Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 61-67

Phorbol ester induces phosphorylation of the 80 kilodalton murine interleukin 1 receptor at a single threonine residue

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Received April 16, 1991

The cytoplasmic domains of some cell surface receptors become phosphorylated in cells treated with phorbol esters. The present study was undertaken in order to determine whether this is also true of the 80 kDa interleukin 1 receptor (IL1R). Recombinant murine IL1R, transfected into chinese hamster ovary (CHO) cells or murine fibroblasts, was immunoprecipitated from [^{32}P]orthophosphate-labelled cells. IL1R phosphorylation was only detected in cells pre-treated with phorbol 12-myristate 13-acetate (PMA) and occurred solely on phospho-threonine. In contrast to a previous report, little or no IL1R phosphorylation occurred in response to IL1. By using a truncated receptor and receptors in which threonine residues were changed to alanines, we established that Thr$_{537}$, near the carboxy-terminus, is the major site of PMA-induced phosphorylation. The human IL1R has a different sequence at this locus, and is apparently not phosphorylated. Binding studies showed that PMA-induced phosphorylation had no discernible effect on ligand binding or internalization.

Tumor-promoting phorbol diesters such as PMA are powerful modulators of cellular function; among their effects is the allosteric modulation of several cell surface receptors including those for epidermal growth factor (1-3), insulin (4,5), α_1-adrenergic agonists (6), and γ-interferon (7). In some cases, decreased ligand binding (1), receptor tyrosine kinase activity (3) or functional desensitization (6) have been correlated with increased receptor phosphorylation, presumeably occuring via PMA-activated protein kinase C (PKC).

Interleukin 1 is a polypeptide cytokine, produced mainly by activated macrophages, which participates in a wide variety of inflammatory and immune responses (see Ref. 8 for review). IL1 receptors (IL1R) are widely distributed and one type, an 80 kDa polypeptide expressed on connective tissue cells and T-cells, was recently cloned from murine (9) and human (10) T-cells. The cloned murine IL1R appears capable of transducing a signal when transfected into CHO cells (11), and a recent report from this laboratory (12) provided

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The abbreviations used are:
IL1, interleukin 1; IL1R, interleukin 1 receptor; Mab, monoclonal antibody; PKC, protein kinase C; PMA, 4ß-phorbol 12-myristate, 13-acetate.

0006-291X/91 $1.50

evidence that such receptors become phosphorylated when occupied by ligand. We carried out the present study to determine if the IL1R is a protein kinase substrate in phorbol ester-treated cells.

MATERIALS AND METHODS

Materials: Carrier-free [^{32}P]orthophosphoric acid was purchased from New England Nuclear. Phosphate-free minimal essential medium (P_i-free MEM) was from Gibco BRL Inc. (Grand Island, NY). Protein G-Sepharose was obtained from Pharmacia. Phorbol myristate acetate, tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin, phosphoaminoacids, proteinase inhibitors and all other reagents were obtained from Sigma.

Cytokines, antibodies and cell lines: Human rIL1α was expressed in *E. coli* and labelled with [^{125}I]Sodium Iodide to 1-3 x 10^{15} cpm/mmol as previously described (9). Characteristics of the rat monoclonal antibody (Mab) RM-5, raised against murine rIL1R expressed in C127 fibroblasts, and of Mabs M1 and M10, raised against similarly-expressed human IL1R have been reported elsewhere (12,13). CHO-K1 cells stably transfected with a murine IL1R cDNA clone containing the entire coding region (105,000 IL1R/cell [11]), and C127muIL1R cells, expressing 1.3 x 10^6 murine IL1R/cell (12, 14) were derived, selected and maintained as already described. A truncated murine IL1R (Δ523) in which a stop codon replaces the GCC at residue 524 (9) resulting in loss of the C-terminal 34 amino acids, and mutants TA537 and TA534 in which Thr_{537} and Thr_{534} are replaced by alanines, were generated by site-specific mutagenesis using established techniques (15). The mutant and wild type (murine and human) IL1Rs were transiently expressed in COS-7 cells as described (9,10). The cells were used three days post-transfection.

Isolation and analysis of phosphorylated IL1 receptors: IL1R-expressing cells in 10 cm petri dishes were rinsed with warm P_i-free MEM and pre-incubated in 3 ml of this medium for 15 mins. Cells were then labeled for 2h in 3 ml of fresh P_i-free MEM/dish supplemented with 0.5% dialysed fetal bovine serum (Intergen Co., Purchase NY) and 0.5-1 mCi/ml [^{32}P]phosphate. During the last 15 minutes of this incubation, IL1α, PMA, or vehicle were added, as indicated in the text. The cell layers were rinsed twice in ice-cold phosphate-saline, scraped into 1 ml/dish of cold lysis buffer (20 mM MES pH 6.0, 1% (v/v) Triton X-100, 400 mM NaCl, 10 mM ATP, 10 mM EDTA, 10 mM EGTA, 50 mM NaF, 1 mM Na_3VO_5 , 10μM pepstatin A, 10 μM leupeptin, 1 mM phenylmethylsulfonylfluoride) and microcentifuged for 10 minutes (13000 rpm). Lysates were precleared by incubating them for 30 mins. with nonimmune rabbit serum (6μl) and protein G-Sepharose (14 μl packed volume). Thereafter, immunoprecipitation with anti-IL1R, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography was carried out as described (12). [^{32}P]-labelled IL1 receptors were eluted from gels, oxidized, trypsin digested and subjected to phosphopeptide mapping as described for the EGF receptor (16). Two-dimensional phosphoaminoacid analysis was performed according to the method of Cooper *et al.* (17).

Measurement of [125I]IL1 binding and internalization: Transfected COS cells in 12- well tissue culture clusters were rinsed with cold RPMI 1640 medium , 20 mM HEPES pH 7.4, 10% (w/v) BSA (binding medium), and were then incubated for 2h at 4°C in the same medium supplemented with 200 pM [^{125}I]IL1α $\pm$ 100 nM unlabeled IL1. The cell monolayers were washed with ice-cold binding medium and lysed in 0.1N NaOH/1% Triton X-100. Aliquots of the lysates were counted in a γ-counter. Alternatively, washed cell monolayers were warmed to 37°C and incubated in fresh binding medium for periods up to 2h. At each time point, the cells were incubated for 5 mins with 0.1M glycine pH 3.0, 0.14M NaCl to extract surface-bound ligand,. The remaining internalized IL1 was recovered by lysis of the cells as described above. All measurements were corrected for non-specific binding in the presence of excess unlabelled IL1.

RESULTS AND DISCUSSION

Murine C127 fibroblasts expressing aproximately 10^6 rmIL1R/cell (C127mulL1R) were labelled with [^{32}P]phosphate and briefly stimulated with PMA or IL1α. Lysates made from these cells were immunoprecipitated with an anti mIL1R Mab or control Mab and the precipitated proteins were analyzed by SDS-PAGE and autoradiography (Fig 1a). Anti-IL1R Mab specifically immunoprecipited a diffuse 80 kDa polypeptide, corresponding to [^{32}P] IL1R, from cells treated with PMA; qualitatively similar results were obtained using CHO-K1mulL1R cells, (100,000 IL1R/cell, Fig 1b). No [^{32}P]IL1R were specifically immunoprecipitated from lysates of untreated cells; furthermore, we were unable to detect increased IL1R phosphorylation in C127mulL1R cells stimulated with IL1 under identical conditions (Fig 1a). In contrast, Gallis et al (12) reported that IL1 induced rapid phosphorylation of IL1R in CHO-K1cells. However, more recent experiments with these cells have generally failed to confirm the initial finding; some very faint phosphorylation of IL1R is occasionally detectable (not shown). We have no samples of earlier passages of CHO-K1mulL1R for comparison, so our conclusion is that the previous report (12) does not reflect a general phenomenon, and that IL1 mediated IL1R phosphorylation may have been peculiar to the initial isolate of CHO-K1mulL1R.

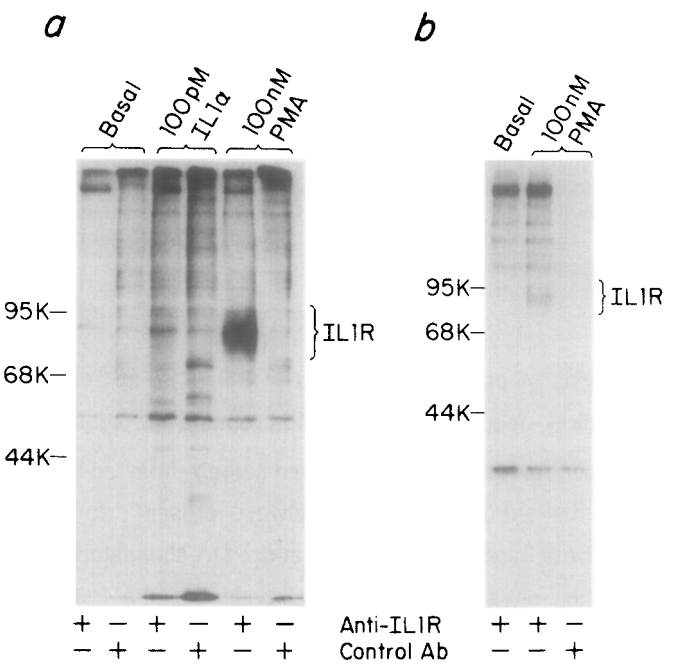

Fig. 1. PMA induces phosphorylation of the murine IL1R. C127mulL1R stably transfected with approximately 10^6 IL1R/cell (*a*) or CHO-K1mulL1R (10^5 IL1R/cell, *b*) were labeled with [32P]orthophosphate for 2h and then treated for 15 mins with IL1α, PMA or vehicle (dimethylsulfoxide, 0.1%) as indicated. Cell lysates were prepared, subjected to immunoprecipitation analysis as described in "Materials and Methods", and resolved on 10% SDS-PAGE gels. Molecular mass (kDa) of marker proteins is indicated on the left of the autoradiographs. Representative of four independent experiments.

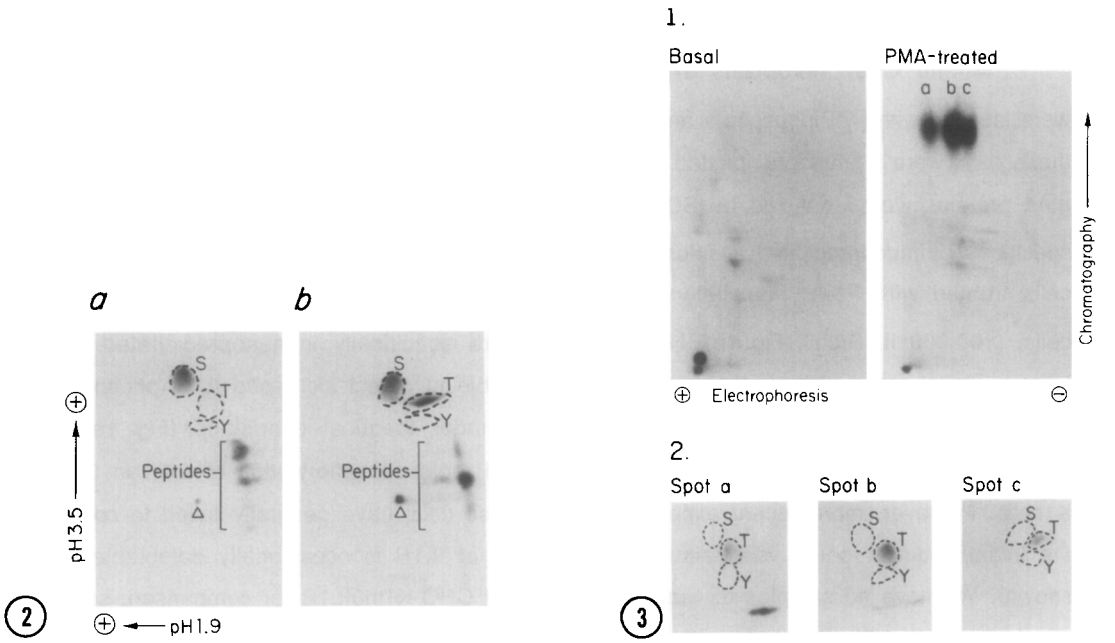

Fig.2. PMA causes phosphorylation of IL1R on threonine residues. The labelled IL1R band from PMA-treated C127mulL1R cell lysates (*b*) or the corresponding region of a gel lane from control cells (*a*) was eluted and hydrolysed as described in "Materials and Methods". Hydrolysates were mixed with unlabelled phosphoserine(S), phosphothreonine (T) and phosphotyrosine (Y,1 μg each) and subjected to 2 dimensional phosphoaminoacid analysis. Δ indicates the point of application. The positions of the authentic phosphoaminoacid markers and partially hydrolysed phosphopeptides are shown. Representative of two independent experiments.

Fig. 3. Two-dimensional phosphopeptide mapping of phosphorylated IL1R. (1) [^{32}P]labelled IL1R from PMA stimulated C127mulL1R cells or corresponding gel slices from untreated cells were eluted, and trypsin-digested (0.28 mg/ml, in two additions, 20h) as described in "Materials and Methods". Phosphopeptides were separated by thin-layer electrophoresis at pH 1.9, followed by ascending chromatography in *n*-butanol: pyridine:acetic acid:water (15:10: 3:12). The origin is at bottom left. (2) The spots a, b, and c from (1) were eluted, hydrolyzed and analysed as descibed in the legend for Fig. 2.

Phosphoaminoacid analysis of partially hydrolysed [^{32}P]IL1R, (Fig 2) revealed that the PMA-stimulated phosphorylation occurred as phosphothreonine [it should be noted that the [^{32}P]phosphoserine in control samples was derived largely from co-migrating proteins non-specifically precipitated by anti-IL1R Mab and always present to some extent (Fig 1)]. Phosphopeptide maps were made from control and PMA-stimulated IL1R samples. Three prominent closely-migrating phosphopeptides were evident in the PMA-treated sample (Fig. 3), each of which contained only phosphothreonine. This pattern could arise from phosphorylation of different threonine residues or, more likely, from incomplete tryptic cleavage at the site of closely-spaced basic amino acids.

The cytoplasmic domain of the murine IL1R contains nine threonine residues (Fig 4) of which three (sequences 7-9 in the figure) are clustered near the carboxy-terminus of the molecule. A mutant IL1R cDNA (Δ523) which terminates prematurely at Pro$_{523}$, together

(1)	Ala	Ser	Asp	Gly	Lys	Thr	Tyr	Asp	Ala	Tyr	Ile	(363-373)
(2)	Ile	Leu	Tyr	Pro	Lys	Thr	Leu	Gly	Glu	Gly	Ser	(373-383)
(3)	Phe	Ser	Asp	Leu	Asp	Thr	Phe	Val	Phe	Lys	Leu	(384-394)
(4)	Tyr	Val	Gly	Glu	Asp	Thr	Ile	Glu	Val	Thr	Asn	(415-425)
(5)	Asp	Thr	Ile	Glu	Val	Thr	Asn	Glu	Asn	Val	Lys	(419-429)
(6)	Pro	Gln	Ser	Ala	Lys	Thr	Arg	Phe	Trp	Lys	Asn	(507-517)
(7)	Lys	His	Arg	Leu	Leu	Thr	Leu	Asp	Pro	Val	Arg	(532-542)
(8)	Asp	Pro	Val	Arg	Asp	Thr	Lys	Glu	Lys	Leu	Pro	(539-549)
(9)	Lys	Leu	Pro	Ala	Ala	Thr	His	Leu	Pro	Leu	Gly	(547-557)

Fig. 4. Threonine residues of the murine T-cell IL1R cytoplasmic domain. The residue numbers, shown in parentheses on the right, correspond to the numbering in Sims *et al.* (9).

with the full length receptor, were transiently expressed in COS cells and tested for their ability to be phosphorylated in response to PMA. As was the case for the other cell lines, anti-murine IL1R Mab specifically precipitated [^{32}P]-labelled wild type IL1R from PMA-treated cells but not from untreated cells (Fig 5a). In contrast, no phosphorylated IL1R were immuno-precipitated from lysates of Δ523-transfected COS cells expressing 1.3 x 10^6 truncated IL1R/cell. It therefore appeared that the phosphorylated threonine residue might be present in the deleted portion of the Δ523 mutant. The most C-terminal threonine (Fig. 4, sequence 9) is not closely flanked by any basic residues and we considered it an unlikely substrate for direct

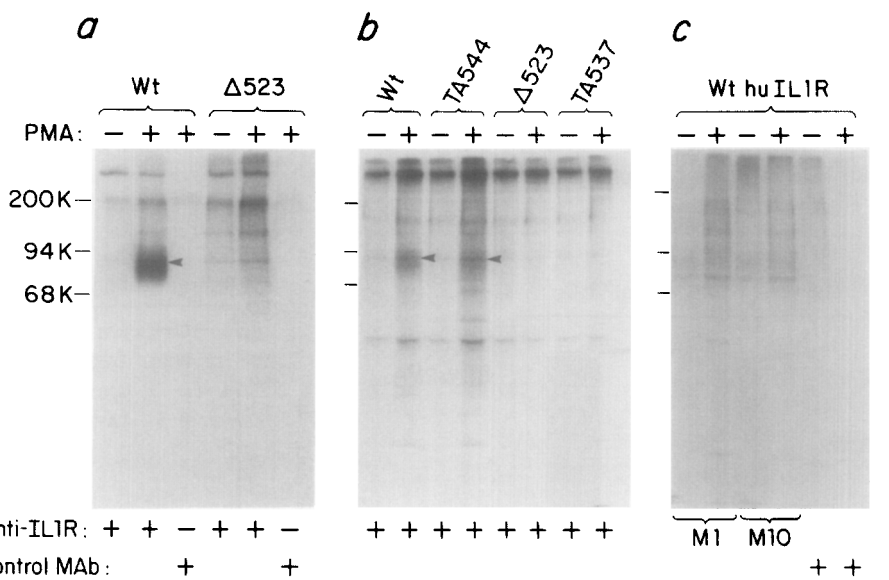

Fig. 5. Phosphorylation analysis of murine IL1R mutants and human IL1R. COS-7 cells were transfected with wild type (Wt) or mutant murine IL1R cDNAs as indicated along the *top* of the figure. Three days later, the cells were labelled with [^{32}P]phosphate, treated with PMA or vehicle (+, -, respectively, as indicated at *top*) and analysed as described in the legend to Fig. 1. M1 and M10 (*panel c*), refer to different anti-human IL1R Mabs. M_r (kDa) is indicated to the *left* of each panel. Scatchard analysis of [^{125}I]IL1α binding revealed, in (*a*) 0.55 x 10^6 IL1R/cell for Wt and 1.3 x 10^6 IL1R/cell for Δ523 transfectants. In (*b*) 23,500, 41,600, 10,050, and 16,070 cpm of [^{125}I]IL1α were specifically bound/dish for Wt, Δ523, TA544, and TA537-transfectants. representative of 2 independent experiments.

phosphorylation by PKC. Accordingly, we constructed IL1Rs bearing threonine to alanine point mutations at Thr_{537} and Thr_{544} (Fig 4., sequences 7 and 8, respectively). When transiently expressed in COS cells, the TA544 mutant receptor, but not the TA537 mutant, was phosphorylated to a similar extent as the wild-type IL1R (fig 5b); we conclude that Thr_{537} is the most likely unique site of PMA-mediated phosphorylation.

Interestingly,using precipitating polyclonal and monoclonal antibodies (13), we have been unable to demonstrate similar phosphorylation of the human rIL1R stably transfected into C127 cells (not shown) or transiently expressed in COS cells (see Fig. 5c). The human IL1R sequence (10) in this region differs from the mouse IL1R: Thr_{537} is replaced by serine, and the subsequent stretch of 6 residues are different in the two receptors. Arg_{534} of murine IL1R is replaced by glutamine in the human receptor, potentially making this a less likely PKC acceptor site (though we cannot exclude the possibility that IL1R phosphorylation is mediated by a different enzyme, indirectly activated by PKC).

We have not been able to demonstrate any functional significance of PMA-induced murine IL1R phosphorylation. Figure 6a shows that, in COS cells, IL1 binding is not affected by PMA-treatment, regardless of whether wild-type or TA537 receptors are expressed. The rate and extent of ligand internalization is similarly unaffected (Fig. 6b). These findings are in agreement with previous reports of the lack of short term effects of PMA on IL1R binding to murine EL-4 thymoma cells (18,19) from which the murine IL1R was cloned. In the B cell lines 70Z/3 [18] and Raji (19), which are known to express a distinct type of IL1R (18,20), PMA caused a rapid decrease in IL1 binding. Further work will be necessary to define the molecular mechanisms underlying this difference and to uncover the significance, if any, of murine 80 kDa receptor phosphorylation.

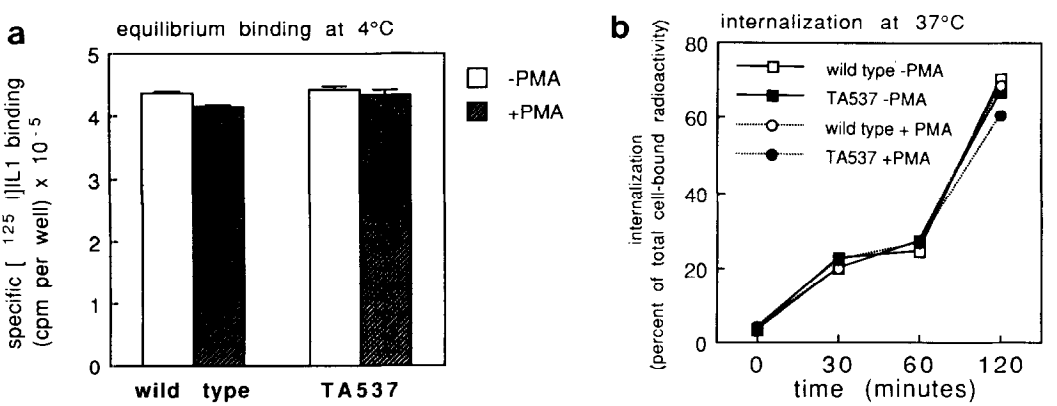

Fig. 6. PMA-treatment does not affect IL1 binding or internalization. (*a*) specific [^{125}I]IL1α binding to monolayers of COS cells transfected with wild type and TA537 murine IL1R. Prior to IL1-binding, cells were treated at 37°C for 30 mins with dimethylsulfoxide vehicle (*open bars*) or 100 ng/ml PMA (*shaded bars*). (*b*) After a 4°C IL1-binding step as in (*a*), monolayers were washed and incubated in fresh medium at 37°C for the indicated times. Surface bound (acid-labile) and internalized ligand were measured as described in "Materials and Methods". Bars and points represent the mean ± standard error of triplicate determinations.

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