EGFR signaling to p120-catenin through phosphorylation at Y228

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Summary

Epidermal growth factor receptor (EGFR) signals to p120^{ctn} (p120), implying a role for EGFR in modulating cell-cell adhesion in epithelial tissues. p120 controls cadherin turnover, and may have other roles that modulate cadherin adhesiveness. To clarify the role for EGFR and other tyrosine kinases in regulating p120 function, we have generated and characterized a new phosphospecific antibody to p120 Y228, as well as a novel siRNA-based reconstitution system for analyzing roles of individual p120 phosphorylation events. In A431 cells, epidermal growth factor induced striking p120 phosphorylation at Y228. Y228-phosphorylated p120 localized to adherens junctions and lamellipodia, and was significantly enhanced in cells around the colony periphery. A screen of carcinoma cell lines revealed that some contain unusually high steady state levels of Y228 phosphorylation, suggesting that disregulated kinase activity in tumors may affect adhesion by constitutive cross talk to cadherin complexes. Despite these observations, mutation of Y228 and other prominent Src-associated p120 phosphorylation sites did not noticeably reduce the ability of E-cadherin to assemble junctions and induce compaction of cultured cells. Although A431 cells display significant activation of both EGFR and Src kinases, our data suggest that these account for only a fraction of the steady state activity that targets p120 Y228, and that Src family kinases are not necessary intermediates for epidermal growth factor-induced signaling to p120 Y228.

Key words: p120 catenin, Cadherin, Src, EGFR, Adherens junction, Adhesion

Introduction

E-cadherin is the primary cell-cell adhesion molecule in epithelial cells and is widely considered to be a master regulator of the epithelial phenotype (Takeichi, 1995). Cadherin function in cell adhesion is regulated by several cytoplasmic binding partners, the catenins, as well as other proteins thought to be more loosely associated with cadherin complexes (for reviews, see Anastasiadis and Reynolds, 2000; Nollet et al., 1999; Yap, 1998). β-catenin binds to the carboxy-terminal domain of E-cadherin and serves to physically link the cadherin complex to the actin cytoskeleton through α-catenin (Aberle et al., 1994; Jou et al., 1995). p120^{ctn} (p120) binds to the cadherin juxtamembrane domain (Navarro et al., 1998; Thoreson et al., 2000; Yap et al., 1998) and regulates surface levels of E-cadherin by modulating cadherin turnover (Davis et al., 2003; Ireton et al., 2002). p120 may have other roles in the cadherin complex or cytoplasm, as suggested by its affects on cadherin clustering and the actin cytoskeleton (Thoreson et al., 2000; Yap et al., 1998) and its ability to regulate Rho GTPases (Anastasiadis et al., 2000; Cozzolino et al., 2003; Goodwin et al., 2003; Grosheva et al., 2001; Magie et al., 2002; Noren et al., 2000). In addition to the catenins, numerous protein tyrosine kinases (e.g. EGFR, VEGFR-2, FGFR) (Hoschuetzky et al., 1994; Lampugnani et al., 2003; Nieman et al., 1999; Rahimi and Kazlauskas, 1999) and phosphatases (e.g. PTPK, PTPμ, PTP-LAR, PTP1B, PCP-2, DEP-1, and SHP-2) (Balsamo et al., 1996; Brady-Kalnay et al., 1998; Fuchs et al., 1996; Holsinger et al., 2002; Kypta et al., 1996; Palka et al., 2003; Ukropec et al., 2000; Yan et al., 2002) reside in adherens junctions, suggesting that phosphorylation of cadherins and associated proteins may provide a means for rapid modulation of cell adhesion.

Recent evidence indicates that a core function of p120 in cadherin complexes is to regulate cadherin stability and turnover (Davis et al., 2003; Ireton et al., 2002; Xiao et al., 2003). The colon carcinoma cell line SW48 is p120-deficient, and as a consequence contains abnormally low levels of E-cadherin. Restoring p120 in these cells stabilizes cadherin levels and rescues epithelial morphology (Ireton et al., 2002). Using short interfering RNA (siRNA), these observations have been extended to other cadherins, including neuronal (N), placental (P) and vascular endothelial (VE) cadherins (Davis et al., 2003; Xiao et al., 2003). Moreover, p120 family members such as ARVCF and δ-catenin can also stabilize cadherin in cells where p120 has been removed (Davis et al., 2003). In p120-deficient cells, cadherins traffic normally to the cell surface, but are then rapidly turned over and removed by a mechanism involving the proteosome and/or lysosomes. These observations indicate that p120 and its relatives regulate adhesion in part by dynamic control of cadherin levels at the cell surface (Davis et al., 2003; Xiao et al., 2003).

The roles of p120 in regulating adhesion are probably controlled by phosphorylation. To date, 16 phosphorylation sites have been identified, eight on tyrosine (Mariner et al., 2001) and eight on serine or threonine (Xia et al., 2003). p120 serine phosphorylation is modulated by events that regulate

protein kinase C (PKC) (Xia et al., 2003), and has been implicated as a negative regulator of adhesion in Colo-205 and HT-29 cells, where adhesion is significantly increased by expression of p120 mutants lacking the amino-terminal phosphorylation domain, or treatment of cells with serine kinase inhibitors such as staurosporine (Aono et al., 1999). p120 tyrosine phosphorylation is elevated in nascent junctions, suggesting that p120 phosphorylation may be involved in junction formation or maturation (Calautti et al., 1998; Lampugnani et al., 1997). The roles of individual p120 phosphorylation events, however, remain to be determined.

p120 is an excellent substrate for Src family kinases and is directly phosphorylated by Src in vitro (Mariner et al., 2001; Reynolds et al., 1992; Reynolds et al., 1989). p120 tyrosine phosphorylation is decreased in Src family kinase Fyn–/– primary keratinocytes (Calautti et al., 1998), and both Fyn and the cytoplasmic tyrosine kinase Fer can bind to p120 constitutively (Kim and Wong, 1995; Piedra et al., 2003). In addition, growth factor-induced activation of several receptor tyrosine kinases, including EGFR, CSFR, PDGFR and VEGFR results in rapid p120 phosphorylation on tyrosine, serine and threonine (Downing and Reynolds, 1991; Esser et al., 1998).

Most of these kinases affect cell-cell adhesion and may act in part through modulation of p120 and other catenins. Though traditionally associated with focal adhesions, Src family kinases are also implicated in the regulation of adherens junctions (reviewed by Frame, 2002; Frame et al., 2002). Interestingly, expression of a phosphorylation-defective mutant of focal adhesion kinase (FAK) in KM12C colon carcinoma cells reverses the effects of elevated Src activity on cell-cell junctions, suggesting that Src might be involved in cross-talk between focal adhesions and adherens junctions (Avizienyte et al., 2002). Additionally, Src activity is required in keratinocytes for transient cell-cell interactions during junction formation (Owens et al., 2000). Exciting relationships are emerging that link receptor tyrosine kinases and Src to p120-induced cell motility and Rho-GTPase signaling (Cozzolino et al., 2003). These activities appear to be coordinated through the p120 amino-terminal end, which contains all of the p120 tyrosine phosphorylation sites (Cozzolino et al., 2003). EGFR partially colocalizes with cadherins in epithelial cells, and is reported to interact directly with the complex through β-catenin (Hoschuetzky et al., 1994; Takahashi et al., 1997). In HaCat cells, a transient, cadherin-dependent spike in EGFR activity occurs upon nascent junction formation (Pece and Gutkind, 2000). Moreover, it is well-established that EGFR activity stimulates Src and vice versa (Mao et al., 1997; Osherov and Levitzki, 1994; Oude Weernink et al., 1994) (reviewed by Frame, 2002). These observations link EGFR and Src to cadherin function and suggest that coordinated activity of these kinases may modulate dynamic changes in adhesion.

Nonetheless, it is not known how receptor or Src-induced p120-phosphorylation modifies cadherin function. We have begun to dissect these events by generating model systems and reagents that make it possible to focus on the role(s) of specific p120 phosphorylation events. In collaboration with Dr Roberto Campos and BD/Transduction Laboratories, we have generated a phospho-specific p120 antibody to Y228, one of several major p120 phosphorylation sites targeted by oncogenic Src. We have used this new tool along with a novel siRNA-based p120 substitution system to examine the EGFR-p120 relationship. We show that Y228 is indeed a major target of EGFR activity and that phosphorylation at this site is constitutively elevated in many carcinoma cell lines. We suggest that EGFR signaling to p120 does not require Src as an intermediate kinase, but may signal to p120 either directly or through non-Src family kinases.

Materials and Methods

Antibodies

The monoclonal antibody (mAb) to p120 phospho-Y228 (clone 21a) was generated in collaboration with BD/Transduction Laboratories and is commercially available. mAb pp120, which detects total p120, was obtained from BD/Transduction Laboratories. Other p120 antibodies used include mAb 15D2 and murine-specific mAb 8D11 (Wu et al., 1998) and rabbit pAb F1αSH (Reynolds et al., 1994). Human-specific E-cadherin mAb HECD-1 was a gift from Dr Masatoshi Takeichi. Phosphotyrosine mAb PY20 and EGFR mAb were obtained from BD/Transduction Laboratories. The α-tubulin mAb (clone DM 1A) was obtained from Sigma. The HA-tag monoclonal antibody 12CA5 is available from Roche.

Cell culture and cell lines

Cells were cultured in DMEM containing L-glutamine (Hyclone), 10% fetal bovine serum (FBS) (Hyclone), and 1% penicillin-streptomycin (Gibco/Invitrogen). FBS for Phoenix cell culture was heat inactivated at 56°C for 30 minutes. A431 cells were obtained from Margaret Wheelock (University of Nebraska Medical Center); 3T3/Src527F cells were a gift from David Shalloway (Cornell University); T47D cells were from Graham Carpenter (Vanderbilt University); MDA-MB-453 cells were from Carlos Arteaga (Vanderbilt University); Her-14 cells were from Joseph Schlessinger (Yale University). SYF cells (SYF + pLXSH-6, SYF + c-Src-4, and SYF + kinase dead c-Src-2) were kindly provided by Jonathan Cooper (Fred Hutchinson Cancer Center) and have been characterized extensively (Cary et al., 2002; Klinghoffer et al., 1999). Phoenix packaging cells for generation of amphotropic retrovirus were obtained from Linda Sealy (Vanderbilt University) by permission of Garry Nolan (Stanford University). Other cell lines were obtained from the American Type Culture Collection: COS-7, HCT116, MCF-7, MDA-MB-231, MDA-MB-468, MIA PaCa-2, NIH 3T3 and SkBr3.

Retroviruses and siRNA

Retroviral vectors used for reintroduction of p120 into A431 cells, EGFR introduction into SYF-family cells, and E-cadherin into SKBr3 and MDA-MB-468 cells were originally derived from Garry Nolan's LZRS retroviral vector (Kinsella and Nolan, 1996) and were subsequently modified by us to allow bicistronic expression (Ireton et al., 2002). Briefly, the gene of interest (e.g. p120, EGFR, or E-cadherin) and a selectable marker [green fluorescent protein (GFP) or a zeocin or neomycin resistance gene, respectively] were linked by an internal ribosomal entry site (IRES), which results in expression of both genes from a single mRNA transcript (Ireton et al., 2002). For expression of p120 siRNA constructs, siRNA sequences were subcloned into the retroviral vector pRetroSuper (pRS, kindly provided by Reuven Agami). Amphotropic retroviruses from pRS and pLZRS vectors were generated in Phoenix amphotropic virus packaging cells, and viral transduction was performed in the presence of 4 μg/ml polybrene (Sigma) for a minimum of 3 hours at either 32°C or 37°C, as described previously (Davis et al., 2003; Ireton et al., 2002). Some cells were subjected to multiple rounds of infection to increase efficiency. Cells with stable retrovirus integration were selected with the appropriate drug or by fluorescence-activated cell sorting (FACS).

A431 cells expressing human p120 short interfering RNA (siRNA) and re-expressing murine p120 were generated by first retrovirally transducing A431 cells with pRS vector alone or with PRS vector containing human-specific p120 siRNA (pRS.h) (Davis et al., 2003), GC CAG AGG TGG TTC GGA TA. Infected cells were selected with puromycin and single-cell cloned by limiting dilution (Davis et al., 2003). p120 was subsequently retrovirally re-introduced into clonal lines Ah5 and Ah10 with LZRS-MS-GFP vector containing murine p120-3A or murine p120-3A/7F (tyrosines 112, 228, 257, 280, 291, 296, 302 mutated to phenylalanine), or with vector alone as a control. Polyclonal populations of cells with normalized expression levels of each construct were obtained by FACS.

Growth factors and drugs

Recombinant human epidermal growth factor (EGF) was obtained from R&D. For growth factor stimulation, cells were serum starved in DMEM containing 0.1% FBS and 1% penicillin/streptomycin for 16-18 hours, or as indicated in figure legends. Growth factor (100 ng/ml EGF) was added to conditioned medium for 5 minutes prior to cell lysis or fixation.

SU6656 was a kind gift from Robert Blake (Sugen, South San Francisco, CA, USA). EKI was generously provided by Bob Coffey (Vanderbilt University). PP1 was obtained from BioMol and AG1478 from Calbiochem.

Pervanadate preparation and treatment

Pervanadate solutions were made fresh before each experiment. Solutions of 0.1 mM Na_3VO_4/0.2 mM H_2O_2 (for cell treatment) or 0.3 mM Na_3VO_4/0.6 mM H_2O_2 (for lysis buffers) were incubated for 20 minutes at room temperature to allow pervanadate to form (Lampugnani and Dejana, 1997). For cell treatment, cells were rinsed once with phosphate-buffered saline (PBS) (10 mM phosphate, pH 7.4, 150 mM NaCl) containing pervanadate (0.1 mM Na_3VO_4/0.2 mM H_2O_2) and incubated in this solution for precisely 2 minutes at room temperature, or as indicated, immediately prior to lysis or fixation. No pervanadate treatment was performed prior to lysis of growth factor-treated cells.

Immunoprecipitation and western blotting

For immunoprecipitation and western blotting, cells were washed once with PBS (with or without pervanadate), then lysed in ice cold radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS)] containing protease inhibitors (1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM EDTA) and pervanadate (0.3 mM Na_3VO_4/0.6 mM H_2O_2) for 5 minutes at 0°C. Lysates were clarified by microcentrifugation for 5 minutes at 4°C, and total protein levels among samples were normalized by bicinchoninic acid (BCA) assay (Pierce), except when transiently transfected cells were used. 1-4 μg monoclonal antibody was added to the supernatant which was incubated for 1 hour at 4°C with end-over-end rotation. Immunoprecipitation antibodies included p120 mAb 15D2, EGFR antibody from BD/Transduction, hemagglutinin (HA) epitope tag mAb 12CA5, and Src mAb 327. Protein A-Sepharose (Amersham-Pharmacia) was coupled to a rabbit anti-mouse bridge antibody (Jackson Immunoresearch, Inc.) and then added to lysates for an additional 1-hour incubation at 4°C. Immunoprecipitates were washed four times with 1 ml lysis buffer, resuspended in 2× Laemmli sample buffer (LSB), and boiled. Denatured proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred to PROTRAN nitrocellulose (Schleicher and Schuell) for western blotting. Nonspecific binding to membranes was blocked for 10 minutes with 3% nonfat milk in Tris-buffered saline (TBS) (10 mM Tris pH 7.4, 150 mM NaCl) for all western blots except for those using phosphotyrosine mAb PY20, which were blocked with 5% bovine serum albumin (BSA) in TBS. Blots were incubated in primary antibody in appropriate blocking solution at 4°C overnight, and subsequently, in secondary antibody (Jackson Immunoresearch, Inc.) diluted in 3% milk/TBS for 1.5 hours at room temperature. Primary antibodies were used at the following concentrations: mAb pY228, 0.5 μg/ml; pp120, 0.1 μg/ml; 8D11, 2 μg/ml; HECD-1, 0.1 μg/ml; PY20, 1 μg/ml; EGFR, 0.25 μg/ml; tubulin, 1 μg/ml. Blots were developed by enhanced chemiluminescence (ECL, Amersham).

In vitro kinase assay

Methodology for in vitro kinase assays has been described previously in detail (Mariner et al., 2001). Briefly, A431 cells were lysed with RIPA containing 1 mM Na_3VO_4 and an equal volume of clarified lysate was used for each assay performed within the same experiment. p120 and c-Src were simultaneously immunoprecipitated from each sample with 2.5 μg mAb pp120 and 2.5 μg mAb 327, respectively, as described above except that only 10 μl of RαM-conjugated protein A-Sepharose bead slurry was added to each sample in order to concentrate the proteins on the bead surface. Following immunoprecipitation and washes, samples were further washed twice with TBS/0.1 mM Na_3V0_4. All buffer was then removed and samples were subjected to in vitro kinase assay for 10 minutes at room temperature, with constant agitation, in 25 μl kinase buffer (20 mM Hepes pH 7.4, 10 mM $MnCl_2$) containing 10 μCi [γ-^{32}P]ATP and the indicated concentration of SU6656 Src inhibitor. Src inhibitor (in DMSO) or DMSO vehicle alone was added at an equal volume to each sample (0.5 μl). The kinase assay was stopped by washing twice with RIPA containing 5 mM EDTA and boiling in 2× LSB.

Immunocytochemistry

Cell fixation and immunocytochemistry procedures have been described previously (Reynolds et al., 1994). Cells were cultured on glass coverslips for 48 hours. Cells were washed once with PBS, then fixed and permeabilized immediately in ice cold methanol for 7 minutes at –20°C and washed twice with PBS. Nonspecific binding was blocked with 3% nonfat milk in PBS for 5 minutes, then cells were incubated in primary antibody diluted in blocking buffer for 30 minutes at room temperature in a humidified chamber. mAb pY228 was used at 1 μg/ml; F1αSH, at 0.5 μg/ml. Following two or three washes in PBS, coverslips were rinsed with blocking buffer and incubated with Alexa^{488} or Alexa^{594}-coupled goat antibodies specific for rabbit IgG or mouse IgG (Molecular Probes, diluted 1:1200) for 30 minutes at room temperature, shielded from light. Following three final washes with PBS, coverslips were mounted onto slides with ProLong Antifade mounting medium (Molecular Probes). Pretreatment with pervanadate for 2 minutes prior to fixation was required for all staining with mAb pY228. Images were collected with OpenLab software (Improvision) and contrast-enhanced with OpenLab or Adobe Photoshop.

Hanging drop assay

This assay has been described in detail previously (Thoreson et al., 2000). Briefly, 30 μl drops of cells at 3×10^5 cells/ml were plated onto the lid of a 48-well plate. The lid was then inverted over the wells of the multiwell plate containing PBS to prevent evaporation of the hanging drops. Cell aggregates were photographed approximately 16 hours after plating.

Calcium switch assay

A431 cells were plated onto glass coverslips 2 days prior to performing calcium switch assays, such that cells were 50-70%

confluent when assayed. One day post-plating, cells were washed twice with sterile PBS, pH 7.4 and serum starved for 16-18 hours in DMEM containing 0.1% FBS, 1% penicillin/streptomycin. Calcium switch assays were initiated by washing cells twice with sterile PBS, pH 7.4 and incubating cells in low calcium medium [DMEM lacking $CaCl_2$ and L-glutamine (Gibco, #21068), 2% dialyzed FBS (Gibco, #10440-014), 1% penicillin/streptomycin, 1% L-glutamine, 5 μM $CaCl_2$ (Sigma, #C-3881)] for 2.5-3 hours. Normal calcium levels (1.8 mM) were then restored to cells by direct addition of 1.8 M $CaCl_2$ stock solution (1000×) to the low calcium medium at the appropriate time point. In typical experiments assaying p120 localization, calcium was restored at 30, 15 or 5 minutes (or not at all) prior to cell fixation and immunofluorescent staining. For 'No Switch' samples, 1.8 mM $CaCl_2$ was included in the low calcium medium for the duration of the experiment.

Cadherin-blocking antibodies were human E-cadherin mAb SHE78-7 (Zymed), 1 μg/ml, and P-cadherin pAb 6A9 (ascites, 1:500), a kind gift from Margaret Wheelock. Blocking antibodies were included in medium at the time of cell plating, and were maintained in the various media used throughout the assay.

Results

Characterization of a novel Y228 phospho-specific p120 monoclonal antibody

Using two-dimensional tryptic mapping, we recently identified the major Src-induced phosphorylation sites in p120 (Mariner et al., 2001). This classic approach to analyzing protein phosphorylation is technically challenging and too cumbersome for routine analyses in cells. Moreover, individual phosphorylation events and their effects on protein localization cannot be tracked in situ. To circumvent these problems, we collaborated with Dr Roberto Campos at BD/Transduction Laboratories to generate phosphospecific antibodies to critical sites. Here, we characterize the best of a panel of monoclonal antibodies that specifically recognize p120 phosphorylated at tyrosine 228, and use this antibody (mAb Y228 clone 21a, hereafter mAb pY228) to directly monitor cellular events associated with p120 phosphorylation at this residue.

Fig. 1 shows the biochemical properties of mAb pY228 in NIH3T3 cells in the presence or absence of activated Src (Src527F). On western blots of whole cell lysates (entire blot is shown), mAb pY228 selectively recognized a phosphorylated form of p120 present at elevated levels in the presence of Src. As expected, the phosphorylated bands migrated slightly slower than the bands identified with the p120-specific antibody, mAb pp120 (Fig. 1A, compare lanes 1, 2 with 3, 4). In contrast, the general phosphotyrosine-specific antibody mAb PY20 recognized several Src substrates. Among these, p120 is a minor band (Fig. 1, lane 6).

To prove the p120 specificity of the bands recognized by mAbY228, immunoprecipitations with mAb pY228, mAb 15D2 (anti-p120) and PY20 (anti-phosphotyrosine) were compared by western blotting with the same antibodies (Fig. 1B). Western blotting of these immunoprecipitates with mAb pY228 revealed that the phosphoprotein recognized by this antibody is identical to the phospho-p120 forms recognized by p120 and anti-phosphotyrosine antibodies (Fig. 1B, top panel). When a mixture of phosphoproteins was immunoprecipitated with mAb PY20, only the bands corresponding to p120 were recognized by mAb pY228 on western blots (lane 4). Likewise, the p120-specific mAb 15D2 efficiently immunoprecipitated the mAb pY228-reactive bands (lane 3).

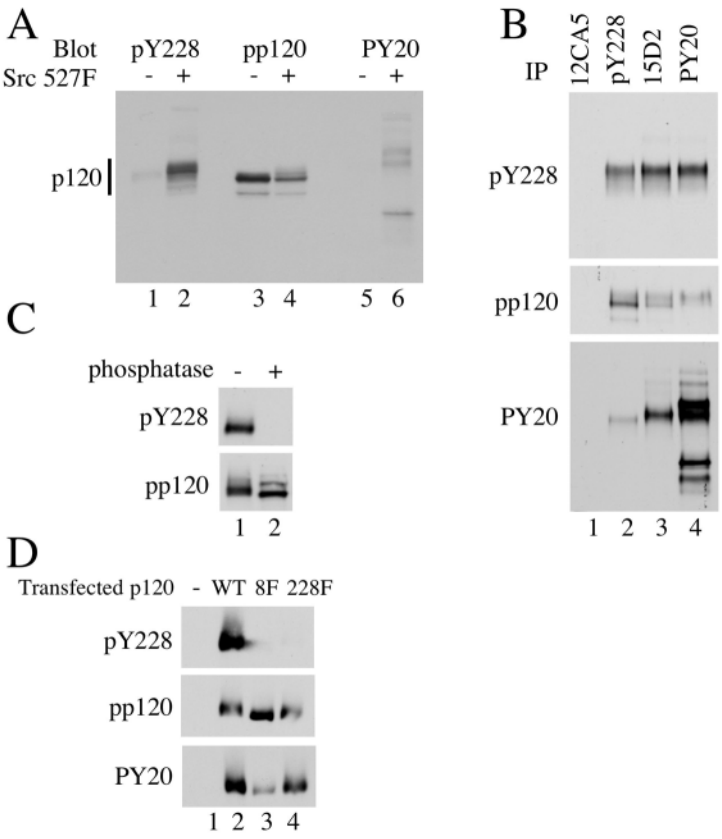

Fig. 1. Characterization of p120 Y228 phosphospecific monoclonal antibody. (A) Detection of phosphorylated p120 on whole cell lysate western blots. 3T3 cells with or without expression of transforming Src 527F were washed for 2 minutes with PBS containing pervanadate, then lysed with RIPA containing pervanadate. Whole cell lysates were separated by SDS-PAGE on a 7% gel and western blotted with mAb pY228 to phosphorylated p120, mAb pp120 to total p120, and mAb PY20, a general phosphotyrosine antibody. The entire blot is shown. (B) mAb pY228 detects p120 by immunoprecipitation. p120, Y228-phosphorylated p120, or various tyrosine phosphorylated proteins were immunoprecipitated from Src-transformed 3T3 cell lysate with mAb 15D2, mAb pY228, or PY20, respectively. Immunoprecipitation with HA-tag antibody 12CA5 served as a negative control. Immunoprecipitates were then western blotted with either mAb pY228, pp120, or PY20. The entire blots are shown for the pY228 and PY20 panels. (C) mAb pY228 is specific for phosphorylated p120. p120 was immunoprecipitated from A431 cells with p120 mAb 15D2. Immunoprecipitates were treated with or without lambda protein phosphatase (New England Biolabs) for 30 minutes at 30°C. Samples were western blotted with mAb pY228 or pp120. (D) mAb pY228 is specific for p120 phosphorylated at Y228. Cos-7 cells were transiently co-transfected with transforming Src (RcRSV c-Src 527F) together with either empty RcRSV vector (lane 1), or RcRSV vector containing mp120-1A (lane 2), mp120-1A/8F (lane 3), or mp120-1A/228F (lane 4), using Superfect reagent (Qiagen). Transfected p120 was specifically immunoprecipitated with murine-specific p120 mAb 8D11, and western blotted with mAb pY228, pp120 or PY20.

Importantly, the reactivity of mAb pY228 for p120 was lost when the immunoprecipitate was treated with phosphatase (Fig. 1C, compare lanes 1 and 2). In addition, western blotting of p120 proteins containing tyrosine to phenylalanine mutations at all eight of the major Src-induced phosphorylation sites present in p120 isoform 1 (Fig. 1D, lane 3), or selective phenylalanine mutation of just tyrosine 228 (Fig. 1D, lane 4), revealed that Y228 is the only phosphorylated residue in p120 that is recognized by this antibody, even though other Y residues in the Y228F p120 mutant were clearly phosphorylated under these conditions (Fig. 1D, PY20 panel, lane 4). Together, these data demonstrate that mAb pY228 selectively binds to p120 phosphorylated at Y228, and is an effective tool for immunoprecipitation and western blotting experiments aimed at monitoring p120 phosphorylation at this residue.

Properties of mAb pY228 for detecting EGFR signaling to p120

We previously reported that epidermal growth factor receptor (EGFR) signals to p120, strongly inducing p120 phosphorylation on tyrosine (Downing and Reynolds, 1991). To determine whether EGFR activation induces p120 phosphorylation at Y228, A431 cells were stimulated with EGF and the phosphorylation status of p120 Y228 was determined by western blotting with the phosphospecific mAb pY228 or a general p120 mAb, pp120 (Fig. 2A). To control for EGFR activity, EGFR was immunoprecipitated and western blotted with antibodies to either phosphotyrosine (PY20) or EGFR (Fig. 2A). Indeed, p120 was rapidly and intensely phosphorylated at Y228 following EGF treatment. To determine the subcellular localization of the tyrosine phosphorylated p120, A431 cells were co-stained with mAb pY228 and a p120-specific polyclonal antibody before and after 5-minute exposure to EGF (Fig. 2B). EGF induced prominent staining by mAb pY228 at cell-cell contacts and in lamellipodia. The phospho-Y228 staining occurred preferentially in cells around the periphery and leading edge of colonies, consistent with previous observations that EGFR activity is decreased in tightly packed cells (Sorby and Ostman, 1996).

To confirm biochemically that phosphorylated p120 remains in association with E-cadherin, the proteins were coimmunoprecipitated with an E-cadherin-specific mAb before and after treatment of cells with EGF. The presence of phosphorylated p120 in the complex was determined by western blotting the co-immunoprecipitated complexes directly with mAb pY228 or mAb pp120 (Fig. 2C). Y228-phosphorylated p120 remained associated with E-cadherin (pY228, lane 2) and the amount of cadherin-associated p120 was not detectably altered by EGF treatment.

Together, these observations indicate that p120 Y228 is significantly phosphorylated in response to EGF treatment and validate the use of mAb pY228 for the study of EGFR signaling to p120 by immunoprecipitation, western blotting and immunofluoresence. Thus, the phosphospecific Y228 antibody makes it possible to directly monitor the phosphorylation status of p120 at Y228 in situ and biochemically.

p120 Y228 phosphorylation levels in carcinoma cell lines

EGFR and various other receptor tyrosine kinase oncogenes are frequently activated in carcinomas, suggesting that p120 phosphorylation might also be elevated in these cancers. To obtain a preliminary assessment of p120 Y228 phosphorylation in carcinoma cells, we surveyed 'basal' p120 Y228 phosphorylation levels (under conditions of normal cell culture in media containing serum but no added EGF) in a panel of E-cadherin-positive and -negative cancer cell lines by western blotting immunoprecipitated p120 with antibodies to p120 or

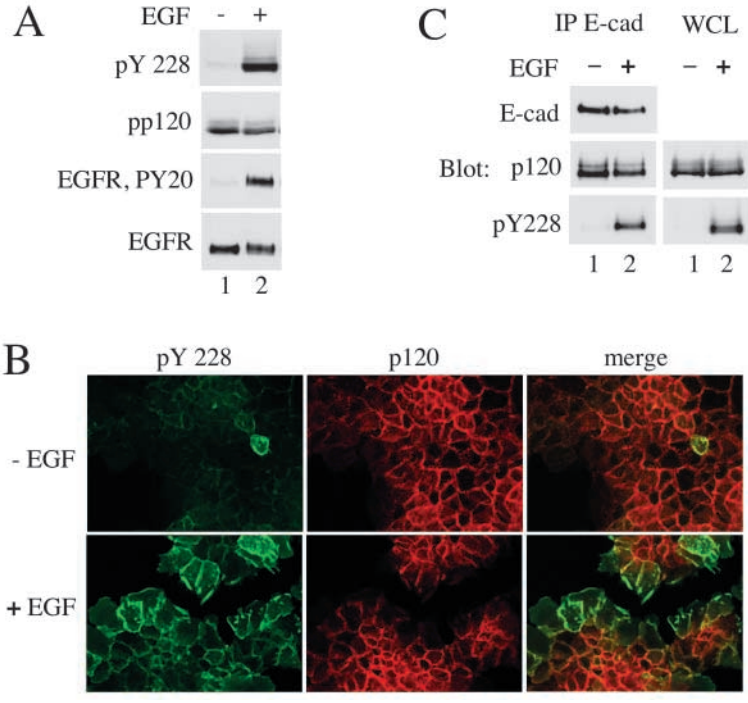

Fig. 2. EGFR activation induces p120 phosphorylation at Y228. A431 cells were serum starved for 16-18 hours, then stimulated for 5 minutes with (+) or without (–) EGF (100 ng/ml). (A) Biochemical characterization of p120 mAB pY228. After EGF treatment, cells were lysed in RIPA buffer containing pervanadate. Lysates were probed by western blotting with a p120 phosphospecific mAB to Y228 (pY 228) or a general p120 mAB (pp120). EGFR immunoprecipitates were blotted with PY20 (EGFR, PY20), a general phosphotyrosine antibody, or with an EGFR monoclonal antibody (EGFR). (B) Immunofluorescent localization of Y228-phospho-p120 before (–) and after (+) EGF treatment. After EGF treatment, cells were washed for 2 minutes with PBS (containing pervanadate), then fixed with methanol. Y228-phosphorylated p120 was stained with mAb pY228 (green). Total p120 was stained with pAb F1αSH (red). The individual images were merged to facilitate comparison (merge). 40× magnification. (C) Coimmunoprecipitation of Y228-phospho-p120 with E-cadherin. E-cadherin was immunoprecipitated with mAb HECD-1 before (–) and after (+) EGF treatment. Immunoprecipitates were probed by western blotting for E-cadherin (E-cad), p120 and Y228-phosphorylated p120 (pY228). Total p120 and Y228-phosphorylated p120 levels in the starting whole cell lysate (WCL) were also analyzed by western blotting.

Y228-phosphorylated p120. E-cadherin expression was also monitored by western blotting cell lysates (Fig. 3A, third panel). p120 Y228 phosphorylation was detectable in most cell lines, though phosphorylation levels varied widely (compare lanes 1, 2, 5 with lanes 3, 4, 6-10) and was substantial in certain cell lines. A431 cells contained the highest levels of p120 Y228 phosphorylation and were subsequently used as a model for further work. The results shown here were identical regardless of whether the tyrosine phosphatase inhibitor pervanadate was included in the PBS wash prior to cell lysis (data not shown). Note that the optimal exposure times of these blots are considerably longer than those for experiments in which cells were stimulated with EGF (e.g. Fig. 2), since the 'high' basal levels of Y228 phosphorylation described here are still lower than those in the same cells optimally stimulated with growth factor. We were unable to establish a clear correlation between EGFR levels and p120 tyrosine phosphorylation in these cell lines, based on published EGFR levels. For example, both A431 and MDA-MB-468 cells have very high EGFR levels, but p120 is not constitutively phosphorylated in the latter cell line. Thus, the cause(s) of elevated p120 phosphorylation may vary from one cell line to the next. Nonetheless, these data suggest that Y228 is prominently phosphorylated in many cancer cell lines and its status in these cells probably represents constitutive tyrosine kinase signaling to p120.

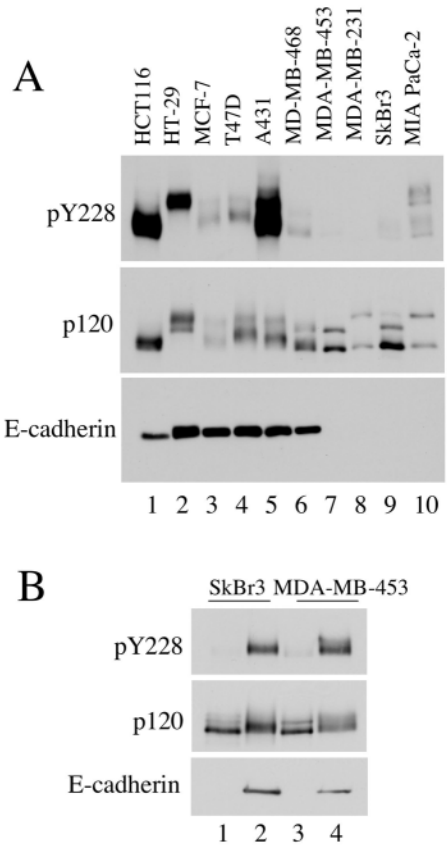

Fig. 3. Constitutive high level phosphorylation of p120 at Y228 in carcinoma cell lines. (A) Variable levels of p120 phosphorylation at Y228. p120 was immunoprecipitated from the indicated human carcinoma cell lines and western blotted with mAb pY228 (pY228) or mAb pp120 (p120). E-cadherin levels were monitored by western blotting of whole cell lysates with mAb HECD-1. (B) Cadherin-dependent p120 phosphorylation. SkBr3 and MDA-MB-453 cells, which lack E-cadherin expression, were infected with control or E-cadherin containing retrovirus. Polyclonal cell lines were generated by selection in G418. Immunoprecipitated p120 was detected by western blotting with mAb pY228 (pY228) or mAb pp120 (p120). E-cadherin was detected in whole cell lysates by western blotting with mAb HECD-1 (E-cadherin). All cells were washed with PBS containing pervanadate for 2 minutes prior to lysis with pervanadate-containing RIPA buffer.

We previously reported that association of p120 with membranes through cadherin-binding is required for p120 phosphorylation (Ozawa and Ohkubo, 2001; Thoreson et al., 2000). Indeed, p120 Y228 phosphorylation was significantly higher in E-cadherin-expressing cells (Fig. 3A, compare lanes 1-6 with 7-10) and reintroduction of E-cadherin into SkBr3 or MDA-MB-453 cells significantly increased basal p120 Y228 phosphorylation levels (Fig. 3B, compare lanes 1 to 2, and 3 to 4). Thus, cadherin association is critical for significant p120 Y228 phosphorylation, but clearly not by itself sufficient to induce it. Instead, such cell lines are likely to also contain activated tyrosine kinases, which may be at least partly responsible for the oncogenic condition of the cells.

p120 Y228 is phosphorylated but not essential during nascent junction assembly

To examine the role of p120 tyrosine phosphorylation in junction assembly, we generated a novel siRNA-based system to significantly reduce levels of endogenous p120 and replace it with p120 mutants containing tyrosine to phenylalanine (Y/F) substitutions, thus forcing the cell to rely on the mutant p120. This approach reduces interference from endogenous p120 while assaying the function of phosphorylation-defective mutants. To establish model cell lines for functional assays of adhesion, we generated A431 clonal cell lines with high-efficiency knockdown of p120. Knockdown was achieved by retroviral (pRetroSuper, pRS) transduction of a human-selective siRNA sequence that we have previously demonstrated to have no effect on ectopically expressed murine p120 (Davis et al., 2003). Infected cells were selected in puromycin. Clonal cell lines were derived by limiting dilution, and cell lines with the highest efficiency of p120 knockdown were selected for further experiments (Davis et al., 2003). Subsequently, p120 was reintroduced in these clonal cell lines by superinfection with virus containing wild type (WT) murine p120 isoform 3A (mp120-3A) or murine p120-3A bearing Y/F mutations at each of the previously identified tyrosine phosphorylation sites (mp120-3A/7F) (Mariner et al., 2001). Isoform 3 p120 was selected since it corresponds to the most abundant isoform naturally expressed in A431 cells. Pools of ectopic p120-expressing cells were isolated by fluorescence-activated cell sorting based on GFP fluorescence. Our retroviral vector is structured such that p120 and GFP are linked on a single mRNA transcript by an internal ribosomal entry site. Thus, GFP and p120 expression are proportional and the resulting cell populations are normalized for p120 expression levels (Ireton et al., 2002; Levenson et al., 1998).

Expression of human-specific siRNA (pRS.h) in A431 cells resulted in knockdown of roughly 90-95% of endogenous p120 (Fig. 4A, compare lanes 1, 2) in two independently isolated clonal cell lines, while murine p120 was re-expressed stably in both clonal lines at approximately endogenous levels present

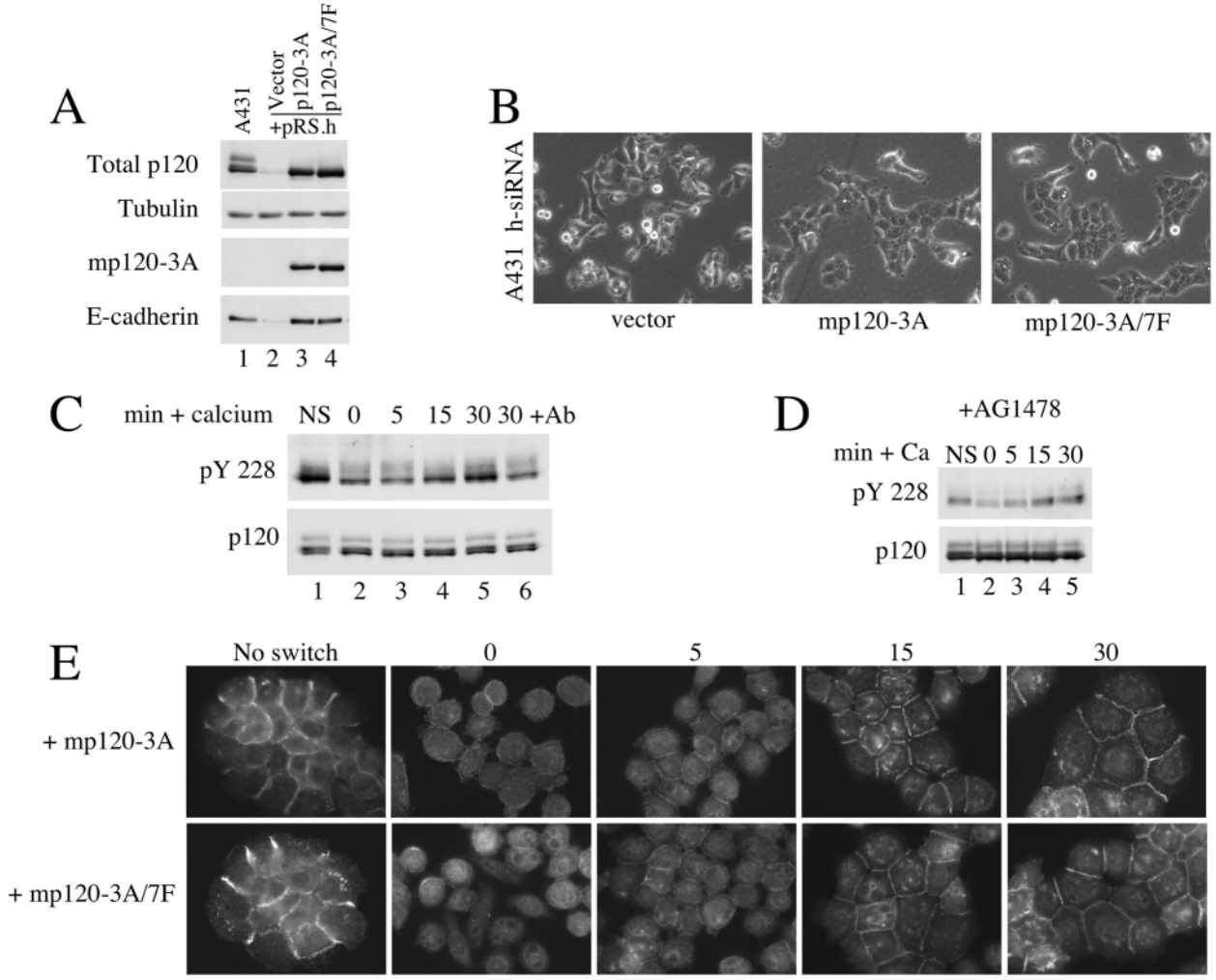

Fig. 4. p120 phosphorylation at Y228 during adherens junction formation. (A) Characterization of a p120 knockdown and add-back model system. Endogenous p120 levels were stably knocked down with retrovirally transduced siRNA specific for human p120 (pRS.h) in parental A431 cells, and clonally derived pRS.h-expressing A431 cell lines were subsequently infected with retroviral vector alone or murine p120 constructs WT-mp120-3A or mp120-3A/7F (which contains phenylalanine mutations at the seven known p120 Y phosphorylation sites). Murine p120 is unaffected by the human p120 siRNA because of mismatches at the nucleotide level. RIPA lysates were probed by western blotting. Human and mouse p120 together were detected by western blotting with mAb pp120 (Total p120). Tubulin was simultaneously detected as a loading control. Murine p120 (mp120-3A) was selectively detected with the murine-specific mAb 8D11. E-cadherin expression was detected with HECD-1. (B) Morphologic effects of WT- and 7F-p120 expression in p120 knockdown cells. The cells lines in A were trypsinized, plated overnight and photographed on a phase contrast microscope (10× magnification). No differences were detected between cells rescued with wild type (mp120-3A) and mutant (mp120-3A/7F) p120. (C) Effects of junction formation on p120 Y228 phosphorylation. p120 Y228 phosphorylation was monitored by western blotting of cell lysates after calcium switch (see Materials and Methods). Total p120 (p120) and Y228 phosphorylated p120 (pY228) were quantitated in the presence of calcium (NS=no switch), or at the indicated times after calcium add-back. (D) Effects of EGFR inhibition. The experiment in C was repeated in the presence of the EGFR inhibitor AG1478 (300 nM). (E) A431 pRS.h (p120 knockdown) cells expressing mp120-3A or mp120-3A/7F were subjected to calcium switch assay as in C, and then examined by immunofluorescence with p120-specific antibody pAb F1αSH at the indicated times after add-back of calcium (63× magnification).

in parental cells (compare lane 1 with 3, 4). Representative data for one clone are shown here and in subsequent figures.

We showed previously that eliminating p120 in A431 cells causes concomitant loss of E-cadherin and that E-cadherin is stabilized by reconstituted expression of murine p120 (Fig. 4A, lanes 3, 4) (Davis et al., 2003). p120 knockdown cells had significantly lower E-cadherin levels (Fig. 4A, lane 2) and both wild-type and phosphorylation-defective p120 effectively restored wild-type levels of E-cadherin expression to the p120 knockdown cells (Fig. 4A, lanes 3, 4). The morphologies of the p120-deficient A431 cell line and the WT mp120-3A and mutant p120-3A/7F reconstituted cell lines are shown in Fig. 4B. p120 knockdown sharply reduced cell-cell adhesion (Fig. 4B, first panel) because of significant loss of E-cadherin, but both WT and Y/F mutant p120 constructs fully restored a normal adhesive phenotype that closely resembled parental A431 cells.

p120 tyrosine phosphorylation has been associated with

nascent junctions (Calautti et al., 1998; Lampugnani et al., 1997). Therefore, we hypothesized that p120 tyrosine phosphorylation is necessary for normal progression of junction formation, particularly in the early stages. To assay the phosphorylation status of p120 Y228 during junction formation, we performed calcium-switch assays using our siRNA-based A431 model cell lines. Cadherin-mediated adhesion was abolished by incubating cells in low calcium medium for 3 hours, and adherens junction formation was initiated by calcium restoration. Phosphorylation of WT p120 at Y228 was monitored by western blotting (Fig. 4C). p120 Y228 phosphorylation was sharply reduced under low calcium conditions and gradually restored over the 30 minutes required for junctions to re-form. This increase in phosphorylation was blocked by pre-incubation of the cells with E- and P-cadherin function blocking antibodies (Fig. 4C, lane 6), suggesting that cadherin engagement is required for this effect. Previously, EGFR activity was found to spike during calcium switch-induced junction reformation in HaCat cells (Pece and Gutkind, 2000). To determine whether EGFR was involved in the alterations in p120 phosphorylation in our cells, we repeated the experiments in the presence of the potent EGFR inhibitor AG1478 (Fig. 4D). The inhibitor had little affect on the p120 Y228 dephosphorylation and rephosphorylation effects associated with calcium-switch. Thus, p120 Y228 phosphorylation is responsive to conditions of junction assembly and responds to cadherin engagement, but despite the high EGFR activity in A431 cells, EGFR does not seem to be the major driver of Y228 phosphorylation under these conditions.

We then asked whether p120 phosphorylation is necessary for junction formation to proceed normally at initial stages. Calcium-induced junction reassembly was monitored by immunofluorescence with a p120 polyclonal antibody (Fig. 4E) or E-cadherin antibodies (not shown) and the rate of junction formation was compared in cells re-expressing either WT mp120-3A or mp120-3A/7F (Fig. 4E). Surprisingly, the rate of junction reassembly, and the recruitment of p120 (Fig. 4E) or E-cadherin (not shown) to nascent junctions, were identical in the WT and mutant p120-expressing cell lines (Fig. 4E, compare top panels to bottom panels). Thus, p120 phosphorylation at Y228 is increased during junction assembly, but is not essential for this process.

We previously reported that cells expressing p120-uncoupled E-cadherin were unable to compact during hanging-drop adhesion assays and were also unable to form strong adhesions capable of withstanding shear force (Thoreson et al., 2000). To determine whether p120 phosphorylation at any of the identified sites is necessary for cell compaction and strong adhesion, we compared the properties of the WT mp120-3A and mp120-3A/7F cell lines by hanging drop aggregation assays (Fig. 5). Allowing these cells to reform junctions overnight in 'hanging drops' after disaggregation with trypsin revealed that both WT-p120 and 7F p120 could efficiently restore the ability of E-cadherin to induce compact cellular aggregates able to withstand shear force, suggesting that p120 tyrosine phosphorylation is not required for the ability of p120 to promote strong cell adhesion.

Contribution of EGFR and Src kinases in steady state signaling to p120 Y228

A431 cells display high levels of both EGFR and Src activity even in the absence of added EGF (Osherov and Levitzki, 1994). Because these cells display high levels of constitutively phosphorylated p120, we evaluated the effects of several EGFR and Src-family kinase inhibitors to identify the relative contributions of these kinases to Y228 phosphorylation under normal cell growth conditions (i.e. cells cultured in serum but lacking added EGF). Interestingly, treatment with AG1478 or EKI, both potent EGFR inhibitors (Fig. 6A, third panel, lanes 2, 3), had relatively moderate (yet reproducible) effects on p120-Y228 phosphorylation (top panel, lanes 2, 3). PP1, a relatively broad-spectrum inhibitor that is widely used to block Src activity (Hanke et al., 1996), reduced Y228 phosphorylation by more than 50% when used at high levels but also significantly reduced EGFR activity (lane 4). The most prominent inhibition resulted from treatment with 10 μM SU6656 (lane 5), a recently developed Src inhibitor that displays better specificity for Src kinases than the classic Src inhibitors PP1 or PP2 (Blake et al., 2000). At 1 and 5 μM, SU6656 is thought be specific for Src kinases, but at 10 μM it appears to affect other kinases as well. Titration of SU6656 (Fig. 6B) revealed approximately 50% inhibition of Y228 phosphorylation at concentrations considered to be reasonably specific for Src kinases (1 and 5 μM) and had no detectable effect on EGFR activity, even at 10 μM. Overall, these data suggest that EGFR activity contributes only moderately to the basal levels of p120 Y228 phosphorylation in A431 cells and Src kinases may have some impact. However, the fact that 10 μM SU6656 was required for significant reduction of Y228 phosphorylation strongly suggests a pronounced role for SU6656-sensitive kinases other than Src relatives and EGFR.

EGF-induced Y228 phosphorylation is independent of Src activity

EGFR can signal through Src (Biscardi et al., 1999), raising the possibility that EGF-induced p120 phosphorylation occurs directly via the receptor, or indirectly through activation of Src,

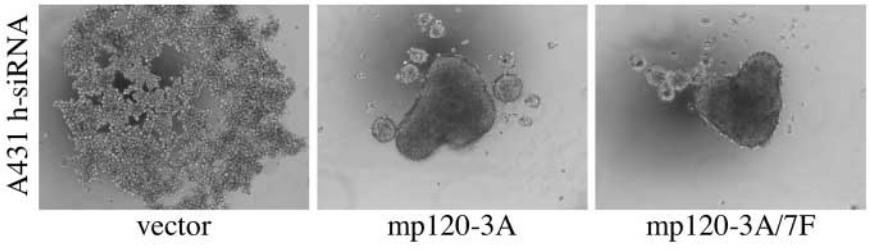

Fig. 5. The major p120 Y phosphorylation sites are not required for E-cadherin-mediated strong cell adhesion. Hanging drop aggregation assays were conducted with p120-deficient cells expressing human p120 siRNA alone (vector), or the same cells reconstituted with murine p120 constructs expressing WT-p120 (mp120-3A) or mutant p120 (mp120-3A/7F). The latter construct contained phenylalanine (F) mutations at the seven major sites of p120 tyrosine phosphorylation. Cells were photographed on a phase-contrast light microscope (10× magnification).

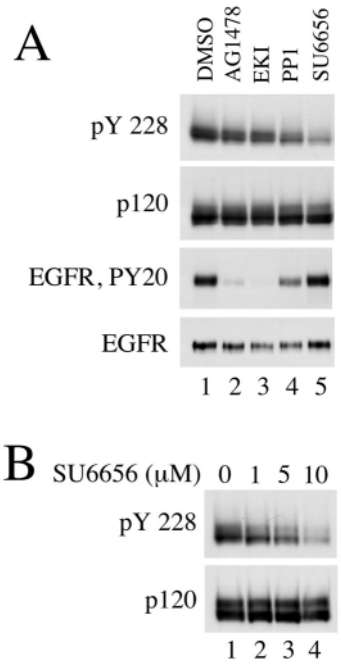

Fig. 6. Effects of EGFR and Src inhibitors on p120 Y228 phosphorylation. (A) A431 cells were treated for 1 hour with DMSO alone or DMSO containing the indicated inhibitors. Drugs concentrations were as follows: AG1478, 300 nM; EKI, 1 μM; PP1, 5 μM; SU6656, 10 μM. Cells were then washed for 2 minutes with PBS containing pervanadate, then lysed with RIPA containing pervanadate. Cell lysates were blotted with mAb pY228 (pY 228) or mAb pp120 (p120). EGFR was immunoprecipitated from whole cell lysates and western blotted with antibodies to phosphotyrosine (EGFR, PY20) or EGFR (EGFR). (B) A431 cells were prepared as described in A, except that cells were treated with increasing concentrations of SU6656 as indicated. Whole cell lysates were western blotted with mAb pp120 or mAb pY228.

or Src-family kinases. To test this hypothesis, A431 cells were EGF-stimulated in the presence of 1-10 μM SU6656. We chose this Src inhibitor over the classic inhibitor PP1 because it is highly potent toward the ubiquitously expressed Src family kinases (IC_{50} 0.28, 0.02, 0.17 μM for Src, Yes and Fyn, respectively) (Blake et al., 2000) while having no detectable activity toward EGFR at the concentrations used (Fig. 6). To validate the activity of the drug in our hands, we first tested its ability to inhibit Src in vitro (Fig. 4A). Endogenous c-Src was immunoprecipitated from A431 cells and assayed in vitro for its ability to phosphorylate itself or p120 (Fig. 7A) in the presence of increasing concentration of SU6656 Src inhibitor. As originally published (Blake et al., 2000), 1 μM SU6656 was sufficient to nearly completely block Src activity (IC_{50} c-Src 0.28 μM) and increasing drug concentrations up to 10 μM only slightly increased the efficacy of the drug.

We then used the drug to determine whether Src activity contributes to tyrosine phosphorylation of p120 Y228 in response to EGFR activation. A431 cells were treated with EGF to induce p120 tyrosine phosphorylation, in the presence or absence of pretreatment with increasing concentrations of SU6656 Src inhibitor (Fig. 7B). To validate the specificity of SU6656, EGFR activation was monitored by phosphotyrosine blotting with mAb PY20. Again, EGFR activation was unaffected by SU6656 at any concentration (Fig. 7B, EGFR, PY20 panel). Nonetheless, even 10 μM SU6656 had only minor effects on EGF-induced p120 Y228 phosphorylation (Fig. 7B, top panel), indicating that Src kinases are not required for the ability of EGFR to induce phosphorylation at this site. These data further suggest that kinases sensitive to high concentrations of SU6656 (e.g. 10 μM, lane 6) are not substantial players in the EGF-induced EGFR signaling to p120. Interestingly, a p120 band shift was consistently observed in the presence of 1 μM SU6656, suggesting that phosphorylation sites other than Y228 may be affected directly or indirectly by the inhibition of SU6656-sensitive kinases.

Experiments in fibroblasts revealed similar results (Fig. 7C,D). Initially, we examined Her-14 cells, a well characterized murine fibroblast line stably transfected with EGFR. As in A431 cells, EGF treatment induced efficient phosphorylation of p120 on Y228, and blocking Src kinases with SU6656 caused only a minor reduction in p120 phosphorylation on Y228 (Fig. 7C, compare lanes 2, 3). We also examined EGF signaling in SYF cells, murine fibroblasts derived from triple knockout mice lacking Src, Yes and Fyn, the only known Src family kinases in these cells (Klinghoffer et al., 1999). SYF fibroblasts re-expressing vector alone or physiological levels of wild-type c-Src or kinase dead c-Src (Cary et al., 2002) were retrovirally transduced with EGFR c-DNA-containing virus, and zeocin-resistant populations of cells were selected. EGFR levels in these cell lines were confirmed by western blotting of immunoprecipitated EGFR. We were unable to successfully generate comparable EGFR-expressing SYF cells that lacked Src entirely (vector-only cells). The EGFR-expressing SYF cells expressing wild-type or kinase dead c-Src were then treated with EGF and signaling to p120 Y228 was assayed by western blotting (Fig. 7D). Signaling to p120 Y228 occurred efficiently in each cell line and was comparable in SYF cells expressing either wild-type or kinase dead c-Src. Together, results from these experiments indicate that Src kinase activity is, at best, a minor contributor in EGFR signaling to p120 Y228. Although other non-Src kinases may be involved, the data are consistent with the possibility that EGFR phosphorylates p120 directly.

Discussion

EGFR signaling to cadherin complexes is known to occur through p120 but the signaling pathway and its consequences in adhesion are unclear. In collaboration with Roberto Campos at BD/Transduction Laboratories, we have derived a high affinity phosphospecific mAb that selectively recognizes p120 phosphorylated at Y228. We find that this site is a major target of EGFR signaling and that it is constitutively phosphorylated in some, but not all, highly malignant carcinoma cell lines. Because p120 tyrosine phosphorylation is tightly regulated in normal cells, it is likely that constitutive p120 phosphorylation at Y228 reflects the presence of activated protein tyrosine kinase oncogenes (e.g. EGFR, HER2), which in turn mediate unscheduled signaling to cadherin complexes through p120.

We also describe an siRNA-based system for knocking down endogenous p120 and replacing it with selected p120 phosphorylation mutants. The system largely solves the problem of competition from endogenous p120, which can be

significant given that tyrosine phosphorylation is a potent signaling event. Such high efficiency knockdown (~95%) was necessary to ensure that data obtained with these cell lines reflected the consequences of the ectopically expressed p120 mutant rather than a masking effect from the remaining endogenous p120. We believe that the cells are essentially forced to use the ectopically introduced p120. The experiments in Figs 4 and 5 were also reconstructed with polyclonal A431 cell lines (exhibiting ~50% p120 knockdown; not shown) with identical results, suggesting that the data obtained here do not represent clonal artifacts.

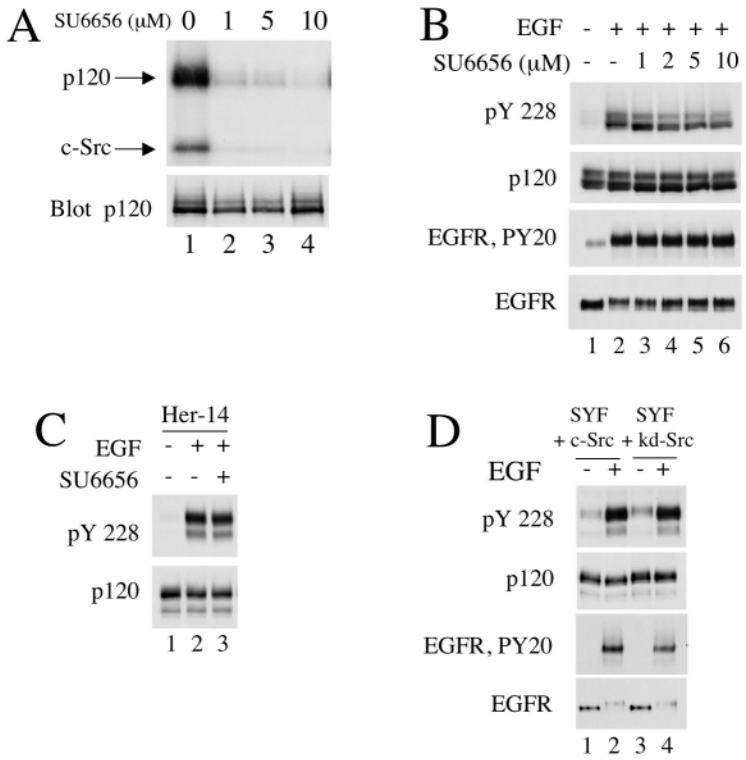

Fig. 7. EGF signaling to p120 Y228 in A431 cells is independent of Src. (A) Validation of SU6656 activity. Phosphorylation of p120 by Src was monitored by in vitro kinase assay in the presence of increasing concentrations of the Src-family inhibitor SU6656. ^{32}P-labeled samples were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography (15-minute exposure). As a control for sample loading, p120 was subsequently quantitated by western blotting with mAb-pp120 (5-second exposure). (B) EGF-induced p120 phosphorylation at Y228 in A431 cells is not blocked by SU6656. A431 cells were serum starved for 18 hours, then treated for 1 hour with DMSO alone or DMSO containing the indicated concentrations of SU6656. Cells were then incubated for 5 minutes with or without EGF (100 ng/ml), and lysed in RIPA containing pervanadate. Total p120 (p120) and Y228-phosphorylated p120 (pY228) levels were quantitated by western blotting. EGFR phosphorylation was determined by western blotting EGFR immunoprecipitates with the phosphotyrosine-specific mAb PY20 (EGFR, PY20) or with an EGFR-specific mAb (EGFR). (C) Src-independent EGF-induced p120 phosphorylation in HER-14 cells. HER-14 cells are murine Swiss 3T3 fibroblasts stably transfected with a human EGFR construct. The cells were serum starved for 12.5 hours in the presence of 4 μg/ml each insulin and transferrin to improve viability, then incubated for one hour with DMSO alone or with DMSO containing 2 μM SU6656. Cells were then treated for 5 minutes with or without EGF (100 ng/ml) prior to lysis in RIPA including pervanadate. p120 immunoprecipitates generated with mAb 15D2 were divided, and then western blotted with mAb pY228 or mAb pp120. (D) EGF induces p120 Y228 phosphorylation in the absence of Src kinase activity. SYF cells are murine embryonic fibroblasts derived from mice that are null for Src, Yes and Fyn. Other Src-family kinases have not been detected in these cells. Polyclonal EGFR-expressing cell lines were generated from SYF cells expressing either c-Src (+c-Src) or kinase dead c-Src (kd-Src). Cells were serum starved for 24 hours, EGF stimulated (100 ng/ml) and analyzed as described in B for p120 phosphorylation at Y228.

In calcium-switch experiments, p120 Y228 phosphorylation was sharply reduced when adhesion was blocked by calcium removal. After calcium add-back, Y228 phosphorylation recovered over 30 minutes, the same time frame associated with recovery of adherens junctions. In addition, the recovery of Y228 phosphorylation was blocked by pre-incubation with E-cadherin function-blocking antibodies. Therefore, it appears that Y228 phosphorylation responds to the adhesive state of cadherins and is dependent on cadherin engagement. These data are consistent with previous observations in keratinocytes (Calautti et al., 1998), although in our experiments, the availability of mAb pY228 allowed direct tracking of phospho-p120, biochemically and in situ, as apposed to indirect monitoring using antibodies to phosphotyrosine.

Despite these observations, Y228 phosphorylation was not essential for junction formation. In fact, simultaneous tyrosine to phenylalanine mutation of the seven previously identified tyrosine phosphorylation sites did not have obvious effects on cadherin-mediated junction formation in calcium switch or aggregation assays, nor did it affect the ability of p120 to stabilize and retain functional E-cadherin at the cell surface (Davis et al., 2003; Ireton et al., 2002). It is possible that by eliminating phosphorylation sites, we removed the ability to dynamically regulate adhesion and motility through modification of p120, but did not otherwise affect the positive role of p120 in promoting adhesion. Indeed, forced expression of p120 isoform 4, which lacks the entire amino-terminal phosphorylation domain, is considerably more efficient at restoring cadherin stability and adhesiveness than the major p120 isoforms which retain the amino terminus (e.g. p120-1A and p120-3A) (Aono et al., 1999; Ireton et al., 2002). These data are consistent with the fact that the amino terminus of p120 is necessary for the motility-inducing effects of p120 in the presence of growth factors (e.g. EGF or HGF) (Cozzolino et al., 2003). Thus, p120 phosphorylation may be necessary for dynamic regulation of adhesion and motility, but the inability to be phosphorylated does not block adhesion. Given the power of this siRNA-based p120 replacement system, an important challenge is to develop assays that discriminate the stabilizing effect of p120 (Ireton et al., 2002) from the more subtle effects that are likely to occur as a result of individual p120 phosphorylation events.

An implication of our data is that under steady state conditions, an SU6656-sensitive kinase besides Src, Yes or Fyn may be involved in signaling to p120 Y228. We examined both steady state signaling and EGF-induced signaling to p120 Y228 in the presence of SU6656. At SU6656 concentrations thought to be selective for Src family kinases, the effect on steady state Y228

phosphorylation in A431 cells was measurable but not substantial. At 10 μM, however, steady state Y228 phosphorylation in the absence of EGF stimulation was drastically reduced. Thus, we suspect that various cell types use both Src-family and non Src-family kinases to signal to p120, and that one or more non Src-family kinases might be central in A431 cells in the absence of added EGF. Candidate kinases based on suspected roles in adhesion and SU6656 sensitivity data include Fer, a known p120 binding partner, Abl (IC_{50} 1.74 μM) and Met (IC_{50} 3.6 μM). Interestingly, activity of Src-kinases was also largely irrelevant under conditions of EGF induction and was clearly not essential for transmission of the EGF-induced signal to p120 Y228. These data were initially surprising because Src phosphorylates p120 with very high efficiency (Mariner et al., 2001; Reynolds et al., 1989) and Src is reported to act downstream of EGFR (reviewed by Biscardi et al., 1999).

One possibility is that the EGFR itself might phosphorylate p120 directly and, in fact, EGFR has been reported to localize to adherens junctions and interact directly with β-catenin (Hoschuetzky et al., 1994; Takahashi et al., 1997). That high concentrations (10 μM) of SU6656 do not affect EGFR activity, yet have little effect on EGF-induced p120 Y228 phosphorylation, is consistent with this hypothesis. In addition, EGFR activation induced efficient p120 Y228 phosphorylation when ectopically expressed in fibroblasts, and in Src/Yes/Fyn-deficient mouse embryo fibroblasts. These are cell types that do not normally express significant levels of EGFR, and yet ectopically expressed EGFR coupled efficiently to p120. All of these data indicate that p120 phosphorylation at Y228 is a potent downstream effect of EGFR activation.

In summary, we have generated a novel Y228 phosphospecific monoclonal antibody to p120 and an siRNA-based system designed to selectively assay the effects of phosphorylation of individual sites. We have found that EGF induces EGFR signaling to the E-cadherin complex through highly efficient phosphorylation at Y228. Moreover, the antibody allowed direct localization of Y228-phosphorylated p120, and revealed selective EGF-induced phosphorylation of p120 in leading edge cells and lamellipodia. Although Y228 phosphorylation was responsive to cadherin ligation (or lack thereof), mutating Y228, along with the other major p120 tyrosine phosphorylation sites, did not inhibit junction formation or strong cell-cell adhesion. Surprisingly, activity of Src-family kinases was not required for EGF-induced phosphorylation at Y228 and, indeed, EGFR might be capable of phosphorylating p120 directly. Finally, several malignant cancer cell lines exhibited high levels of constitutive p120 Y228 phosphorylation, indicating that cancers caused by dysregulated tyrosine kinase activity may constitutively crosstalk to cadherin complexes via p120 phosphorylation.

We extend thanks to Roberto Campos and James Brooks for collaborative efforts in generating the p120 Y228 antibody, to Jonathan Cooper and Leslie Cary (Washington University) for providing the SYF family of cell lines, to Margaret Wheelock for P-cadherin blocking antibodies and to James Staros and Rich Stein (Vanderbilt University) for providing EGFR cDNA. This work was supported by NCI RO1 (CA55724) to A.B.R., the SPORE in GI Cancer Grant 1P50CA95103 and NCI Vanderbilt-Ingram Center Support Grant P30CA68485.

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