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The catalytic activity of the CD45 membrane-proximal phosphatase domain is required for TCR signaling and regulation

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Cell surface expression of CD45, a receptor-like protein tyrosine phosphatase (PTPase), is required for T cell antigen receptor (TCR)-mediated signal transduction. Like the majority of transmembrane PTPases, CD45 contains two cytoplasmic phosphatase domains, whose relative *in vivo* function is not known. Site-directed mutagenesis of the individual catalytic residues of the two CD45 phosphatase domains indicates that the catalytic activity of the membrane-proximal domain is both necessary and sufficient for restoration of TCR signal transduction in a CD45-deficient cell. The putative catalytic activity of the distal phosphatase domain is not required for proximal TCR-mediated signaling events. Moreover, in the context of a chimeric PTPase receptor, the putative catalytic activity of the distal phosphatase domain is not required for ligand-induced negative regulation of PTPase function. We also demonstrate that the phosphorylation of the C-terminal tyrosine of Lck, a site of negative regulation, is reduced only when CD45 mutants with demonstrable *in vitro* phosphatase activity are introduced into the CD45-deficient cells. These results demonstrate that the phosphatase activity of CD45 is critical for TCR signaling, and for regulating the levels of C-terminal phosphorylated Lck molecules.

Key words: LCA/Lck/mutagenesis/transmembrane phosphatase/tyrosine kinase

Introduction

Reversible protein phosphorylation on tyrosine residues is a critical regulatory mechanism in controlling cellular effector functions, differentiation and proliferation (Cantley *et al.*, 1991). The overall level of cellular protein tyrosine phosphorylation is a result of the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). Analogous to the PTK family of enzymes, the PTPases can be classified into two structurally distinct groups: intracellular enzymes and transmembrane receptor-like proteins (reviewed in Fischer *et al.*, 1991). The cytosolic PTPases contain a single phosphatase domain of ~300 amino acids, flanked by unique amino- and/or C-terminal domains of variable size, which may regulate the function (Cool *et al.*, 1990) and/or subcellular localization of the cytosolic enzymes (Frangioni *et al.*, 1992). The transmembrane PTPases all express structurally unique and diverse extracellular domains which may interact with very distinct ligands. The cytosolic region of the majority of the transmembrane PTPases consist of two tandemly arranged phosphatase domains. Only PTPβ (Kreuger *et al.*, 1990) and the *Drosophila* phosphatase, dPTP10D (Tian *et al.*, 1991; Yang *et al.*, 1991), contain a single cytoplasmic phosphatase domain. While the majority of transmembrane PTPases contain two phosphatase domains, the function of this tandem arrangement is not understood. Moreover, the individual contribution of the two phosphatase domains to cellular functions is not known.

CD45 (leukocyte common antigen), a member of the transmembrane receptor-like subclass of PTPases, is exclusively expressed on nucleated cells of hematopoietic origin. Multiple isoforms of CD45 are expressed on cells as a result of developmental and cell type specific regulation of mRNA splicing of three exons which encode portions of the extracellular domain (reviewed in Trowbridge and Thomas, 1994). The complex regulation of the structure of the extracellular domain of CD45 suggests that this domain may have an important regulatory function. A putative ligand for CD45, CD22, has been identified; however, the specificity and functional consequences of the interaction of CD45 with CD22, if any, are not known (Stamenkovic *et al.*, 1991; Aruffo *et al.*, 1992).

A requisite function for CD45 was demonstrated from analysis of CD45-deficient T cells, which fail to proliferate or produce cytokines in response to stimulation of the T cell antigen receptor (TCR) (Pingel and Thomas, 1989; Weaver *et al.*, 1991). Moreover, CD45 expression has been shown to be required on B cells (Justement *et al.*, 1991) and natural killer cells (Bell *et al.*, 1993) for competent signal transduction through their respective antigen receptors. Furthermore, CD45 expression appears to be necessary for normal T cell development, as mice which fail to express CD45 have a severe defect in T cell ontogony (Kishihara *et al.*, 1993). Although B cell development does not appear to be affected in CD45-deficient mice, signal transduction through the B cell antigen receptor is abrogated.

In CD45-deficient cells, signal transduction through the TCR is blocked at the level of tyrosine phosphoprotein induction (Koretzky *et al.*, 1991). Since the TCR lacks intrinsic tyrosine kinase activity, it is thought that the TCR couples to at least one cytoplasmic PTK. The Fyn, Lck and ZAP-70 kinases are believed to participate in TCR signaling (reviewed in Chan *et al.*, 1994). While little is known regarding the regulation of ZAP-70, members of

the Src family of kinases can be regulated by tyrosine phosphorylation (Cooper and Howell, 1993). There are two known sites of regulation: a positive regulatory autophosphorylation site, and a C-terminal tyrosine, a site of negative regulation. The C-terminal tyrosine is not a site of autophosphorylation, but is instead thought to be phosphorylated *in vivo* by the ubiquitously expressed Csk kinase (Nada *et al.*, 1991). In T cells lacking CD45, the Lck and Fyn kinases are hyperphosphorylated on the C-terminal tyrosine, relative to Lck and Fyn isolated from cells expressing CD45 (Ostergaard *et al.*, 1989; Hurley *et al.*, 1993; McFarland *et al.*, 1993; Sieh *et al.*, 1993). Thus, a model for the signaling defect in CD45-deficient cells emerges in which CD45 maintains Lck and Fyn in a state in which they can be 'activated', by maintaining the C-terminal tyrosine in a dephosphorylated state.

CD45, like the majority of transmembrane PTPases, contains two tandem phosphatase domains; the *in vivo* functions of these domains are not known. Herein, the membrane-proximal phosphatase domain will be referred to as the first phosphatase domain or domain I, while the distal phosphatase domain will be referred to as the second phosphatase domain or domain II. *In vitro* enzymatic studies with bacterially expressed, or *in vitro* translated mRNA encoding the CD45 cytoplasmic domain suggest that only domain I has phosphatase function (Streuli *et al.*, 1990; Johnson *et al.*, 1992). In contrast, a CD45 mutant in which the catalytic cysteine of domain I was deleted appeared to be enzymatically active *in vitro* against the substrate myelin basic protein (MBP) (Tan *et al.*, 1993). Thus, it is not clear whether both phosphatase domains of CD45 are active. Moreover, the *in vivo* role of the two phosphatase domains of CD45 in TCR signaling processes has not been established.

We have previously demonstrated that a chimeric molecule consisting of the epidermal growth factor receptor (EGFR) extracellular and transmembrane domains fused to the cytoplasmic region of CD45, when introduced into a CD45-deficient cell, was able to restore TCR signaling functions (Desai *et al.*, 1993). Moreover, the function of the EGFR/CD45 chimera was negatively regulated by ligands to the EGFR, demonstrating that the function of the receptor-like PTPases (R-PTPases) could be regulated by ligands. This negative regulatory effect may represent the effect that the cognate ligand for CD45 would have. The regulatory effect of ligand on EGFR/CD45 function required dimerization of two full-length chimeric molecules, indicating a critical regulatory role for the cytoplasmic domain of the EGFR/CD45 chimera. The relative contribution of the two phosphatase domains in ligand-mediated regulation of CD45 function is unknown.

In order to determine the *in vivo* function of the CD45 phosphatase domains in regulating TCR signal transduction and in ligand-mediated negative regulation of CD45 function, we mutated individually and in combination the catalytic amino acid in the two phosphatase domains of CD45 and analyzed the ability of the various constructs to restore TCR signaling. In this report, we demonstrate that the function of the first phosphatase domain of CD45 is both necessary and sufficient for TCR-mediated signal transduction and for ligand-mediated regulation of CD45 function.

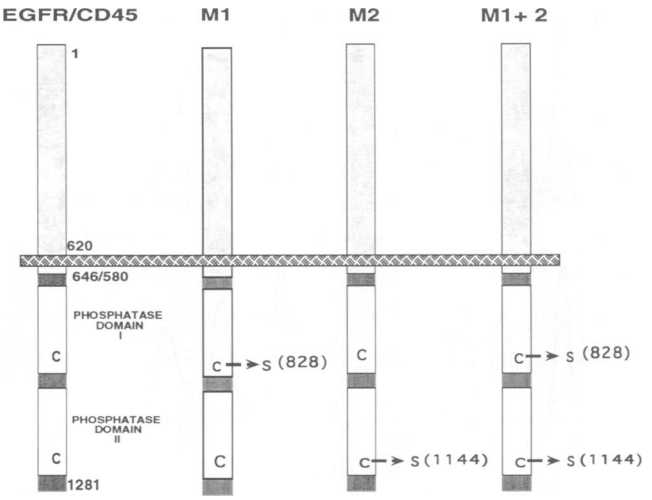

Fig. 1. Schematic representation of the EGFR/CD45 chimera and phosphatase domain point mutants. The numbers correspond to the amino acids from the mature EGFR and CD45 proteins which contribute to the formation of the EGFR/CD45 chimera. The sites of the catalytic cysteine to serine point mutations are indicated.

Results

Construction and characterization of EGFR/CD45 chimera point mutants

The phosphatase domains of both the cytosolic and transmembrane PTPases share an 11 amino acid consensus motif located near the C-terminus of the phosphatase domain [(I/V)HCXAGXXR(S/T)G] (Charbonneau and Tonks, 1992). Biochemical studies have demonstrated that the cysteine residue within this conserved motif acts as a nucleophile and forms a covalent thiol-phosphate bond with the phosphorylated tyrosine residue of the substrate (Guan and Dixon, 1991; Pot and Dixon, 1992). Thus mutation of this catalytic cysteine results in complete abolition of phosphatase activity. To characterize further the requirements for the two phosphatase domains of CD45 we introduced individual point mutations in the catalytic cysteine within the first (C828S) and second (C1144S) phosphatase domains of human CD45, resulting in the generation of the M1 and M2 mutants, respectively. In addition, a double point mutant was also created, referred to as M1+2 (Figure 1). The CD45 phosphatase domain mutations were introduced into a chimeric molecule, in which the extracellular and transmembrane domains of CD45 had been replaced with those of the EGFR. We have previously demonstrated that the EGFR/CD45 chimera functions analogously to wild-type CD45 with regard to reconstitution of proximal TCR signal transduction function (Desai *et al.*, 1993). The three mutant constructs were introduced into a CD45-deficient variant of the HPB-ALL leukemic cell line (H45). Multiple clones expressing each construct were isolated, analyzed and compared with H45 cells previously transfected with the wild-type EGFR/CD45 chimera. The phenotypes of representative clones expressing each construct are shown in Figure 2. Cell surface expression of the TCR, CD45 and the EGFR/CD45 chimera variants was quantified by immunofluorescence and flow cytometry. Importantly, the CD45-deficient cells expressed similar levels of the wild-

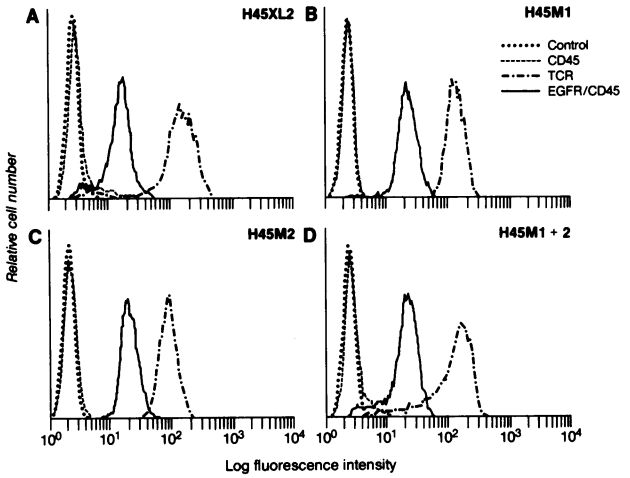

Fig. 2. Cell surface expression of the TCR, CD45 and the EGFR/CD45 chimera mutants. Cells were stained with a control mouse IgG2b mAb (dotted line), Leu4 (anti-CD3ε) for the TCR (alternating dashes and dots), GAP 8.3 mAb (anti-pan CD45; broken line), and LA22 (anti-EGFR, solid line). (**A**)–(**D**) depict CD45-deficient (H45) clones expressing the wild-type (H45XL2), M1, M2 and M1+2 EGFR/CD45 chimeras respectively.

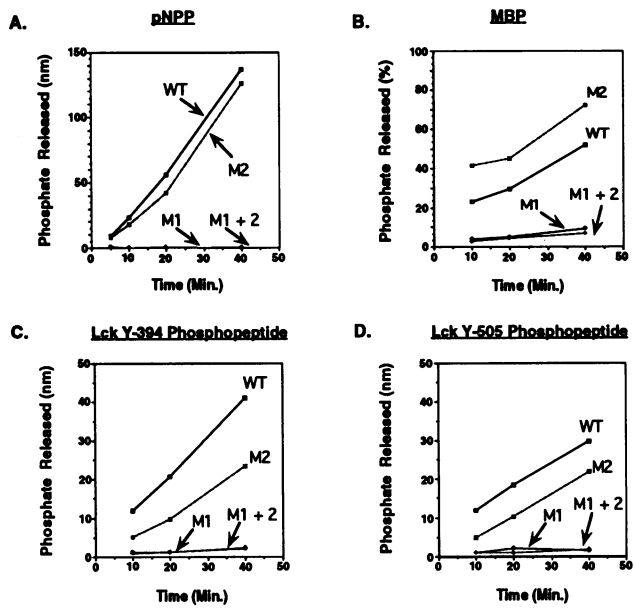

Fig. 3. Time-dependent dephosphorylation of different substrates by the wild-type and mutant EGFR/CD45 chimeras. The wild-type and mutant EGFR/CD45 chimera immunoprecipitates were divided into five aliquots. One was immunoblotted with an anti-CD45 cytoplasmic domain antiserum and the other four aliquots were incubated with phosphatase assay buffer plus (**A**) 10 mM pNPP; (**B**) 50 μM MBP; (**C**) 250 μM Lck autophosphorylation (Y394) phosphopeptide; and (**D**) 250 μM Lck C-terminal (Y505) phosphopeptide for the indicated times. The absorbance of the pNPP was measured at 405 nm. ^{32}P release from MBP was quantified by scintillation counting, while inorganic phosphate release from the phosphopeptides was quantified using the ascorbic acid/molybdenate reaction. Points shown represent values obtained following normalization for protein amount and subtraction of background.

type EGFR/CD45 chimera (H45XL2; Figure 2A), phosphatase domain I mutation (H45M1; Figure 2B), domain II mutation (H45M2; Figure 2C), and a double mutation (H45M1+2; Figure 2D). While none of the clones expressed CD45, they maintained high level expression

Table I. Phosphatase activities of wild-type and M2 EGFR/CD45 chimeras

Enzyme	K_m (μM)		V_{max} (μmol/min/unit)	
	pNPP	Y505 peptide	pNPP	Y505 peptide
Wild-type EGFR/CD45	15.5	0.39	55.3	91.3
M2 EGFR/CD45	14.5	0.31	46.0	84.5

The wild-type EGFR/CD45 or M2 EGFR/CD45 chimeras were immunoprecipitated from equivalent numbers of cells expressing similar levels of chimeras. The immunoprecipitates were assayed for enzymatic activity, which was normalized for the relative amount of each protein by immunoblotting a portion of the sample with an anti-CD45 mAb.

of the TCR (Figure 2A–D). To confirm that the various clones did indeed express the correct CD45 mutation, RNA was isolated from each clone, and RT-PCR fragments containing the catalytic cysteines of domains I and II were sequenced (data not shown).

Introduction of either the individual cysteine or double cysteine mutations did not have any apparent effect on the size or structure of the EGFR/CD45 chimera (data not shown). To characterize the effect of mutating the conserved cysteines in domains I and II on the enzymatic activity of CD45, the various chimeras were immunoprecipitated and analyzed for phosphatase activity utilizing a number of substrates. The wild-type EGFR/CD45 chimera displayed time-dependent phosphatase activity against *para*-nitrophenyl phosphate (pNPP) (Figure 3A), MBP phosphorylated with purified c-Src (Figure 3B), and phosphopeptides encompassing the autophosphorylation (Y394) or negative regulatory (Y505) sites of the Lck protein tyrosine kinase (Figure 3C and D). Mutation of the catalytic site within the first phosphatase domain (M1) rendered the EGFR/CD45 chimera inactive against all substrates, and was virtually indistinguishable from the double phosphatase domain mutant (M1+2). Mutation of the catalytic cysteine in the second phosphatase domain resulted in a molecule which appeared to have catalytic activity comparable with that of the wild-type molecule as indicated by the similar slopes observed in Figure 3. Similar data were obtained utilizing v-Abl-phosphorylated MBP as the exogenous substrate (data not shown). These results on the *in vitro* enzymatic function of the wild-type and phosphatase domain point mutants of CD45 are in agreement with those determined for bacterially produced (Streuli *et al.*, 1990) or *in vitro* translated (Johnson *et al.*, 1992) cytoplasmic domain variants of CD45.

To determine more precisely the effect of the M2 mutation on the enzymatic function of the EGFR/CD45 chimera, kinetic analysis was performed using two different substrates. The K_m and V_{max} values for dephosphorylation determined for the wild-type and M2 mutant using pNPP and the Lck Y505-containing phosphopeptide were similar, indicating that the putative catalytic function of the second domain does not appear to significantly influence the function of the first domain *in vitro* with the substrates utilized (Table I). The Lck Y394-containing phosphopeptide was utilized equally well by both the wild-type and M2 EGFR/CD45 chimeric molecules (data

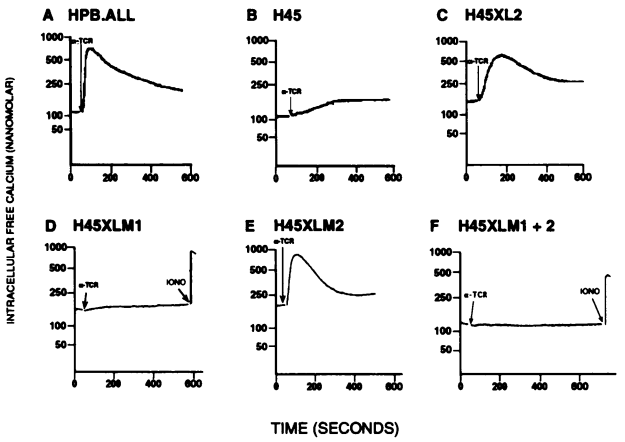

Fig. 4. TCR-mediated mobilization of intracellular free calcium in wild-type and mutant EGFR/CD45 chimera expressing cells. HPB-ALL ($CD45^+$; **A**), H45 ($CD45^-$; **B**), H45XL2 ($CD45^-$, EGFR/$CD45^+$; **C**), H45M1 ($CD45^-$, EGFR/CD45-$M1^+$; **D**), H45M2 ($CD45^-$, EGFR/CD45-$M2^+$; **E**), H45M1+2 ($CD45^-$, EGFR/CD45-$M1+2^+$; **F**) were treated at the indicated time with either 1 μg/ml anti-CD3 (Leu4) antibody or with a calcium ionophore, ionomycin (Iono; 1 μm). Intracellular free calcium levels were measured using the calcium-sensitive dye Indo-1.

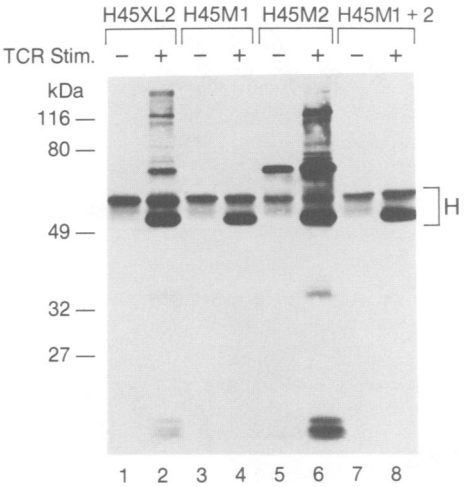

Fig. 5. TCR-mediated induction of tyrosine phosphoproteins. H45XL2 (lanes 1 and 2), H45M1 (lanes 3 and 4), H45M2 (lanes 5 and 6) and H45M1+2 (lanes 7 and 8) were treated with medium (−) or with a 1:1000 dilution of ascitic fluid containing anti-CD3 mAb (+) for 2 min. Induction of phosphotyrosine-containing proteins was assessed by immunoprecipitation followed by immunoblotting with an anti-phosphotyrosine mAb. The immunoglobulin heavy chain of the immunoprecipitating and stimulatory antibody is indicated by an H.

not shown). The K_m values obtained here with the EGFR/CD45 chimera are slightly higher than those previously reported utilizing a molecule consisting solely of the CD45 cytoplasmic domain (Cho *et al.*, 1992). It is not known whether this difference reflects an effect of the EGFR transmembrane and extracellular domains or whether it simply reflects experimental variation.

Function of the CD45 phosphatase domain mutants in TCR signaling

In CD45-deficient cells, the TCR is uncoupled from the intracellular signaling machinery, resulting in a loss of TCR-mediated tyrosine phosphoprotein induction and mobilization of intracellular free calcium ($[Ca^{2+}]_i$). We have previously shown that expression of the EGFR/CD45 chimera in CD45-deficient cells restores TCR signal transduction in a manner indistinguishable from wild-type CD45 (Desai *et al.*, 1993). In addition, TCR signal transduction can also be restored by targeting the cytoplasmic domain of CD45 to the plasma membrane (Volaravic *et al.*, 1993).

To examine the functional requirements of the two phosphatase domains of CD45 in TCR signaling, we analyzed the ability of the various point mutants to restore TCR-mediated $[Ca^{2+}]_i$ mobilization. Engagement of the TCR on wild-type HPB-ALL cells ($CD45^+$) resulted in a rapid mobilization of intracellular calcium (Figure 4A), by a mechanism which requires CD45 expression (Figure 4B). Expression of the wild-type EGFR/CD45 chimera or the M2 mutant in CD45-deficient cells restored intracellular calcium responses (Figure 4C and E). Examination of multiple clones of CD45-deficient cells expressing either the wild-type or M2 EGFR/CD45 chimera indicates no significant difference in the ability of these molecules to regulate TCR-mediated $[Ca^{2+}]_i$ mobilization. However, neither the M1 nor the M1+2 mutant was capable of restoring the TCR-regulated increase in $[Ca^{2+}]_i$ (Figure 4D and F).

In addition to intracellular calcium mobilization, another hallmark of TCR engagement is the induction of tyrosine phosphoproteins. Therefore, we examined the ability of the various mutants to restore TCR-mediated tyrosine phosphoprotein induction. Engagement of the TCR with anti-CD3 mAb on the wild-type EGFR/CD45 chimera and M2 EGFR/CD45 chimera-expressing cells resulted in a qualitatively similar pattern of tyrosine phosphoprotein induction (Figure 5, lanes 1, 2, 5 and 6). The 70 kDa tyrosine phosphoprotein seen in the unstimulated H45M2 cell, as well as the greater degree of tyrosine phosphoprotein induction in H45M2, was not reproducibly observed in this or two other independently derived CD45-deficient cells transfected with the M2 mutant. The M1 and M1+2 mutants were unable to restore TCR function (Figure 5, lanes 3, 4, 7 and 8). These results demonstrate for the first time that CD45 phosphatase activity is necessary for proximal signal transduction events mediated by the TCR. Moreover, the catalytic function of the first phosphatase domain of CD45 appears to be essential for this process. If the second domain has catalytic activity, which was not revealed in the *in vitro* assays, its activity would appear not to be required for TCR-mediated intracellular calcium mobilization or tyrosine phosphoprotein induction.

In vivo tyrosine phosphorylation state of Lck in cells expressing EGFR/CD45 chimera phosphatase domain mutants

Two potential substrates of CD45 are the Src-family kinases Lck and Fyn. Previous studies have demonstrated that Lck and Fyn are hyperphosphorylated on the C-terminal negative regulatory tyrosine (Y505) in cells deficient in CD45 expression (Ostergaard *et al.*, 1989; Hurley *et al.*, 1993; McFarland *et al.*, 1993; Sieh *et al.*, 1993). Moreover, Fyn and Lck appear to be specific substrates since the phosphorylation state of the C-terminal tyrosine residue of c-Src is not affected by the absence of

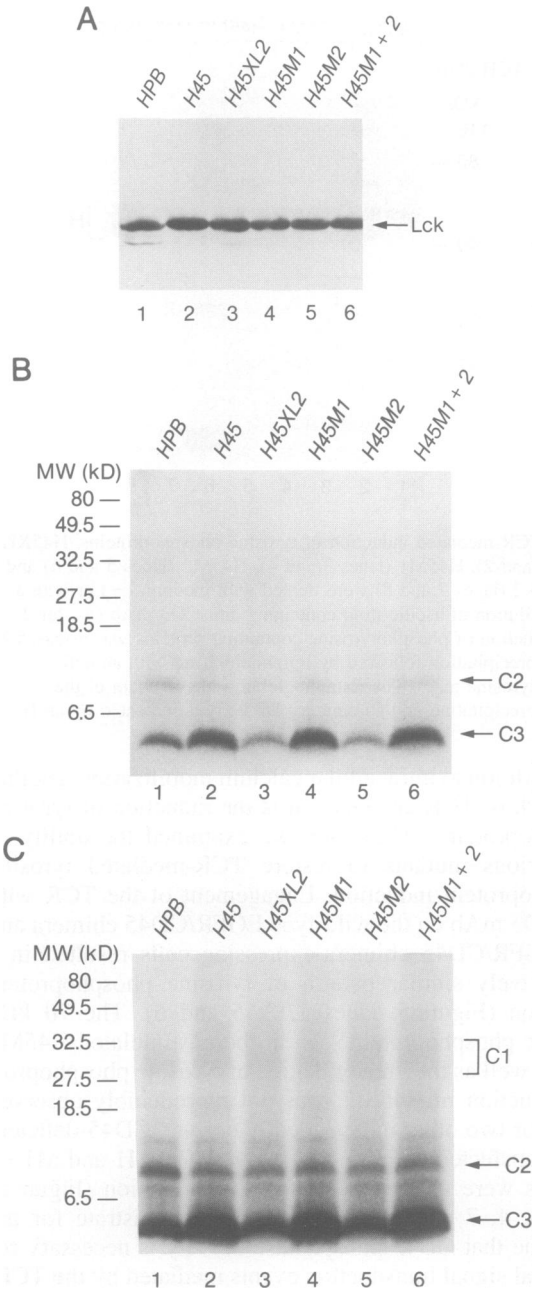

Fig. 6. *In vivo* phosphorylation status of the C-terminal tyrosine residue (Y505) of Lck. Lck from HPB-ALL, H45, H45XL2, H45M1, H45M2 and H45M1+2 cells labeled with [^{32}P]orthophosphate was immunoprecipitated, separated by SDS−PAGE, transferred to nitrocellulose and immunoblotted (**A**) to ensure that approximately equal amounts of Lck were present in each immunoprecipitate. The nitrocellulose immobilized Lck was treated with CNBr and the resulting peptide fragments were separated on a 19% polyacrylamide gel. Band C1 contains multiple sites of serine/threonine phosphorylation, while the autophosphorylation and C-terminal tyrosine-containing bands are contained in bands C2 and C3, respectively. (**B**) and (**C**) represent 16 and 48 h exposures, respectively. Comparable results were obtained in three separate experiments.

CD45 (Hurley *et al.*, 1993). The phosphorylation state of the Lck C-terminal tyrosine (Y505) in cells expressing the various phosphatase domain mutants was examined by immunoprecipitation of Lck from cells labeled with [^{32}P]orthophosphate, followed by immunoblotting to determine the relative levels of Lck isolated from each cell line (Figure 6A), prior to treatment with cyanogen bromide (CNBr). Lck contains two sets of nested CNBr cleavage sites, resulting in the generation of three predominant fragments (Veillette *et al.*, 1988; Abraham and Veillette, 1990; Luo *et al.*, 1990; Veillette and Fournel, 1990): C1, a ~30 kDa peptide, encompasses the N-terminal region of the molecule and also contains numerous serine phosphorylation sites. C2, a ~12–14 kDa peptide, contains the autophosphorylation site (Y394). The ~4 kDa C3 fragment encompasses the C-terminal negative regulatory site (Y505). The C3 fragment was phosphorylated to a greater extent in the H45 ($CD45^-$) cells compared with the $CD45^+$, HPB-ALL cells (Figure 6B, lanes 1 and 2). Introduction of the wild-type EGFR/CD45 chimera or the M2 version, both of which restored TCR signaling function, resulted in a reduction in Y505 phosphorylation (Figure 6B, lanes 3 and 5). However, expression of the M1 and M1+2 mutants of the EGFR/CD45 chimera did not diminish the phosphorylation status of Lck Y505 (Figure 6B, lanes 4 and 6). Longer exposure of Figure 6B demonstrates relatively equivalent levels of serine/threonine phosphorylation of Lck (the diffuse band of the C1 fragment) among the different mutant clones, indicating similar levels of Lck labeling and turnover in the different clones (Figure 6C). Thus, there is a correlation between CD45 phosphatase activity *in vitro*, restoration of TCR signaling and dephosphorylation of Lck on Y505. We were not able to assess satisfactorily the phosphorylation status of Fyn in these cells due its relatively low level of expression.

Phosphatase domain 1 is required for ligand-mediated regulation of EGFR/CD45 function

We have previously demonstrated that ligands to the EGFR/CD45 chimera can negatively regulate the function of the chimera, resulting in inhibition of TCR-mediated calcium mobilization and tyrosine phosphoprotein induction (Desai *et al.*, 1993). We were interested in assessing the relative importance of the two CD45 phosphatase domains in this process. Stimulation of the TCR resulted in a mobilization of intracellular calcium, which could be rapidly attenuated with the addition of ligand to the EGFR/CD45 chimera (Figure 7A−C). As shown above, mutation of the catalytic cysteine in the second phosphatase domain resulted in an EGFR/CD45 chimera competent in restoring TCR signaling (Figure 7D). Moreover, the M2 mutant also could still be negatively regulated by EGFR ligands, resulting in attenuation of TCR-mediated calcium mobilization (Figure 7E). As with the wild-type chimera, engagement of the M2 chimera prior to TCR stimulation prevented the antigen receptor-mediated calcium increase (Figure 7F).

Since tyrosine phosphoprotein induction is an early and requisite event in T cell activation, we were interested in determining if mutation of the second phosphatase domain of CD45 had a differential effect on the pattern of protein dephosphorylation following engagement of the M2 chimera. Stimulation of the TCR for 2 or 3 min on both H45XL2 and H45M2 resulted in the qualitatively similar induction of a number of tyrosine phosphoproteins (Figure 8, lanes 1–3 and 6–8). The addition of EGF protein concurrently with anti-TCR monoclonal antibodies

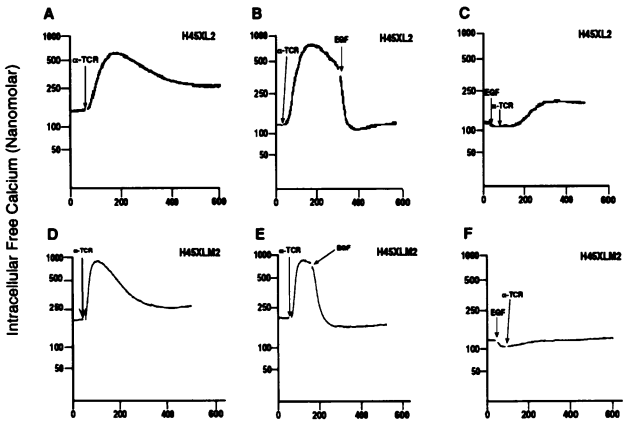

Fig. 7. Modulation of TCR-mediated mobilization of intracellular free calcium by EGFR/CD45 ligand. H45XL2 or H45M2 cells were treated at the indicated times with either 1 μg/ml anti-TCR mAb or 100 ng/ml EGF.

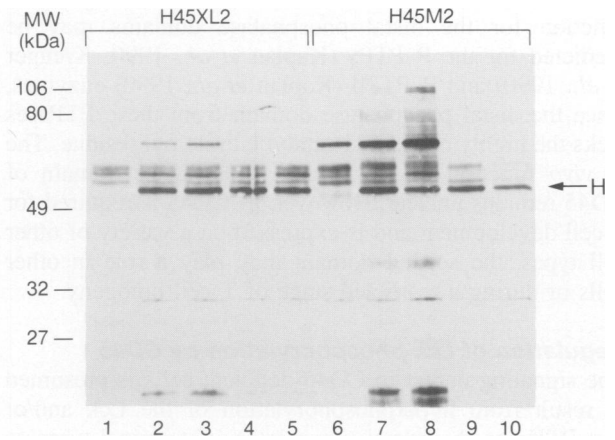

Fig. 8. Modulation of TCR-mediated tyrosine phosphoprotein induction by EGFR/CD45 ligand. Tyrosine phosphoproteins were immunoprecipitated from H45XL2 (lanes 1–5) or H45M2 (lane 6–10) cells treated for 2 min with medium (lanes 1 and 6), 2 min with anti-TCR mAb (lanes 2 and 7), 3 min with anti-TCR mAb (lanes 3 and 8), 2 min with anti-TCR mAb plus EGF simultaneously (lanes 4 and 9), or 2 min with anti-TCR mAb, followed by 1 min with EGF (lanes 5 and 10).

Table II. Cell surface expression of the EGFR/CD45 chimera subsequent to EGF treatment

Time (min)	Percent cell surface EGFR/CD45 chimera
1	94.0
2	92.4
5	92.1
10	93.9

Values are expressed as a percentage of remaining cell surface EGFR/CD45 chimeric molecules compared with control treated cells. 1×10^6 H45XL2 cells were treated at 37°C with 100 ng/ml EGF for the indicated times. Control H45XL2 cells were treated at 4°C as above. Cells were stained for cell surface EGFR/CD45 chimera using an anti-EGFR extracellular domain mAb. Percentages reflect changes in mean fluorescence intensity.

(mAbs) almost completely inhibited the induction of tyrosine phosphoproteins in cells containing the wild-type and M2 EGFR/CD45 chimeras (Figure 8, lanes 4 and 9). Moreover, subsequent to TCR stimulation for 2 min, the addition of EGF for 1 min resulted in a rapid dephosphorylation of the majority of the induced tyrosine phosphorylation events (Figure 8, lanes 5 and 10). Thus it appears that mutation of the catalytic site within the second phosphatase domain does not have any appreciable effect on the ability of EGFR ligands to regulate negatively the function of the EGFR/CD45 chimera and subsequent TCR signal transduction.

Since ligand binding to the EGFR/CD45 chimera resulted in a phenotype reminiscent of CD45-deficient cells, we assessed whether addition of EGF resulted in internalization of the EGFR/CD45 chimera. Analysis of cell surface expression of the EGFR/CD45 chimera at various times following the addition of EGF indicated that there was no substantial change in surface expression of the EGFR/CD45 chimera (Table II). Therefore, the ability of EGF to modulate negatively TCR signaling function was not the result of reducing the cell to a state of CD45 deficiency.

Discussion

The catalytic function of the first phosphatase domain of CD45 is sufficient for TCR signal transduction

Numerous studies have defined the importance of CD45 expression in regulating an essential component(s) in the signal transduction pathway utilized by hematopoietic cell antigen receptors [reviewed in Chan *et al.* (1994)]. In most CD45-deficient cells, signals emanating from the stimulated TCR appear to be blocked at an early stage in the signaling pathway. These TCR-mediated responses can be restored by introducing any one of multiple CD45 isoforms (Koretzky *et al.*, 1990, 1992; Shiroo *et al.*, 1992; Desai *et al.*, 1993). Moreover, the extracellular and transmembrane domains of CD45 are not required to restore TCR function (Desai *et al.*, 1993; Hovis *et al.*, 1993; Volaravic *et al.*, 1993). Therefore, the cytoplasmic domain of CD45 is sufficient to permit TCR signaling.

CD45, like the majority of transmembrane R-PTPases, contains two tandem phosphatase domains. However, the relative *in vivo* contribution of the two domains to CD45 function in TCR signal transduction was not known. Experiments in which individually mutating the catalytic cysteinyl residue in either or both domains and introducing the resulting mutant forms of the EGFR/CD45 chimera into CD45-deficient cells demonstrate that the phosphatase activity of CD45 is critical for proximal signaling through the TCR. Moreover, it appears that the catalytic function of domain I is necessary to restore TCR signaling in the HPB-ALL leukemic cell line model system. Mutation of the cysteine residue in the catalytic site of the first phosphatase domain resulted in a molecule that was not functional *in vitro* against a number of structurally unique substrates, including a small molecule (pNPP), a tyrosine-phosphorylated protein (MBP) and a tyrosine phosphopeptide encoding the C-terminal 15 amino acids of Lck, including the negative regulatory Y505, a putative *in vivo* substrate of CD45. Mutation of the catalytic site in domain I also rendered the EGFR/CD45 chimera non-functional *in vivo*, as it was unable to restore TCR signaling. Mutation of the catalytic site in the second phosphatase domain

resulted in a molecule with *in vitro* and *in vivo* properties similar to that of the wild-type molecule: (i) similar rates of substrate dephosphorylation (Figure 3), (ii) similar K_m and V_{max} values (Table I), (iii) similar ability to reconstitute TCR-proximal signal transduction events (Figures 4 and 5) and (iv) comparable *in vivo* capacity to reduce the phosphorylation of Lck Y505. These results suggest that the potential catalytic activity of the second phosphatase domain is not necessary for TCR-mediated calcium mobilization and tyrosine phosphoprotein induction.

Our results are in agreement with previous studies, in which no apparent phosphatase activity was present when the cytoplasmic domain of CD45 containing a mutation in the first domain catalytic site was expressed *in vitro* or in bacteria (Streuli *et al.*, 1990; Johnson *et al.*, 1992). In these studies, the first domain was responsible for all observed *in vitro* enzymatic activity. In a recent study, though, expression of a CD45 molecule containing a partial deletion within the first phosphatase domain exhibited activity toward v-Abl-phosphorylated MBP (Tan *et al.*, 1993). However, in those experiments the residual phosphatase activity was never formally demonstrated to originate from the second phosphatase domain. In contrast, we could not attribute any phosphatase activity to the second phosphatase domain in the M1 EGFR/CD45 chimera, even when v-Abl-phosphorylated MBP was utilized as the substrate. Regardless of whether the second phosphatase domain has activity against MBP, the second domain does not appear to have *in vivo* function with regard to restoration of proximal TCR signaling. Moreover, the catalytic function of the second phosphatase domain, if it has any, was not required for restoration of TCR signaling, since the enzymatic function of the proximal domain was sufficient to restore TCR signal transduction.

We have demonstrated that alteration of the catalytic cysteine residue of the second phosphatase domain did not result in any observable difference in the *in vitro* or *in vivo* function of the EGFR/CD45 chimera. The distal phosphatase domain of the R-PTPase, LAR, also does not appear to be functional *in vitro* against the substrates tested to date (Streuli *et al.*, 1990; Pot *et al.*, 1991). However, the distal phosphatase domain of R-PTPα, a ubiquitously expressed PTPase, does have enzymatic activity, albeit at a substantially reduced level compared with that of the first domain (Wang and Pallen, 1991). In addition, the *in vitro* substrate specificity of the two domains appears to be different, which suggests that the two domains may have distinct cellular functions.

Comparison of the amino acid sequences around the catalytic site in the second phosphatase domains of CD45 and R-PTPα indicates that a number of critical and absolutely conserved amino acids in the 11 amino acid phosphatase motif are absent in CD45. However, changing the catalytic core sequence of the second phosphatase domain of CD45 to that found in the proximal domain did not generate an enzymatically functional phosphatase domain capable of dephosphorylating angiotensin or MBP (Johnson *et al.*, 1992), suggesting that other structural features are necessary to create an active phosphatase domain. However, the second domain may possess exquisite substrate specificity not detectable *in vitro* with the artificial substrates utilized. Alternatively, the activity of the second domain may be regulated by post-translational modifications such as phosphorylation, or may require the association of an activator protein, as in the case of $p80^{cdc25}$ and cyclin B (Galaktionov and Beach, 1991). Regardless of the mechanism by which the potential enzymatic activity of the second phosphatase domain of CD45 may be regulated, the studies presented here suggest that the potential (if existent) phosphatase function of the second domain is not required for coupling the TCR to the intracellular signaling machinery.

The fact that the majority of the R-PTPases contain two phosphatase domains, in which the second domains are as well conserved as first phosphatase domains (Kreuger *et al.*, 1990), suggests a function for the second domain. Mutational analysis suggests that the second phosphatase domains of CD45 and LAR, rather than directly dephosphorylating tyrosine phosphoproteins, may facilitate and regulate the activity of the first domain. Deletion of the second domain results in an enzymatically inactive proximal domain *in vitro* (Streuli *et al.*, 1990; Cho *et al.*, 1992; Johnson *et al.*, 1992). A regulatory function for the distal phosphatase domains may be predicted for the R-PTPγ (Kaplan *et al.*, 1990; Kreuger *et al.*, 1990) and R-PTPβ (Kaplan *et al.*, 1990) enzymes, since the distal phosphatase domain from these PTPases lacks the highly conserved catalytic cysteinyl residue. The *in vivo* function of the second phosphatase domain of CD45 remains unclear. However, as CD45 is required for T cell development and is expressed on a variety of other cell types, the second domain may play a role in other cells or during a restricted stage of T cell ontogeny.

Regulation of Lck phosphorylation by CD45

The signaling defect in CD45-deficient cells is presumed to result from hyperphosphorylation of the Lck and/or Fyn PTKs on the negative-regulatory C-terminal tyrosine residue (Chan *et al.*, 1994). The phosphorylation status of the negative regulatory sites of these PTKs has correlated better with their function in signaling than their kinase activities. Indeed, the kinase activities of Lck and or Fyn in CD45-deficient cells have been reported to be decreased (Mustelin *et al.*, 1989; McFarland *et al.*, 1993) or increased (Burns *et al.*, 1994). Although our studies do not demonstrate a direct effect of CD45 on Y505 of Lck *in vivo*, this site can be dephosphorylated by CD45 *in vitro* (Mustelin *et al.*, 1989) and CD45 and Lck have been co-immunoprecipitated suggesting a direct interaction may occur *in vivo* (Schraven *et al.*, 1991; Koretzky *et al.*, 1993). Thus, in CD45-deficient cells the action of Csk, the kinase demonstrated to phosphorylate the C-terminus of Src-like kinases (Okada *et al.*, 1991), would be unopposed, resulting in accumulation of phosphate on the C-terminal tyrosine of Lck. However, introduction of a functional CD45 molecule should reverse the state of Lck phosphorylation. Our results with the phosphatase domain point mutants support the notion that restoration of TCR signaling function correlates with a reduction in the level of phosphorylation of Lck on the negative regulatory site (Y505). Moreover, only the wild-type and M2 EGFR/CD45 chimeras, which have demonstrable *in vitro* phosphatase activity, resulted in a reduction in phosphorylation of Lck Y505, in addition to restoring TCR signal transduction. Additionally, overexpression of Csk has been demonstrated to result in down-modulation of TCR signaling

(Chow *et al.*, 1993). Thus, taken together, these results suggest that there is a dynamic equilibrium between Csk and CD45 in the regulation of the Lck and Fyn PTKs.

Function of the dual phosphatase domains in ligand-mediated negative regulation

Previously, we had shown that ligands to the EGFR/CD45 chimera could negatively regulate its function (Desai *et al.*, 1993). Negative regulation required dimerization of the full-length chimera, since co-expression of a truncated molecule consisting of only the EGFR extracellular and transmembrane domains prevented the ligand effect. The inhibitory effect of the truncated EGFR molecule is attributable to its ability to form heterodimers with the wild-type EGFR/CD45 chimera. Moreover, the identification of CD45 dimers (Takeda *et al.*, 1992) suggests an intermolecular mechanism of regulation of CD45 function in which either or both phosphatase domains may play a role. The finding that under some circumstances CD45 exists as a phosphoprotein (Stover *et al.*, 1991; Autero *et al.*, 1994) suggests a model in which one of the phosphatase domains may regulate CD45 function by influencing its phosphorylation status. In this model, ligand would induce dephosphorylation of CD45.

In the current study, we demonstrate that the potential phosphatase function of the distal phosphatase domain is not required for ligand-mediated negative regulation. If tyrosine phosphorylation of CD45 is a physiological occurrence and a means of regulation, then the proximal phosphatase domain may be involved in *trans*-dephosphorylation. However, we have been unable to observe tyrosine phosphorylation of the EGFR/CD45 chimera (data not shown). Even the M1+2 EGFR/CD45 chimera, which would be incapable of autodephosphorylation, does not become tyrosine-phosphorylated following stimulation of the TCR when expressed in $CD45^+$ cells (D.M.Desai and A.Weiss, unpublished observations). Thus, CD45 tyrosine phosphorylation has only been observed *in vivo* following treatment of cells with tyrosine phosphatase inhibitors, or by treatment of purified CD45 *in vitro* with a variety of kinases (Stover *et al.*, 1991; Autero *et al.*, 1994). The tyrosine phosphorylation and consequently, the phosphatase activity of CD45 acting upon such a phosphorylation may not be involved in ligand-mediated regulation Alternatively, dimerization of the EGFR/CD45 chimera may result in steric inhibition of substrate access to the catalytic site or alter phosphatase activity.

CD45 is expressed in multiple isoforms and on a variety of cell types and as such it is not surprising that regulation of CD45 function is complex and may vary depending on the cellular context or developmental stage of the cell. The identification of CD45 ligands should yield valuable information on when and where CD45 function may be regulated.

Materials and methods

Constructs and antibodies

The EGFR/CD45 chimera has been described previously (Desai *et al.*, 1993). The cysteine to serine point mutations were created in the EGFR/CD45 chimera backbone by oligonucleotide-mediated, site-directed mutagenesis (Clonetech) and confirmed by nucleotide sequencing. The EGFR/CD45 chimera mutants were expressed by the pRK7 vector driven by the cytomegalovirus long terminal repeat. The pAW-Neo3 vector has been described previously (Desai *et al.*, 1993).

Antibodies used for flow cytometry were control mouse immunoglobulin γ2b (Zymed Lab), Leu4 (anti-CD3ε, generously provided by R.Evans and the Memorial Sloan Kettering Institute, New York, NY), GAP8.3 (pan-anti-CD45, ATCC) and LA22 [anti-EGFR, Upstate Biotechnology Inc. (UBI)]. mAb 108.1 recognizes an epitope on the extracellular domain of the EGFR and has been described elsewhere (Honegger *et al.*, 1989). The anti-Lck polyclonal antiserum and the anti-phosphotyrosine-specific mAb 4G10 were purchased from UBI. The 4G10 mAb was used for immunoprecipitation and immunoblotting.

Cells and transfections

The human leukemic T cell line HPB-ALL and its derivative H45 (CD45-deficient) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Intergen), penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mM) (Irvine Scientific). Cells expressing the EGFR/CD45 chimera were passaged in the above medium containing 2 mg/ml geneticin (Gibco).

Transfections were performed as described earlier (Desai *et al.*, 1993) with the exception that 25 μg of the expression vector containing the EGFR/CD45 chimera mutants was co-transfected with 2.5 μg of pAW-Neo vector containing the neomycin resistance gene.

Flow cytometry and measurement of intracellular calcium

Cells were stained at 4°C with saturating concentrations of primary antibody followed by fluorescein-conjugated goat anti-mouse antibody (Caltag). Cells were analyzed on a FACScan (Becton-Dickinson) as described by Irving and Weiss (1991). Intracellular free calcium levels were measured using the calcium-sensitive dye Indo-1 (Molecular Bioprobes) as described by Grynkiewicz *et al.* (1985). Cells (3×10^6/ml) were stimulated with the mAb Leu4 (1 μg/ml) or EGF (100 ng/ml) as indicated.

TCR stimulation

Cell were harvested, washed twice with PBS and resuspended at 1×10^8/ml in PBS. Cell were incubated at 37°C for 15 min. Leu4 ascites (anti-CD3ε) at a 1:500 dilution was added to the cells, followed by a 2 min incubation at 37°C. Cells were pelleted in a microfuge and lysed as described below.

Immunoprecipitations, SDS–PAGE and immunoblotting

Cells are lysed at 1×10^8/ml in 1% Nonidet-P40 (Boehringer Mannheim), 150 mM NaCl and 10 mM Tris (pH 7.8) supplemented with 1 mM phenylmethylsulfonylfluoride, 2 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 mM sodium pyrophosphate, 5 mM EDTA, 10 mM sodium fluoride and 1 mM sodium orthovanadate. Lysates were incubated at 4°C for 30 min, followed by centrifugation at 13 000 *g* for 15 min to remove insoluble material. Lysates were subjected to immunoprecipitation with antibody coupled to protein A–Sepharose beads (Pharmacia) for 2 h at 4°C. Immune complexes were washed, resolved by SDS–PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Towbin *et al.*, 1979). Immunoblotting was performed as described (Straus and Weiss, 1992) using either alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies with NBT/BCIP (Zymed Laboratories) or ECL substrates (Amersham), respectively.

Tyrosine phosphatase assay

Cells were harvested and lysed as above, except that sodium orthovanadate and sodium pyrophosphate were omitted from the lysis buffer. The wild-type and mutant EGFR/CD45 chimeras were immunoprecipitated using 1–2 μg of mAb 108 as described above. The immune complexes were washed five times with 1 ml of lysis buffer followed by two washes in 25 mM HEPES (pH 7.5) and 5 mM EDTA (pH 8.0). The samples were then divided into five equal fractions. 2 × SDS sample buffer was added to one fraction for immunoblotting, and the other four fractions were resuspended in 50 μl phosphatase assay buffer (as above with 10 mM DTT) plus substrate. The MBP substrate was prepared using purified c-Src (kindly provided by D.Morgan, University of California at San Fancisco) or v-Abl (Oncogene Science). PNPP was purchased from Sigma and used at a final concentration of 10 mM. The phosphopeptides Lck Y394 (IEDNEYTAREG) and Lck Y505 (EDDFTATEGQYQPQP) were synthesized using standard Fmoc chemistry by C.Turck (University of California at San Francisco). The reactions were placed at 37°C for the indicated times and then stopped by the addition of 50 μl 50 mg/ml BSA and 150 μl 25% trichloroacetic acid. The samples were vortexed and pelleted. The supernatant was assayed for free phosphate by either

scintillation counting or by a colorimetric assay as previously described (Mustelin *et al.*, 1989).

In vivo [^{32}P]orthophosphate labeling

Cells were washed twice with phosphate-free RPMI 1640 (Irvine Scientific) containing 10% dialyzed FCS. Cells were incubated at 2×10^6/ml in phosphate-free medium at 37°C for 1 h. Cells were washed twice more with the above medium and resuspended at 1×10^7/ml in phosphate-free medium containing 1 mCi/ml [^{32}P]orthophosphate (ICN). They were then incubated for 6 h at 37°C (maximal ^{32}P incorporation occurred at 5–6 h; data not shown). The cells were harvested and washed once with serum- and phosphate-free medium. Cells were lysed as above. Lysates were incubated overnight with fixed *Staphylococcus aureus* (Calbiochem) and immune complexes were isolated as above (similar results were obtained if lysates were precleared for 2 h). Immune complexes were separated by SDS–PAGE and transferred to nitrocellulose. Samples were immunoblotted to determine the relative levels of Lck protein. The bands of interest were excised and treated for 3 h with 100 mg/ml cyanogen bromide in 70% formic acid. Samples were lyophilized, washed twice with deionized water and run on a 19% SDS–polyacrylamide gel containing tricine (Sigma). The bands were visualized by autoradiography.

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