NaHCO_3$, pH 9.2, as seen on the sensorgrams as vertical spikes. The time during which protein is passed over the sensor chip is indicated by the brackets in Fig. 3*B*.

RESULTS AND DISCUSSION

Identification of the $Y(X)_2L(X)_7Y(X)_2L$ Motif in IL-2 Production. We generated CD8α–ζΔ and a series of alanine scanning mutants within the CD3ζ component of the chimera as indicated in Fig. 1 *Left*. The CD8α–ζΔ chimera consists of the extracellular and transmembrane region of human CD8α (amino acids −19 to 187; numbered as in ref. 19) connected with 35 CD3ζ membrane-proximal amino acids (amino acids 52–86 of the CD3ζ protein; numbered as in ref. 7). The $CD3\zeta^-\eta^-$ mouse T-cell hybridoma MA5.8 was then transfected with individual chimeric constructs and stable trans-

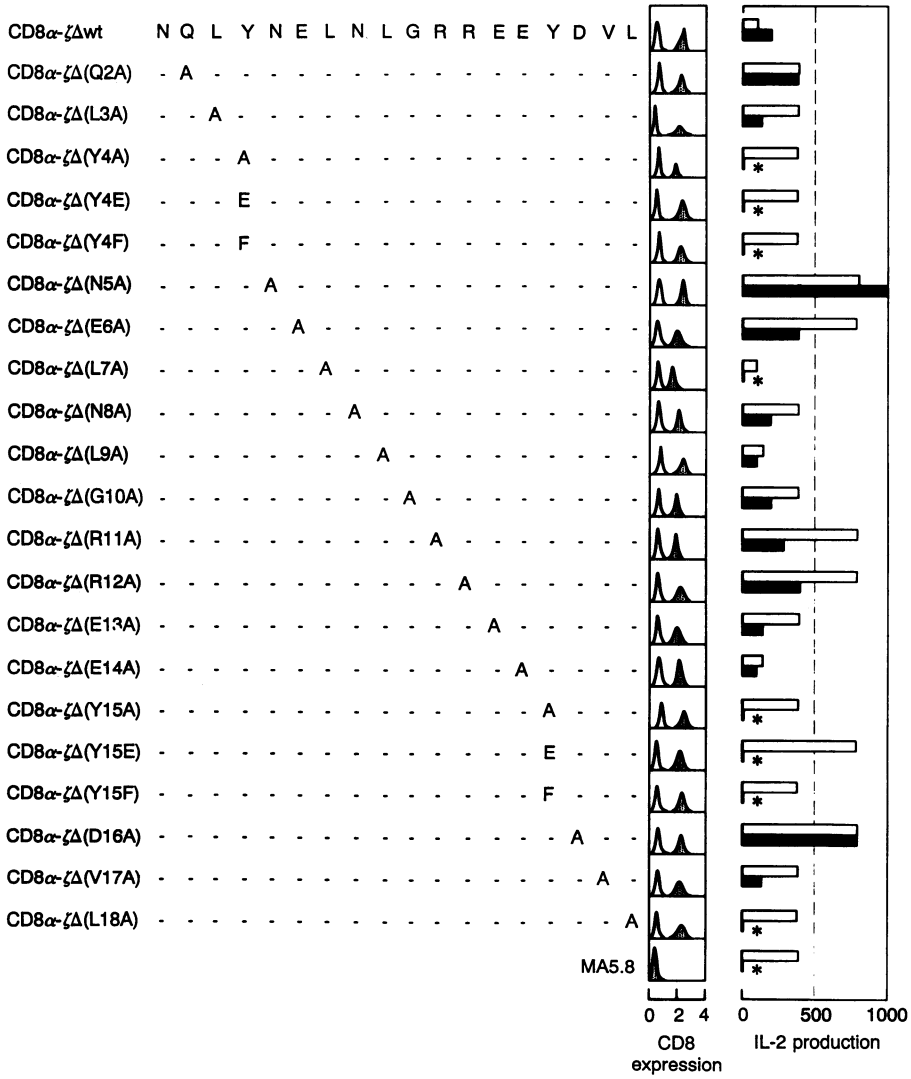

FIG. 1. Analysis of IL-2 production by transfectants containing CD8α–ζΔ chimeras mutated at CD3ζ amino acid residues. (*Left*) The sequence of the C-terminal 18 amino acids of the wild-type CD8α–ζΔ chimera (residues 69–86, according to ref. 7) and the name and sequence of each mutant are shown. −, Wild-type sequence. (*Center*) Flow cytometric analysis of chimera expression in MA5.8 cells. A representative clone of each mutant was stained with anti-human CD8α mAb 21Thy2D3 (stippled curve) or an irrelevant mAb (open curve) followed by fluorescein isothiocyanate-conjugated goat anti-mouse Ig(H+L), and 10,000 cells were analyzed on a FACScan (Becton Dickinson). The staining profile of the parental MA5.8 cells is at the bottom. CD8 expression is presented as the logarithm of fluorescence intensity. (*Right*) IL-2 production of transfectants. Cells were stimulated with immobilized 145-2C11 (anti-mouse CD3ε; open bars) or 21Thy2D3 (solid bars) for 24 h 1 μg per well. Culture supernatants were examined for IL-2 activity with an indicator cell line CTLL-20. IL-2 production is presented as unit activity per ml per 10^6 cells. At least two clones of each type were examined in three experiments and a representative result is shown. Essentially the same results were obtained with 10 μg of 21Thy2D3 per well. An asterisk indicates that no IL-2 activity (<1 unit/ml) was detected.

fectants of each type expressing similar copy numbers of the chimeric receptors were established after G418 selection (Fig. 1 *Center*). Prior studies indicated that chimeric CD8α–ζ receptors form mainly disulfide-linked trimers on the T-cell surface (2, 6). As shown in Fig. 1 *Right*, when crosslinked with immobilized anti-CD8α mAb 21Thy2D3, the CD8α–ζΔwt chimera transmits signals leading to IL-2 production. Among transfectants expressing various mutant chimeras, cells transfected with CD8α–ζΔ(Y4A), CD8α–ζΔ(L7A), CD8α–ζΔ(Y15A), and CD8α–ζΔ(L18A) were unable to produce IL-2 after anti-CD8 mAb-mediated crosslinking. In contrast, IL-2 production was stimulated through molecules with mutations in all the other amino acid positions to a level ≥50% of CD8α–ζΔwt. Further mutations at Y4 and Y15 showed that phenylalanine or glutamic acid was unable to substitute for tyrosine at either position. Lack of IL-2 production from the above transfectants was not due to an intrinsic inability of the cells to produce IL-2 because all transfectants produced IL-2 in response to immobilized anti-CD3ε mAb, which crosslinks the endogenous TCR on MA5.8. The importance of tyrosine residues in TCR (CD3ζ, CD3ε) and Fc receptor (FcεRIγ) signal transduction has been observed (4, 20–22). Our results clearly demonstrate that $Y(X)_2L(X)_7Y(X)_2L$ is the functional motif of the CD3ζ cytoplasmic segment and that the amino acids designated X are not critical for this activity. Of note, this cytoplasmic domain motif is present three times in CD3ζ, twice in CD3η, and once in CD3γ, δ, and ε, Igα and β, and FcεRIγ (1, 23). Similar findings were observed for IL-2 production with chimeras transfected into the human T-cell line 31.13, a TCR^- variant of Jurkat (data not shown). Thus, the motif is operative in human and murine T cells.

PTK Pathway Activation and Ca^{2+} Flux. Activation of the PTK pathway and the resulting Ca^{2+} flux are critical for T-cell activation (24–27). To examine triggering of these events through the chimeric proteins, a number of the constructs listed in Fig. 1 and CD8α–ζ (containing the entire CD3ζ cytoplasmic tail) (6) were transfected into 31.13 (Fig. 2*A*). Crosslinking of the extracellular domain of CD8α–ζΔwt with biotinylated anti-CD8α mAb 21Thy2D3 plus avidin triggered an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Fig. 2*B*). In contrast, Ca^{2+} flux was impaired in the transfectants carrying tyrosine and leucine mutations; little or no increase in $[Ca^{2+}]_i$ was observed in CD8α–ζΔ(Y4A) or CD8α–ζΔ(L7A) transfectants while CD8α–ζΔ(Y15A) and CD8α–ζΔ(L18A) transfectants consistently showed only a modest increment in $[Ca^{2+}]_i$ in a fraction of cells. These findings suggest that the function of Y4 is not identical to Y15 and, likewise, that L7 and L18 are not interchangeable. The increase in $[Ca^{2+}]_i$ in CD8α–ζ cells upon stimulation through the chimera did not require secondary crosslinking (Fig. 2*B*). Perhaps this is because the multiple copies of the motif in the full-length CD3ζ cytoplasmic domain chimera (28) make its signaling function more efficient than that of CD8α–ζΔwt. In addition, the CD8α–ζΔ(N5A) transfectant was also stimulated to undergo an increase in $[Ca^{2+}]_i$ after chimera crosslinking without any further requirement for a secondary reagent (Fig. 2*B*).

To next examine the PTK pathway, the 31.13 transfectants were stimulated by the anti-CD8 mAb for 5 min and tyrosine phosphorylation of cellular proteins was analyzed on Western blots with anti-phosphotyrosine mAb 4G10 (Fig. 2*C*). Consistent with the results of IL-2 and Ca^{2+} assays, tyrosine phosphorylation of cellular proteins was induced upon chimera crosslinking of CD8α–ζ, CD8α–ζΔwt, CD8α–ζΔ(N5A), and CD8α–ζΔ(E6A) transfectants, whereas CD8α–ζΔ(Y4A), CD8α–ζΔ(L7A), CD8α–ζΔ(Y15A), and CD8α–ζΔ(L18A) transfectants showed no inducible tyrosine phosphorylation, either in total cellular lysates (Fig. 2*C*) or in anti-CD8 mAb 7Pt3F9 immunoprecipitates of the chimera (Fig. 2*D*). The absence of inducible tyrosine phosphorylation was not due to an alteration in kinetics of the reaction; no phosphorylation was seen even 30 min after stimulation (data not shown).

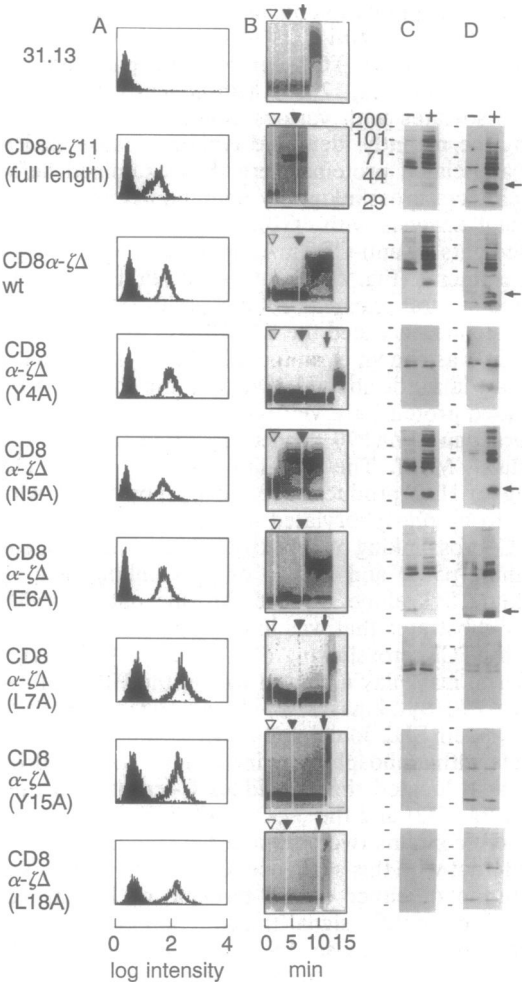

FIG. 2. Signal transduction function of the CD8α–ζΔ chimera and derivative mutants. (*A*) Flow cytometric analysis of chimera expression in 31.13 cells, a TCR^- variant of the Jurkat human T-cell line. CD8α–ζ11 cells transfected with a chimera containing the full-length CD3ζ tail were as described (6). Cells were stained with 21Thy2D3 (open curve) or an irrelevant (solid curve) mAb followed by fluorescein isothiocyanate-conjugated goat anti-mouse Ig(H+L) and analyzed on a FACScan. Flow cytometry data are presented as the logarithm of fluorescence intensity. (*B*) Ca^{2+} flux triggered by crosslinking of the CD8α extracellular domain of each transfectant. Changes in cytosolic Ca^{2+} concentration were monitored in cells loaded with the Ca^{2+}-sensitive dye Indo-1. Sequential additions to cell suspensions were biotinylated 21Thy2D3 (open arrowhead; 15 μg/ml), avidin (solid arrowhead; 25 μg/ml), and 2 μM A23187 (arrow). Results are expressed as the 410/480-nm emission ratio in arbitrary units (*y* axis) vs. time in minutes (*x* axis). Results are representative of at least four experiments. (*C*) Tyrosine phosphorylation of cellular proteins induced by crosslinking of the chimeras. Cells were unstimulated (−) or stimulated (+) with biotinylated 21Thy2D3 and avidin for 5 min. Cells were then lysed in a buffer solution containing 1% Triton X-100 and the postnuclear supernatant was resolved by SDS/PAGE. Tyrosine-phosphorylated proteins were detected by Western blot analysis with 4G10. Numbers on the left side indicate the migration positions of molecular mass markers in kDa. At least two clones were examined and results shown are representative of at least three experiments. (*D*) Analysis of tyrosine-phosphorylated proteins associated with the chimeras. Chimeric molecules were immunoprecipitated with another anti-human CD8α mAb 7Pt3F9 from the lysates prepared in *C*, resolved by SDS/PAGE, and analyzed on a Western blot with anti-phosphotyrosine mAb 4G10. Migration positions of molecular mass markers in kDa are shown to the left of *C*. Arrows indicate the phosphorylated chimeras.

After TCR stimulation by antigen and major histocompatibility complex or anti-CD3 mAb, the homodimeric CD3ζ subunits in a native TCR complex are phosphorylated on tyrosine residues (11, 12, 29). Subsequently, phosphorylated CD3ζ associates with various cellular phosphoproteins including the recently identified tyrosine kinase ZAP70 (14). Motif-associated proteins were thus examined before and after chimera crosslinking by immunoprecipitation and immunoblot analysis with 4G10. Stimulation of the "active" transfectants by anti-CD8 mAb induced the phosphorylation of the chimeras (Fig. 2*D*, arrows) and 7Pt3F9 coimmunoprecipitated several phosphoproteins of 70–180 kDa. The ≈70-kDa protein(s) included the ZAP70/Syk kinase family member ZAP70 as shown by immunoblot analysis with a specific antisera (data not shown), whereas the identities of other associated proteins are yet to be defined.

Interaction of ZAP70 Tandem SH2 Domains with the Phosphorylated Motif. The signaling capacity of the chimeras leading to IL-2 production perfectly correlated with their ability to be phosphorylated and associate with other proteins. Cocrosslinking of the extracellular domain of chimeras containing $p56^{lck}$ and ZAP70 or crosslinking of a chimera containing Syk alone induced tyrosine phosphorylation of cellular substrates that was indistinguishable from that triggered by TCR crosslinking (30). Thus, ZAP70/Syk family member(s) may play a central role in the PTK pathway. Of note, ZAP70/Syk kinases are characterized by the presence of two tandem SH2 domains (14, 31). Because an SH2 domain interacts with a phosphotyrosine residue and a hydrophobic amino acid located three residues C-terminal to the phosphotyrosine (32) and the $Y(X)_2L(X)_7Y(X)_2L$ motif we identified here contains two potential SH2 binding sites, ZAP70 may interact with this sequence. Consistent with this notion, substitution of either of the tyrosine or leucine residues impaired the motif's signal transduction capabilities as described above. Likewise, the inability of phenylalanine or glutamic acid to substitute for tyrosine can be understood in view of the important roles of both the phosphate moiety and hydrophobic phenol ring in the interaction with the tyrosine pocket of structurally characterized SH2 domains (33–35). To address the possibility that ZAP70 binds to this motif, we have produced the recombinant ZAP70 tandem SH2 domains as either a GST fusion protein (termed GST–NC) or a native protein (termed NC) (Fig. 3*A*) and synthesized four peptides corresponding to nonphosphorylated, monophosphorylated, and diphosphorylated forms of the motif. We then employed biospecific interaction analysis (36) on the BIAcore (Pharmacia) to examine the interaction of the SH2 domains with these peptides coupled to dextran matrices on the surface of a sensor chip. As shown in Fig. 3*B*, the recombinant tandem SH2 domains as a native protein or a GST fusion protein bound to the diphosphopeptide. The apparent difference in the off-rate of NC vs. GST–NC proteins may be a consequence of the GST component of the fusion protein. In contrast, little or no binding was seen to Y15 monophosphorylated (Fig. 3*B*), Y4 monophosphorylated (data not shown), or nonphosphorylated (Fig. 3*B*) motifs under comparable experimental conditions. Note that phosphotyrosine residues are intact in both monophosphopeptides and diphosphopeptides as judged by 4G10 anti-phosphotyrosine mAb reactivity (Fig. 3*B*). As expected, GST protein alone shows no binding to any peptides.

Implications. These results unequivocally demonstrate that both tyrosine residues in the motif must be phosphorylated for the binding of ZAP70. In agreement with these data, Wange *et al.* (37) have recently shown that both SH2 domains are required for the binding of ZAP70 to phosphorylated CD3ζ prepared from activated T cells and that individual SH2 domains are unable to stably bind to phosphorylated CD3ζ. Our findings now show that both SH2 domains interact with

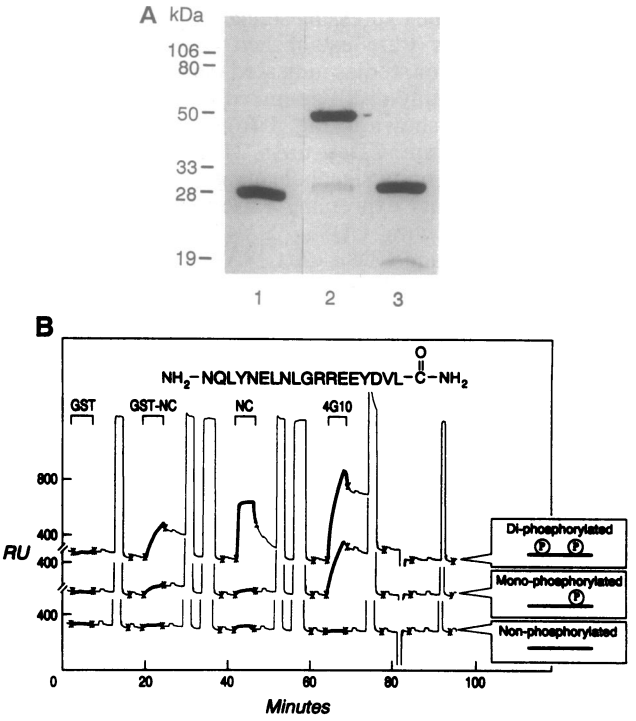

FIG. 3. Binding of the ZAP70 tandem SH2 domains requires a motif phosphorylated on both tyrosine residues. (*A*) Recombinant proteins expressed from various constructs with or without the ZAP70 tandem SH2 domains were analyzed by SDS/PAGE under reducing conditions and stained by Coomassie brilliant blue. Lanes: 1, GST protein; 2, GST–NC fusion protein; 3, NC protein. Numbers on the left indicate the migration positions of molecular mass markers. Prestained molecular mass markers (Bio-Rad) used here are phosphorylase *b* (106 kDa), bovine serum albumin (80 kDa), ovalbumin (50 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (28 kDa), and lysozyme (19 kDa). (*B*) Binding of the recombinant proteins to the peptide surface analyzed by BIAcore. In each sensorgram, 20 μl of the protein solution was passed at 5 μl/min across the indicated peptide surface. The peptide surface was regenerated between protein injections with the order of injections being GST (20 μg/ml), GST–NC (10 μg/ml), NC (12 μg/ml), and 4G10 (7 μg/ml). Diphosphorylated (Y4+Y15), monophosphorylated (Y15), and nonphosphorylated peptides were coupled to the sensor chip (top, middle, and bottom curves, respectively). RU, resonance unit.

the two PO_4-$Y(X)_2L(X)_2L$ sites in a single motif. The interaction of the two PO_4-$Y(X)_2L(X)_2L$ sites need not be restricted to the SH2 domains of ZAP70. A recent study demonstrated the functional coupling of CD3ζ expressed in COS cells with $p59^{fyn}$ in the absence of ZAP70 kinase (38). Presumably endogenous Syk or another kinase with tandem SH2 domains functions in lieu of ZAP70 to initiate phosphorylation events and Ca^{2+} flux. As noted below, it is also possible that the nonphosphorylated motif served as a critical binding site for *fyn*.

Evidence continues to accumulate as to the importance of Src family kinases in the TCR signaling process (38–41). However, the PTK that initially phosphorylates the $Y(X)_2L(X)_7Y(X)_2L$ motif is unknown. Among the Src family kinases, $p56^{lck}$ and/or $p59^{fyn}$ are candidates (38–41). In this regard, an unexpected finding of the present study is the effect of the L7 and L18 mutations; alteration of the leucines abolishes the tyrosine phosphorylation of the chimera, suggesting that these residues are essential not only for binding to SH2-containing proteins but also for the interaction with the PTK that phosphorylates the motif. Although binding of kinase domains to $Y(X)_2L$ has not been demonstrated, it is possible that such an interaction is critical for triggering the

phosphorylation of the $Y(X)_2L(X)_7Y(X)_2L$ motif. Alternatively, binding of SH2-containing proteins to the phosphorylated motif may sequester protein tyrosine phosphatases away from the motif and, hence, permit maintenance of a phosphorylated state. Rapid dephosphorylation may occur in the absence of the leucines because of a weakened SH2 interaction, thereby preventing further downstream signaling.

Ligand binding to the extracellular domain of the TCR alone is insufficient for cellular activation; receptor crosslinking is always required in addition (42, 43). The role of crosslinking is, however, still poorly understood. One possibility is that crosslinking may increase the local concentration of the receptors leading to a higher concentration of substrates available to the primary PTK(s). Alternatively or in addition, crosslinking may induce conformational changes within the motif which, in turn, provide the PTK(s) with optimally configured substrates.

In summary, this study advances our understanding of T-cell signaling as follows. (*i*) $Y(X)_2L(X)_7Y(X)_2L$ is directly shown to be the transduction motif in CD3ζ and, by inference, in other receptor signaling components that express this motif. Each of the residues at the other positions (X) is dispensable. (*ii*) The motif binds simultaneously to both SH2 domains of ZAP70 and presumably to other ZAP70/Syk family members containing tandem SH2 domains. For this binding to occur, both tyrosine residues in a single motif must be phosphorylated. This observation could not have been predicted *a priori*. In addition, consistent with the important ligand specificity determining role of the PO_4-Y+3 pocket (18) in Src-like SH2 domains, the leucine located three residues C-terminal to each tyrosine is apparently critical for the SH2 interaction. (*iii*) Both leucine residues are also involved in the primary reaction leading to detectable phosphorylation of the motif. This finding raises the interesting possibility that the leucines may be components of a recognition motif for a primary kinase. The precise role of the leucines in this activation process is now important to ascertain.

Note Added in Proof. Since submission of this manuscript, an independent report (44) has observed that both tyrosine residues of a single activation motif must be phosphorylated for ZAP70 binding. In addition, an important role for leucine residues in the motif has been reported by others (45).

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