Biochemical Evidence That the Phosphorylated Tyrosines, Serines, and Threonines on the Aggregated High Affinity Receptor for IgE Are in the Immunoreceptor Tyrosine-based Activation Motifs*

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Activation of cells mediated by the high affinity receptor for IgE leads to rapid phosphorylation of tyrosines (and later other residues) on the receptor’s β and γ subunits, and there is circumstantial evidence that the tyrosines modified are in the so-called immunoreceptor tyrosine-based activation motifs (ITAMs). We identified and quantitated the residues phosphorylated on the subunits of the native receptor by comparing the properties of peptides derived from the receptors radiolabeled in vivo or in vitro with those of synthetic peptides. Our results with receptors labeled in vivo confirm that only the tyrosines in the ITAMs of β and γ became phosphorylated, and preferentially, those in the canonical YXX(L/I) sequences. The extent of phosphorylation of the canonical tyrosines was of the same order of magnitude, but the amino-terminal canonical tyrosine in the ITAM of the β subunit was consistently phosphorylated to a lesser degree. The non-canonical ITAM tyrosine in the β subunit was considerably less phosphorylated. Phosphorylation of serine (on β) and threonine (on γ) also occurred mainly in the ITAMs, but selectively at some positions whose characteristics seem to be conserved among other receptors containing ITAMs. The studies with receptor complexes isolated and radiolabeled in vitro gave similar results for phosphorylation of tyrosines, suggesting that the latter, much simpler system is a useful model for more detailed studies.

The receptor with high affinity for IgE, FcεRI, belongs to a family of “multichain immunorecognition receptors” (MIRR) (1). Members of this family share important functional and structural features. Upon interaction of the bound immunoglobulin (Fc receptors) or the immunoglobulin-like part of the receptor (B- and T-cell antigen receptors) with a multivalent antigen, each of them initiates cellular responses through a process dependent upon aggregation of the receptors (2, 3). Following such aggregation, one of the earliest events is phosphorylation of protein tyrosines, even though all these receptors lack an intrinsic kinase activity. Instead, they initiate such phosphorylation through their association with one or more non-receptor Src family tyrosine kinase(s) (4, 5). The cytoplasmic extensions of one or more of the subunits of each of these receptors contain a highly conserved sequence (6), now referred to as the immunoreceptor tyrosine-based activation motif (ITAM) (7), which contains a tyrosine residue in two canonical YXX(L/I) sequences. There is evidence that these tyrosines are substrates for the Src family tyrosine kinases associated with the receptors, and that their phosphorylation initiates signal propagation by recruiting proteins containing SH2 domains; in particular, the related tyrosine kinases Zap and Syk, phosphoinositide-related enzymes such as phospholipase C and phosphoinositide-3 kinase, and adapter proteins such as Vav, Crk, and Grb-2, which connect the receptors to several metabolic pathways (8, 9).

The molecular details of the events triggered by FcεRI and by related receptors have been explored using genetically engineered chimeric proteins and mutational analysis. The results from such studies have varied somewhat, depending on the system studied and compared with what is observed with the intact receptor. For example, Jouvin et al. (10) found that upon aggregation, chimeric constructs of the individual cytoplasmic domains of the β and γ subunits of FcεRI elicited some cellular responses, and that mutation of the tyrosine residues in the ITAMs inhibited the binding of Lyn kinase to β and of Syk kinase to γ. Notably, however, no phosphorylation of the unmutated chimeras was observed, even though this is observed when intact receptors are aggregated. In the B-cell antigen receptor, a chimera containing the ITAM of the Igα subunit was sufficient to elicit cellular responses upon aggregation, and mutation of either of the two ITAM tyrosines resulted in the loss of the response (11, 12). However, when these mutations were expressed in the context of the whole B-cell receptor, the loss of activity was much less dramatic (12). In that study, phosphorylation of only one of the ITAM tyrosines seemed to suffice for certain responses. In a related system, chimeras of the CD3-ε subunit of the T-cell receptor were found to interact with different kinases, depending on which tyrosine residue was phosphorylated in the ITAM (13, 14). In still another study, phosphorylation of the CD3-ε chimeras upon aggregation was not detected, even when downstream proteins became phosphorylated (15).

It must be recalled that despite the power of protein engineering, the evidence that is derived from those approaches is largely circumstantial particularly for mutants showing a loss of function. For example, substituting tyrosine with an alanine or phenylalanine not only substitutes a residue that can be phosphorylated for one that cannot, but also for one that has very different hydrophaticity and hydrogen bonding properties; significant conformational changes in the protein produced by such a substitution might contribute to or even account for the functional alterations observed. Second, genetic approaches can only indirectly give clues about the quantitative aspects of any modifications. We decided that to complement the pub-
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liahed mutational studies, it was important to examine the phosphorylation of the different subunits in the native FcεRI directly, a kind of analysis that has not been previously reported for this or any of the other MIRR.

We recently compared the phosphorylation of the receptors and other substrates in vivo and after an in vitro assay. Those results supported a model in which receptors constitutively associated with kinase become transphosphorylated upon aggregation and are thereby able to recruit additional molecules of kinase (16, 17). A further rationale for undertaking the present study was to determine whether the receptor aggregates isolated under the special conditions we used would show a pattern of phosphorylation similar to what is observed in the much more complex situation in vivo.

EXPERIMENTAL PROCEDURES

General Procedures—RBL-2H3 cells were grown (18), sensitized with mouse monoclonal anti-2,4-dinitrophenyl (DNP) IgE (19), and stimulated with 0.5 µg/ml DNP-bovine serum albumin (25 mol of DNP/mol of protein) for 2 min as described previously (16). When appropriate, the cells were activated in buffer supplemented with 0.4 mM MgSO4 and 1 mM CaCl2.

Phosphorylation of Receptors—For studies in vivo, cells were incubated with 1–3 µCi/ml [32P]orthophosphate (acid-free, carrier-free, Amersham) for 3–4 h in a medium that was otherwise free of phosphate. This extended incubation in the absence of phosphate did not affect the antigen-induced phosphorylation of receptor tyrosines, as assessed with an anti-phosphotyrosine antibody by Western blotting of immunoprecipitated receptors (data not shown). Cells were lysed as described (20), except that 1 µl haptens (DNP-N-A-aminocaproate) was added after lysis to ensure by disaggregation, more complete immunoprecipitation of the receptors aggregated during activation of the cells (16). The immunoprecipitated receptors were isolated and washed as described (20). For the in vitro labeling of the receptor, the immunoprecipitates were isolated and incubated as described (16). The immunoprecipitates from in vivo and in vitro labeled receptors were submitted to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as reported previously (16, 20).

Proteolytic Cleavage of Subunits—The radioactive bands corresponding to the β and γ subunits of the receptor, identified by autoradiography, were excised from the nitrocellulose membrane and submitted to proteolytic digestion as described (21). Alternatively, the proteolysis was performed on specimens obtained by high pressure liquid chromatography (HPLC) (below) after drying the fractions and resuspending the material in the appropriate buffer. The buffers and temperatures of incubation employed were as follows: (i) for 1-tosylamido-2-phenyl-ethyl chloromethyl ketone-treated trypsin (Worthington), 50 mM NH4HCO3, pH 8.0, 37 °C; (ii) for Staphylococcus aureus V8 (ICN Pharmaceuticals Inc., Costa Mesa, CA), 50 mM NH4HCO3, pH 7.6, 37 °C; (iii) for proline-specific endopeptidase (ICN Pharmaceuticals Inc.), 50–100 mM sodium phosphate, pH 7.2, 30 °C; (iv) for thermolysin (Calbiochem), 100 mM NH4HCO3 and 1 mM CaCl2, pH 8.0, 55 °C.

Synthetic Peptides—Peptides were synthesized on a model 431A peptide synthesizer (Applied Biosystems). Amino acid analyses of the purified synthetic peptides were performed with a model 420A amino acid analyzer (Applied Biosystems). Phosphoamino acid analysis was performed as described (22, 23).

Separation of Peptides—The released peptides were dried in a Speed-Vac, resuspended in 0.2–0.5 ml of H2O, and analyzed by HPLC. The samples, in a volume ranging from 50 to 500 µl, were applied to a reverse phase column (Delta Pak 150×30 mm, 3.0 × 300 mm, Waters Corp., Milford, MA) after injection of the sample into a 1-ml loop. The column was developed with a discontinuous linear gradient from Buffer A (0.1% v/v trifluoroacetic acid in water) to 0.1% v/v trifluoroacetic acid in 60% v/v acetonitrile in water (Buffer B) at a flow rate of 0.7 ml/min. The gradient was (in percent buffer B): 0–0.06 min, (10%); 7.3 min, (15%); 20 min, (30%); 35 min, (40%); 40 min, (100%); 50 min, (100%); 60 min, (10%); 70 min, (10%). The Dynamax HPLC System (Rainin Instrument Co., Woburn, MA) was employed to process the column. Throughout these studies, the fraction size was 0.49 ml (0.7 min), except for the thermolysin digest, for which 0.21-ml (0.3 min) fractions were used to improve the separation of the peptides.

Analysis of Peptides—Radioactivity in the HPLC fractions was measured with scintillation liquid for direct quantitation, or by Cerenkov counting when the peaks required further processing. HPLC peaks identified by absorbance at 214 nm (synthetic peptides) or by radioactivity (from the phosphorylated receptor subunits) were dried in 1.5-ml polypropylene tubes in a Speed-Vac overnight, at the low or intermediate temperature setting. Higher temperatures resulted in poorer subsequent solubilization of some peptides.

In some instances we needed to determine whether specific cleavage products of a synthetic peptide contained non-radioactive phosphotyrosine or not. Lyophilized peptide was resuspended in H2O, adjusted to neutral pH, and its absorbance scanned between 220 and 320 nm. The solution was then adjusted to pH 11 with NaOH and resanned. Phosphotyrosine-containing peptides show a peak of absorbance at 285 nm, which is unchanged at basic pH; peptides containing unphosphorylated tyrosine show a peak of absorbance at 274 nm at neutral pH, and at 290 nm at basic pH.

RESULTS

Identification and Characterization of the 32P-Labeled Peptides

The number of receptors we analyzed was too small to allow for conventional direct compositional analysis of the peptides generated by proteolysis and separated by HPLC. Instead, we identified the phosphorylated peptides by comparing their properties to those of synthetic peptides both with respect to their behavior in HPLC and by their sensitivity to various endoproteinases. This approach, combined with analysis of which amino acid had become phosphorylated, allowed us to identify and quantitate the phosphorylated tyrosines and, in some instances, serines and threonines.

Choice of Synthetic Peptides

We selected proteases that would be likely to generate peptides that would be useful for distinguishing in particular between different tyrosines. We then prepared appropriate synthetic peptides based on the sequence of the receptors and on the particular proteases selected. Because our preliminary data suggested that the ITAMs were the main or exclusive sites of tyrosine phosphorylation, only peptides including all or part of those motifs were synthesized.

The β subunit contains five tyrosines in the cytoplasmic portions of the receptor: one in the loop between the second and third transmembrane domains (Tyr125), and four in the COOH-terminal extension (Tyr201, Tyr218, Tyr224 and Tyr228) (24) (Fig. 1). Two of the latter, Tyr218 and Tyr228, are in the canonical YXXL sequences of the β chain’s ITAM, and a third, Tyr224 falls between them at a non-canonical site. We selected the endopeptidase Glu-C from S. aureus (V8) to hydrolyze this subunit. This enzyme hydrolyzes peptide bonds carbonyl-terminal to glutamic and aspartic acids, but in appropriate buffers cleaves exclusively to glutamic acid residues (25). Under the latter conditions, four peptides containing the five cytoplasmic tyrosines of the β subunit are expected: two containing the ITAM’s tyrosines, and two others containing transmembrane fragments. The non-canonical Tyr224 would be present in peptide β-2 together with the canonical Tyr228. Appropriate phosphotyrosine peptides were synthesized based on these considerations (Fig. 1 and Table I).

In the γ chain, three of the four tyrosines are in or proximal to the chain’s cytoplasmic extension (Tyr25, Tyr47, and Tyr248), and only the latter two are in the canonical sequence. Trypsin is expected to yield three tyrosine-containing peptides (Fig. 1).

Characterization of Synthetic Peptides

We synthesized the phosphotyrosine analogues of the expected peptides shown in Fig. 1 (Table I). To assess which if any of the two tyrosines in peptide β-2 (Fig. 1) becomes phosphorylated, three alternative peptides were prepared (β-2a, β-2b, and β-2c). For each of the peptides used in this study, the major peak from a preparative HPLC was isolated and re-purified by...
same characterizations were performed with receptors labeled with synthetic peptides characterized in Table I, in which the tyrosines were open arrows indicated by a solid arrow. The critical tyrosines are indicated by a. From the ITAM motif, the designations between the sequences refer to the expected sites of proteolytic cleavage. The detailed composition shows the number of residues of each amino acid relative to an integral number of leucines. The number in the parentheses shows the average of all those residues expected to be present in duplicate relative to the average of all those expected to be present singly.

**Evidence for Phosphorylation of Tyr^{218}**

A peptide 11 residues in length containing the NH$_2$-terminal YXXL motif of the ITAM would be expected to be generated by digestion of the β subunit by V8 (Fig. 1). We identified the radioactive component in fraction 37 as the phosphorysine analogue of this peptide (β-1 in Table I) on the basis of the following observations. (a) The synthetic phosphorysine analogue of β-1 eluted in the same fractions as this radioactive component. (b) Fraction 37, isolated from receptors labeled either in vivo or in vitro, contained phosphorysine but no other phosphoamino acid. (c) The radioactive material was sensitive to trypsin (Fig. 1 and Table I), and upon rechromatography of the tryptic digest, the counts shifted to the same fractions (fractions 34 and 35) as those containing the phosphorysine (assessed by UV spectral analysis) when the synthetic peptide β-1 was similarly treated with trypsin. Because of the low levels of phosphorylation in vivo, it was impractical to perform the latter analysis on the β chains labeled in vivo.

The small shift in the elution fraction is unexpected if tryptic cleavage had occurred predominantly after Arg$^{216}$ resulting in the peptide LpYE (Fig. 1). Indeed, when a synthetic peptide of the latter composition was prepared, it eluted at fraction 24. It appears that cleavage occurred after Arg$^{209}$ or Lys$^{211}$ preferentially, possibly because of the cluster of acidic residues (Asp$^{214}$, Asp$^{215}$, Tyr(P)$^{218}$, and Glu$^{219}$) surrounding Arg$^{216}$ (Fig. 1). These alternative cleavage sites will produce a peptide only one or three amino acids shorter than the parental NH$_2$-terminal peptide. Similar results were obtained by treatment of a tryptic digest of β with V8 (below). Only phosphorysine was identified in the peak at fraction 35 when the latter treatment was performed on receptors labeled in vivo and in vitro.

**Evidence for Phosphorylation of Tyr^{228}**

A dodecapeptide containing the phosphorylated COOH-terminal YXXL motif of the ITAM would also be expected after digestion of the β subunit by V8 (Fig. 1). The radioactive peak at fraction 64 obtained from the same V8 digest was identified as the phosphorysine analogue of this peptide as follows. (a) By HPLC it eluted in the same fractions as the alternative synthetic phosphopeptides β-2a and β-2b (Table I), both of which eluted in the same fraction. (b) This fraction, isolated from receptors phosphorylated in vivo or in vitro, contained phosphorysine. Similarly, when this peak was isolated after treatment of the tryptic digest with V8 (below) phosphorysine was identified. (c) It was sensitive to prolylendopeptidase. This enzyme should cleave at Pro$^{218}$ to yield the peptides LHVYSP and IYSALE in which one or the other tyrosine would be phosphorylated. The corresponding radioactive component was identified in fraction 37 when the latter treatment was performed on receptors labeled in vivo and in vitro.

**TABLE I**

| Trivial name | Sequence$^a$ | Observed composition$^b$ | HPLC fraction | Expected cleavage by |
|--------------|--------------|--------------------------|---------------|---------------------|
| β-1          | RSVPEDRLpYE  | D$_1$.e$_1$.s$_{0}$.r$_{1}$.p$_{0}$.d$_{0}$.v$_{0}$.o$_{0}$.l$_{1}$.e$_{1}$.k$_{0}$.k$_{7}$ | 0.08          | -              +  +
| β-2a         | LHVYSPiPYSbALE | E$_{0}$.s$_{2}$.h$_{1}$.a$_{1}$.p$_{0}$.d$_{1}$.v$_{0}$.o$_{1}$.l$_{1}$.e$_{2}$.o$_{2}$ | 1.93          | 64         +  +
| β-2b         | LHVYSPiPYSbALE | E$_{0}$.s$_{1}$.h$_{1}$.a$_{1}$.p$_{1}$.d$_{1}$.v$_{0}$.o$_{1}$.l$_{2}$.o$_{1}$.l$_{2}$.o$_{0}$ | 1.92          | 64         +  +
| γ-1          | SDAVpYGLNTR  | D$_{0}$.s$_{2}$.s$_{0}$.g$_{1}$.r$_{0}$.t$_{1}$.a$_{0}$.l$_{0}$.y$_{0}$.o$_{0}$.l$_{1}$.k$_{0}$.k$_{7}$ | 2.06          | 36         -  -  -
| γ-2          | NQETpYETLK   | D$_{0}$.e$_{2}$.t$_{1}$.s$_{1}$.l$_{1}$.o$_{1}$.k$_{0}$.k$_{7}$ | 2.04          | 30         -  -  -

$^a$ These peptides are analogous to those shown in Fig. 1 but with particular tyrosines phosphorylated.

$^b$ The detailed composition shows the number of residues of each amino acid relative to an integral number of leucines. The number in the parentheses shows the average of all those residues expected to be present in duplicate relative to the average of all those expected to be present singly.
radioactive material in fraction 64 of the combined trypsin plus V8 digest (Fig. 2A, bottom panel).

The digesta of material phosphorylated in *vivo* sometimes revealed an additional peak at fractions 65–66 (Fig. 2B, top panel). The relative intensity of this peak was variable, and usually this component was only evident as a widening of the major peak with a maximum at fraction 64. We think it likely that this component arises by a partial alternative cleavage by the V8 protease at Glu236 instead of Glu232. In support of this suggestion, a synthetic peptide comprising residues Leu221 to Tyr228 was considerably greater than 5–15% of the total radioactivity of the V8 and tryptic eluates. No additional peaks were observed before fraction 15. The profiles for hydrolysis with the different enzymes, and for the HPLC analysis are described under “Experimental Procedures.” The void volume of the HPLC column was 4.0 ml (fraction 8) and for the HPLC analysis are described under “Experimental Procedures.” The fractions containing the phosphorylated or unphosphorylated peptide were determined from the UV absorbance (see “Experimental Procedures”).

**Relative Phosphorylation of Canonical Tyrosines in β Subunit**

The radiolabeled peptides isolated by HPLC corresponding to the COOH-terminal portion of the ITAM were digested with prollylendopeptidase and re-chromatographed by HPLC. The elution pattern of radioactive counts was compared to the elution pattern of synthetic peptides.

The band on the nitrocellulose membrane. Furthermore, analysis of the digest radiolabeled in *vivo* and *in vitro* contained 5–15% of the total radioactivity of the V8 and tryptic eluates. The relative intensity of this peak was variable, and usually this component was only evident as a widening of the major peak with a maximum at fraction 64. We think it likely that this component arises by a partial alternative cleavage by the V8 protease at Glu236 instead of Glu232. In support of this suggestion, a synthetic peptide comprising residues Leu221 to Tyr228 was considerably greater than 5–15% of the total radioactivity of the V8 and tryptic eluates. No additional peaks were observed before fraction 15. The profiles for hydrolysis with the different enzymes, and for the HPLC analysis are described under “Experimental Procedures.” The void volume of the HPLC column was 4.0 ml (fraction 8) and for the HPLC analysis are described under “Experimental Procedures.” The fractions containing the phosphorylated or unphosphorylated peptide were determined from the UV absorbance (see “Experimental Procedures”).

### Evidence for Phosphorylation of Non-canonical Tyr224

As already noted, as illustrated in the bottom panel of Fig. 2A and the top panel of Fig. 2B, a radioactive component was also observed in fractions 60 and 61, albeit less consistently. This is the same position in which the bisphosphorylated synthetic peptide β-2e elutes (Table I). Phosphoamino acid analysis of this radioactive peak revealed only phosphotyrosine after labeling in *vivo* and *in vitro*. When the prollylendopeptidase digest of this peak was subjected to HPLC, radioactive peaks were observed at fractions 36 and 45. Fraction 36 is where the phosphotyrosine-containing peptide derived from the synthetic peptide β-2a elutes, whereas fraction 45 is where the phosphotyrosine-containing peptide derived from the synthetic peptide β-2b (Table II) is found. The amount of radioactivity in the two peaks was similar. The radioactivity was too low to permit us to perform the same analyses on receptors labeled in *vivo*. These results indicate that the non-canonical as well as the canonical tyrosine in the ITAM of the β subunit can become phosphorylated. Similar results with prollylendopeptidase were obtained when this peak was isolated from the V8 digest of the β subunit, or after V8 treatment of the tryptic digest (Fig. 2A, bottom panel).

### Analysis of Tyrosines Phosphorylated in γ Subunit

Tryptic cleavage of γ was performed on reduced samples of the receptor, to promote the accessibility to the proteolytic enzyme. However, similar results were obtained with or without reduction. Tryptic released 85–95% of the radioactivity from the region of the nitrocellulose membrane containing the γ chains. Analysis of the digest radiolabeled in *vivo* and *in vitro*...
by HPLC consistently revealed only two discrete radioactive components, with peaks at fractions 30 and 36 (Fig. 3, top and bottom). For the receptors labeled in vitro the peak at fraction 30 consistently contained a larger percent of the radioactivity, whereas the opposite was true for the receptors labeled in vivo. However, the difference was smaller than that observed with the peptides derived from β. Except as noted, the same characterizations were done for the receptors labeled in vivo and in vitro.

**Evidence for Phosphorylation of Tyr47**

The following observations indicate that the canonical tyrosine at position 47 became phosphorylated during both in vitro and in vivo incubation with 32P. (a) By HPLC the synthetic phosphorylated analogue of the tyrosine fragment γ-1 (Fig. 1) eluted in fraction 36: the same fraction in which the principal radioactive components released by trypsin from the γ chain were found. (b) Phosphoamino acid analysis of the material in fraction 36 from receptors labeled in vivo and in vitro identified tyrosine as the main phosphorylated residue (see also below). (c) Brief treatment with thermolysin of the receptor-derived material in fraction 36 generated three new radioactive peaks by HPLC. This would be expected for cleavage NH2-terminal to Val46 or Leu50, or both, yielding VpYTGLNTR, SDAVpYTG, and VpYTG (Fig. 1). Treatment of the synthetic γ-1 peptide under these conditions produced five peptides, three of which contained phosphotyrosine, as assessed by UV spectral analysis, in the same positions as the radioactive peptides (data not shown). On the other hand, more complete digestion led to an increase in the radioactive peak that eluted first at the expense of the two others, as would be expected from a hydrophobic reverse phase column. (d) Under the conditions we used, V8 cleaves only after glutamic acid; therefore, as expected, the synthetic γ-1 was resistant to the action of V8. The material in fraction 36 was likewise insensitive to the action of V8. (e) By digesting γ first with V8, one expects a 15-residue peptide containing the canonical tyrosine 47 and the tripeptide containing the other canonical tyrosine at position 58 (Fig. 1). The V8 digest of labeled γ revealed a single radioactive peak at fraction 35, likely because the highly polar TyPE tripeptide, containing Tyr(P)47 (below) was only poorly retained on the column. Subsequent digestion of the 15-mer with trypsin should yield a peptide identical to γ-1 (Fig. 1). When the radioactive component in fraction 35 was treated with trypsin, this is exactly what was observed. It was only practical to perform the analysis described in (e) with the peptides derived from receptors labeled in vitro.

**Evidence for Phosphorylation of Tyr58**

The evidence that the other canonical tyrosine, Tyr58, becomes phosphorylated is based on the following observations. (a) The synthetic peptide γ-2 eluted in the same fraction as the other major component derived from the digest, i.e. fraction 30 (Fig. 3). (b) By phosphoamino acid analysis, tyrosine was the only phosphorylated residue identified in fraction 30 in the digest of the γ chains derived from receptors labeled in vivo and in vitro. (c) In addition, the radioactive material in fraction 30 showed the expected sensitivity to V8. Treatment with the latter led to a shift of the radioactivity from fraction 30 to the void volume, as expected for the polar peptide TyPE (Fig. 1) generated by hydrolysis at Ghu28 and Ghu30.

**Kinetics of Phosphorylation**

The in vivo and in vitro phosphorylation of the receptors was analyzed multiple times. The extent of in vitro incorporation of 32P made it practical to examine the kinetics at which the four canonical tyrosines in the ITAMs of β and γ became phosphorylated. Such an experiment is shown in Fig. 4 for the β (A) and γ (B) subunits. The data are plotted to show the degree of labeling relative to the levels achieved at 60 min. Several points are notable. The two canonical tyrosines within the ITAM of an individual subunit achieve their final levels of phosphorylation at more or less equivalent rates. Similarly, the two types of subunits reach their final values at more or less equivalent rates. Finally, in agreement with our previously reported results, in both cases a plateau is achieved even though the kinase remains active (as assessed by its activity...
toward an exogenous substrate (16). The low level of incorporation of $^32$P in the labeling carried out in vivo made it impractical to pursue a similar kinetic study of such labeling. All the results reported here are for receptors that had been aggregated by antigen for 2 min, a time at which the phosphorylation in vivo and the association of tyrosine kinase activity to the receptor, reach a maximum under the conditions of activation employed (16).

### Relative Phosphorylation of Canonical Tyrosines

The ratios of phosphorylation of the COOH-terminal and NH$_2$-terminal peptides and the individual tyrosines in the ITAMs of the $\beta$ and $\gamma$ subunits are summarized in Table III. Several aspects of these results are notable. With respect to the ITAM in the cytoplasmic domain of $\beta$, it is apparent that in vivo, the relative incorporation of $^32$P into the membrane distal COOH-terminal peptide is larger than in vitro (column 3). However, when the radioactivity is corrected for incorporation of $^32$P into other amino acids (below), the ratio for the in vivo incorporation into tyrosines falls to approximately 3–4 (column 6). This ratio is close to that observed for the incorporation observed in vitro, where phosphotyrosine accounted for at least 95% of the incorporation of $^32$P (below).

With respect to the ITAM of $\gamma$, the ratio of phosphorylation of the COOH-terminal peptide relative to the NH$_2$-terminal peptide was roughly unity in vivo and in vitro (Table III, column 4). In the case of the $\gamma$ chain, the fraction of the counts that were incorporated in vivo that were not due to tyrosine was sufficiently small that it did not change significantly the ratio for the incorporation into tyrosines shown in Table III, columns 7 and 8. However, the relative incorporation into tyrosines on $\gamma$ in vivo was consistently 2–4 times larger than in vitro (Table III, columns 7 and 8, first versus second row).

We noted a roughly inverse relationship between the extent of labeling observed in vivo and in vitro. This would be expected if there were a limited number of tyrosines within an aggregate that could be phosphorylated, if the level of phosphorylation in vivo at any one time was determined by a balance between the activity of kinase(s) and of phosphatase(s) and if the phosphorylation in vitro mimicked that in vivo except for the absence of phosphatase. We reasoned that if the action of phosphatases was also suppressed in vivo, then the level of phosphorylation achieved should approximate that observed in vitro. Fig. 5 shows the result of such an analysis using the phosphatase inhibitor phenylarsine oxide. In this experiment, we assessed phosphotyrosine by Western blotting to be able to compare phosphorylation in vivo and in vitro. Although the reactivity of the anti-phosphotyrosine antibody with phosphorylated $\beta$ and phosphorylated $\gamma$ may not be identical (20), the correlation between phosphorylation and antibody binding should allow rough comparisons to be made between levels of phosphorylation on the individual subunits.

### In Vivo Phosphorylation of ITAM Serines and Threonines

The principal purpose of our studies was to analyze the phosphorylation of tyrosines on the receptor that occurs as an early response to its aggregation. However, analysis of the $\beta$ and $\gamma$ subunits labeled in vivo also allowed us to identify and in some cases to localize, other phosphorylated residues. Similar results were obtained in the presence or absence of extracellular Ca$^{2+}$ or Mg$^{2+}$. Earlier work had indicated that the $\beta$ subunit became phosphorylated on one or more serines and the $\gamma$ subunit on one or more threonines (27–29). In the present study, all of the radioactive peaks contained phosphotyrosine. It is possible that serines and threonines outside the ITAM also become phosphorylated, but at least in our studies, their steady state level is likely to be less than 5% of the total phosphorylation because we should have been able to observe larger amounts as discrete additional peaks.

### Phosphatase Inhibitor

The phosphatase inhibitor resulted in an increased level of phosphorylation as shown previously (20, 26). The new finding is that this enhanced phosphorylation achieved in vivo approximates the maximum level achieved in vitro when the aggregated receptors are incubated under conditions that are permissive for further phosphorylation by the receptor-associated kinase.

### Table III

| Labeling | COOH-terminal/NH$_2$-terminal$^a$ | Relative phosphorylation$^c$ |
|----------|----------------------------------|-----------------------------|
|          | $\beta$                           | $\gamma$                    |
| In vivo  | $2.07 \pm 0.14$                   | $8.86 \pm 2.13$             |
|          | $0.58 \pm 0.03$                   | $3.66 \pm 0.27$             |
| In vitro | $1.22 \pm 0.08$                   | $1.29 \pm 0.05$             |

$^a$ The ratios are based on the absorbance values obtained by densitometry of the autoradiographs of the same nitrocellulose membranes that were then submitted to proteolytic digestion.

$^b$ These ratios are based on the counts of $^32$P in the appropriate HPLC fractions from digests of $\beta$ with V8 and of $\gamma$ with trypsin.

$^c$ These values were calculated from columns 3 and 4, using the ratios in column 2. The value for $\beta$ Tyr$^{228}$ and $\gamma$ Tyr$^{57}$ in vivo, and of $\beta$ Tyr$^{228}$ in vitro were corrected for the fact that about 50%, 90% and 95% of the radioactivity present in the corresponding peptides was phosphotyrosine. The values for $\gamma$ represent the phosphorylation in each chain and would have to be multiplied by 2 to determine the relative amounts of phosphotyrosine in the $\gamma$ chains versus the $\beta$ chains in the receptor as a whole.
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from in vivo labeled subunits was reacted with prolylendopeptidase, a single radioactive peak was observed at fraction 36. This result is consistent with the predominant if not exclusive phosphorylation of Ser^239 (in addition to Tyr^228).

γ Subunit—About 10% of the radioactivity in the NH2-terminal peptide derived from tryptic digestion of the γ subunit (γ-1 in Fig. 1) from receptors labeled in vivo was found in phosphothreonine with the remainder in phosphotyrosine, whereas less than 1% was in phosphothreonine in the COOH-terminal peptide. No incorporation of ^32P into threonine was observed in either peptide after labeling in vitro.

After treatment with thermolysin, phosphoamino acid analysis of the two more hydrophobic peptides derived from the NH2-terminal γ peptide (fraction 36) revealed only phosphotyrosine, whereas the remaining undigested peptide contained both phosphotyrosine and phosphothreonine. These results favor Thr^32 as the principal and possibly exclusive threonine that becomes phosphorylated in the γ chain.

DISCUSSION

Phosphorylation of β Subunit—Both canonical tyrosines present in the ITAM of β were phosphorylated, although not to the same degree. The severalfold greater phosphorylation of Tyr^228 than of Tyr^214, was observed both in vivo and in vitro (Fig. 2) and to a similar degree (Table III, column 6). In vivo, the level of phosphorylation is the result of a dynamic process of phosphorylation and dephosphorylation (20, 26, 30). It might be supposed that in vivo the different levels of phosphorylation result from a different susceptibility of the two tyrosines to a phosphatase. However, this would not explain why a similar differential was observed in vitro in the presence of the inhibitor vanadate, where all tyrosine phosphatase activity was eliminated. Alternatively, the differential might reflect a different accessibility of the two tyrosines to the receptor-bound kinases(s). If so, then the difference must be more or less absolute for some of the aggregated receptors because the phosphorylation in vitro reached a plateau value at similar rates for both tyrosines (Fig. 4). We have no additional data to support other explanations. Recently, various downstream signaling molecules were shown to interact with a diphosphorylated β ITAM peptide (31). Possibly, alternative phosphorylations of the β ITAM tyrosines lead to differential interactions with such distal components.

The analyses reported here cannot determine what fraction (if any) of the β ITAMs were simultaneously phosphorylated on both tyrosines. It has been shown that both tyrosines must be phosphorylated simultaneously in order for an ITAM to interact with either Syk and ZAP-70 to propagate an activation signal (9). Although those kinases are thought to interact chiefly with the γ subunit (below), diphosphorylation may also favor interaction of SH2-containing molecules with β (31). The results on the β subunit resemble those found with the Iga chain of the B-cell antigen receptor, where only the canonical tyrosines in the ITAM were phosphorylated, and to different extents (12). The level of phosphorylation in Iga were minimally affected when one of the canonical tyrosines was mutated to phenylalanine (12).

Our data show that the non-canonical Tyr^224 (which is conserved in the human, mouse, and rat β ITAM) can also become phosphorylated. This tyrosine is at position –4 from the COOH-terminal canonical tyrosine. Among the members of the MIRR family, only marine CD3 γ has a non-canonical tyrosine within the ITAM, at position –3 from the COOH-terminal canonical tyrosine. In neither instance is the tyrosine surrounded by a sequence that is typically found in substrates for Src family kinases (32). Phosphorylation of Tyr^224 was found only in the bisphosphorylated COOH-terminal peptide, suggesting that its phosphorylation occurs only after the canonical tyrosine has been phosphorylated. In those receptors in which the COOH-terminal peptide eluting at fraction 64 contained phosphoserine, the bisphosphorylated peptides at fraction 60 contained only phosphotyrosine. It is as if the double phosphorylations were mutually exclusive. We are unaware of other reports of in vivo or in vitro phosphorylation at non-canonical tyrosines in the ITAMs.

An unexpected finding was that the principal serine(s) that became phosphorylated were also in the ITAM. On HPLC we observed no radioactive fractions that did not contain phosphorysine. Such fractions should have been observed in the V8 or tryptic digest if any of the numerous non-ITAM serines in the carboxyl- and/or amino-terminal cytoplasmic domains of the β had been phosphorylated. In vivo, the phosphorylation of Ser^229 accounts for about 50% of the radioactivity found in the COOH-terminal peptide of the ITAM. It is potentially significant that serines at an equivalent position (+1 from the COOH-terminal tyrosine) are found also in T-cell receptor ζ and CD3 γ, δ, ε, and in the viral protein BLV gp30a (see Fig. 1 in Ref. 7). When phosphorylated, the serine would have a negative charge, and it is noteworthy that the corresponding residue in all the other ITAM-containing subunits of the multichain immune recognition receptor family is a negatively charged glutamic or aspartic acid (Fig. 1 in Ref. 7). Interestingly, in the receptor for transforming growth factor β, mutation of a phosphorylatable threonine to an aspartic acid or glutamic acid residue constitutively activates the receptor (33). Again, our current studies do not reveal whether any of the β subunits are phosphorylated on tyrosine and serine simultaneously. It is possible that in the V8 digest, the additional peak at fraction 66, frequently found as a widening of the peak at fraction 64 was only observed on receptors labeled in vivo (Fig. 2, B, top panel) because the presence of phosphoserine at position 229 affects the hydrolysis by V8 at Glu^32 (Fig. 1). Indeed, when we were able to isolate this peak, its relative content of phosphoserine was larger than in peak 64.

Phosphorylation of γ Subunit—The phosphorylation of the canonical tyrosines in the ITAM of γ was more equivalent than in the case of β. In vivo, the relative phosphorylation of Tyr^47 (NH2-terminal peptide) was always larger than Tyr^228 (COOH-terminal peptide), and the opposite was true in vitro (Fig. 3), but in both cases the ratio was reasonably close to 1. The extent of phosphorylation of the γ tyrosines relative to β Tyr^214 was 2–3 times larger in vivo than in vitro (Table III, columns 7 and 8, top versus bottom row). If one takes into account that about 90% of the radioactivity in the NH2-terminal γ peptide in vivo is phosphothreonine, the average of the ratios in vivo and in vitro of phosphorylation of the two ITAM tyrosines in γ is roughly 1 (Table III, column 4). This is consistent with the results shown in Fig. 5.

Several reports suggest that it is the γ subunit that interacts with Syk kinase. Thus, Syk as well as its isolated SH2 domains preferentially bind to the isolated γ compared with the β subunit (10, 31, 34). It has also been shown that peptides containing the γ ITAM bind and activate Syk kinase (35). Syk, and the related ZAP-70 tyrosine kinases, contain two consecutive SH2 domains whose integrity is required for binding to ITAMs and activation (9). Furthermore, Syk and the related ZAP-70 used by other MIRR family members interact only with bisphosphorylated ITAMs (9). Our observations, that the phosphorylation of the two tyrosines in the ITAM of γ are approximately equivalent, is consistent with the mechanistic model these data imply.

Our data indicate that the phosphorylation of threonine in γ also occurs at sites proximal to a canonical tyrosine as was the case of serine in β. However, whereas in β the phosphoserine
generates the negative charge that in virtually all other known ITAMs is conferred by a highly conserved aspartic or glutamic acid residue (above), the residues homologous to Thr\textsuperscript{32} are polar but not necessarily charged, and to a similar extent the same is true for those homologous to Thr\textsuperscript{32}. Nevertheless, in all ITAMs either one of these two positions is polar or charged. These residues are conserved in all γ subunits analyzed so far, and its phosphorylation may regulate the interaction of the γ ITAMs with proteins containing SH2 domains.

Comparison of Phosphorylation in Vitro and in Vivo—Aggregated receptors that were incubated under conditions permissive for tyrosine kinase activity (and in the presence of phosphatase inhibitor) became phosphorylated on their ITAM tyrosines (Figs. 2 and 3). The kinetics were similar for the canonical tyrosines within each ITAM and for the β compared with the γ subunits (Fig. 4). As noted previously for the intact subunits, a plateau level was reached relatively rapidly even though under the conditions used, phosphorylation of an exogenous substrate continues unabated (16). Furthermore, under these conditions only a fraction of the receptors are phosphorylated as judged by their precipitability by anti-phosphotyrosine.\textsuperscript{2} Notably, the level of phosphorylation achieved in vitro approximates that achieved in vivo when phosphatase activity is inhibited under both conditions (Fig. 5). These new data are consistent with the following formulation. 1) At a given point in time, phosphorylation of aggregated FcεRI results from a finite number of receptor tyrosines being accessible to a finite number of receptor-associated kinases. 2) In vivo, the steady state level of phosphorylation results from the opposing actions of phosphatases and the associated kinase. 3) In the presence of phosphatase inhibitors, the level of phosphotyrosine achieved in vitro does not substantially exceed the level observed in vivo (Fig. 5). This observation suggests that under the conditions used for these studies, we recovered most of the kinase that was associated with FcεRI in vivo. Together, these aspects suggest that with respect to the initial phosphorylations of receptor tyrosines, the immunoprecipitates of the aggregated receptors isolated under conditions that maintain their associated kinase(s) are a reasonable analogue of the in vivo complex.

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